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**SEÑALIZACIÓN POR VARIANTES ALÉLICAS ASOCIADAS A
MELANOMA DEL RECEPTOR DE MELANOCORTINAS 1**

Memoria presentada por
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ÍNDICE.

ABREVIATURAS.....	1
INTRODUCCIÓN.....	5
1. LA PIEL: ESTRUCTURA Y COMPOSICIÓN.....	7
2. MELANOCITOS.....	8
2.1. Melanocitos. Formación y función: síntesis de melaninas.....	8
2.2. Formación de melanosomas.....	11
3. REGULACIÓN DE LA MELANOGÉNESIS.....	11
3.1. Efectos de la radiación UV sobre la pigmentación.....	12
3.2. Las melanocortinas.....	14
4. RECEPTORES ACOPLADOS A PROTEÍNAS G.....	15
4.1. Características estructurales y clasificación.....	15
4.2. Señalización a través de la proteína G.....	17
4.3. Glicosilación de los GPCRs.....	19
5. RECEPTOR DE MELANOCORTINAS 1.....	20
5.1. Subfamilia de receptores de melanocortinas.....	20
5.2. Características generales del MC1R.....	22
5.3. Estructura del MC1R.....	23
5.3.1. Extremo N-terminal.....	24

5.3.2. Los bucles extracelulares.....	24
5.3.3. Los bucles intracelulares.....	25
5.3.4. Los fragmentos TM y la zona de unión al agonista.....	25
5.3.5. El extremo citosólico C-terminal.....	25
5.4. Vías de señalización del MC1R.....	26
5.5. Actividad constitutiva del MC1R.....	28
6. REGULACIÓN DE LA FUNCIÓN DEL MC1R.....	28
6.1. Variantes alélicas del MC1R.....	29
6.2. Tráfico de MC1R a través de la vía secretora.....	32
6.3. Desensibilización e internalización del MC1R.....	34
7. LA SUPERFAMILIA DE LAS QUINASAS ACTIVADAS POR MITÓGENOS.....	36
7.1. Características funcionales de las MAPKs y clasificación.....	36
7.2. Vía de las ERKs.....	38
7.3. Mecanismos de activación de las ERK iniciados por GPCRs.....	41
7.3.1. Mecanismos dependientes de segundo mensajero.....	42
7.3.2. Activación por el dímero $\beta\gamma$ de la proteína G.....	44
7.3.3. Regulación por proteínas adaptadoras.....	44
7.3.4. Transactivación.....	45
7.3.4.1. Transactivación de RTKs por mecanismos independientes de sus ligandos.....	46
7.3.4.2. Transactivación de RTKs por mecanismos dependientes de su ligando.....	49
7.4. Receptor tirosina quinasa c-KIT.....	50

8. Melanomas.....	52
8.1. Melanomas: etiología y subtipos.....	52
8.2. Melanoma familiar: genes de alta penetrancia.....	53
8.3. Melanoma esporádico.....	55
8.4. Relación entre el MC1R y el cáncer de piel.....	56
 BIBLIOGRAFÍA DE LA INTRODUCCIÓN	58
 OBJETIVOS.....	71
 MATERIALES Y MÉTODOS.....	75
 1. REACTIVOS Y MATERIALES.....	77
 2. LÍNEAS CELULARES, CULTIVOS CELULARES Y PREPARACIÓN DE EXTRACTOS.....	78
 3. ACTIVADORES E INHIBIDORES.....	80
 4. TRANSFECCIONES TRANSITORIAS.....	82
4.1. Transfección transitoria de ADN plasmídico.....	82
4.2. Transfección transitoria de ARN pequeño de interferencia.....	83
 5. ELECTROFORESIS ANALÍTICA DE PROTEÍNAS.....	84
 6. TRANSFERENCIA WESTERN.....	84

7. ENSAYO DE UNIÓN HORMONA-RECEPTOR.....	86
7.1. Ensayos de unión y de competición.....	86
7.2. Ensayos de internalización.....	87
8. ENSAYO DE ACOPLAMIENTO FUNCIONAL.....	88
8.1. Estimulación y extracción de AMPc.....	88
8.2. Ensayo de AMPc.....	88
9. DETERMINACIÓN DE LA VIDA MEDIA DE PROTEÍNAS.....	89
10. TRATAMIENTO CON ENDOGLICOSIDASAS.....	89
11. EXTRACCIÓN DE ADN GENÓMICO.....	90
12. EXTRACCIÓN DE ARN.....	90
13. SÍNTESIS DE cDNA.....	91
14. CLONACIÓN DE GENES.....	91
14.1. Obtención de mutantes de glicosilación del MC1R mediante mutagénesis dirigida.....	91
14.2. Subclonación de c-Kit en pcDNA3.....	93
15. SECUENCIACIÓN DE N-RAS, B-RAF Y MC1R EN LÍNEAS DE MELANOMA.....	94
16. MICROSCOPIA.....	96
16.1. Microscopía óptica.....	96

16.2. Microscopía confocal.....	96
16.2.1. Tinción inmunquímica.....	96
16.2. 2. Adquisición de imágenes.....	97
RESULTADOS Y DISCUSIÓN.....	99
Capítulo 1.	
N-glicosilación del receptor de melanocortinas 1. Ocupación de los sitios de glicosilación y papel funcional.....	101
Capítulo 2.	
Mutaciones en el receptor de melanocortinas 1 tienen un impacto diferencial a través de la vía del AMPc y a través de la vía de las proteínas quinasas activadas por mitógenos ERK.....	129
Capítulo 3.	
La señalización del receptor de melanocortinas 1 a las proteínas quinasas activadas por mitógenos ERK1 y ERK2 implica la transactivación de c-KIT.....	145
Capítulo 4.	
Relaciones funcionales de las vías del AMPc y de las proteínas quinasas activadas por mitógenos ERK1 y ERK2 mediadas por el receptor de MC 1 en células de melanoma humano.....	183
CONCLUSIONES.....	213
SUMMARY.....	217
APÉNDICE.....	237

ABREVIATURAS.

A continuación se detalla por orden alfabético una lista de las abreviaturas utilizadas en la Memoria.

AC: adenilato ciclasa	EndoH: endoglicosidasa H
ACTH: hormona adenocorticotropa	ERK: quinasa regulada por señales extracelulares
AGRP: proteína relacionada con el gen <i>Agouti</i>	ET-1: endotelina 1
AMPc: adenosina 3',5'-monofosfato cíclico	FGFR: receptor del factor de crecimiento de fibroblastos
AP2: proteína adaptadora 2	FSK: forskolina
ARN: ácido ribonucleico	G418: geneticina
ARNm: ARN mensajero	GFP: proteína verde fluorescente
ASP: agouti	GPCR: receptor acoplado a proteína G
bFGF: factor de crecimiento básico de fibroblastos.	GRK: quinasa de receptores acoplados a proteína G
CRE: elemento de respuesta al AMPc.	IBMX: 1-metil-3-isobutilxantina
CREB: proteína de unión a elementos sensibles a AMPc	MAPK: proteína quinasa activada por mitógenos
cpm: cuentas por minuto	MCR: receptor de melanocortina
DCT: dopacromo tautomerasa	β-ME: β-mercaptoetanol
DHI: 5,6-dihidroxiindol	MITF: microphthalmia
DHICA: ácido DHI-2-carboxílico	MMP: metaloproteasa de matriz
DMEM: medio mínimo esencial modificado por Dulbecco	αMSH: hormona estimulante del melanocito alfa
dpm: desintegraciones por minuto	NDP-MSH: [Nle ⁴ , D-Phe ⁷] αMSH
DQ: dopaquinona	PBS: tampón fosfato salino
EDTA: ácido etilendiaminotetracético	PDE: fosfodiesterasa
EGFR: receptor del factor de crecimiento epidérmico	PNGasa F: N-glicosidasa F
	PMSF: fluoruro de fenilmetilsulfonilo

PKA: proteína quinasa A

PKC: proteína quinasa C

POMC: proopiomelanocortina

PMA (TPA): forbol-12-ministato-13-acetato

QC: control de calidad

RE: retículo endoplasmático

RGP: fase de crecimiento radial

RHC: fenotipo "Red Hair Colour"

RTK: receptor con actividad tirosina quinasa

SBF: suero bovino fetal

SCF: factor de células madre

SDS: dodecil sulfato sódico

SFK: quinasa de la familia Src

siRNA: ARN pequeño de interferencia

TAE: tampón acético-EDTA

TBS: tampón tris salino

TEMED: N,N,N',N'-tetrametilendiamina

TGS: tampón tris glicina SDS

TM: fragmento transmembrana

TYR: tirosinasa

UV: ultravioleta

VGP: fase de crecimiento vertical

INTRODUCCIÓN.

1. LA PIEL: ESTRUCTURA Y COMPOSICIÓN.

La piel es un órgano complejo, que cubre toda la superficie del cuerpo. Constituye el 15% del peso total del adulto y, por tanto, es el órgano más extenso del cuerpo. Cumple múltiples funciones vitales gracias a su estructura elaborada, que se encuentra organizada en tres capas: la epidermis (y sus apéndices asociados, folículos pilosos y folículos sebáceos, y glándulas sudoríparas); la dermis (separada de la epidermis por la unión dermal-epidermal); y la hipodermis (Figura 1). Embriológicamente, la epidermis y sus apéndices son de origen ectodérmico, mientras que la dermis y la hipodermis son de origen mesodérmico.

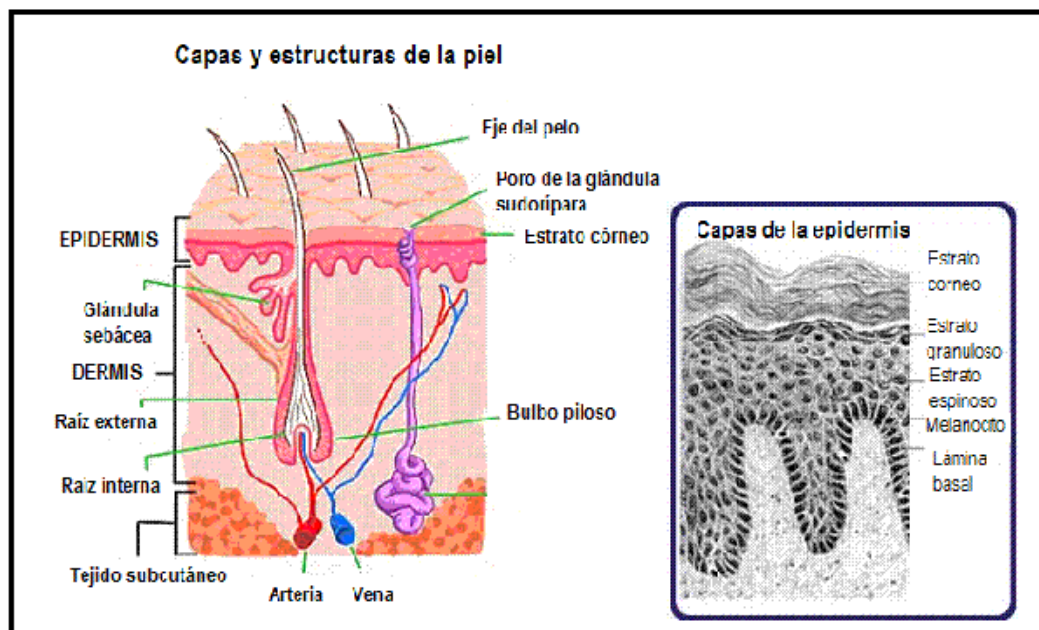


Figura 1. Estructura de la piel y capas de la epidermis.

La **hipodermis** es un tejido conjuntivo adiposo, que representa la parte más profunda de la piel y conecta la dermis con los órganos. Tiene una función importante en la termorregulación, aislamiento, aprovisionamiento de energía (almacén nutricional) y protección frente a lesiones mecánicas.

La **dermis** es un tejido conjuntivo grueso elástico y compresible, que protege la epidermis, sus apéndices y los plexos vascular y nervioso que la recorren. Está formada por células (fibroblastos, células dendríticas, mastocitos) y moléculas fibrosas. Contiene receptores táctiles y del dolor, cuyas ramificaciones llegan hasta la superficie de la piel y a diversas glándulas: glándulas sudoríparas, glándulas sebáceas y folículos pilosos. También, en el interior de la dermis, se encuentran vasos sanguíneos que proporcionan nutrientes y calor a la piel, así como nervios que se ramifican entre las distintas capas de la misma.

La **epidermis** es un epitelio estratificado, que se renueva continuamente. Está formada por cinco capas de células: el estrato corneo es la capa más externa y el estrato germinativo es la más profunda. La superficie del estrato corneo está formada por 25-30 subcapas de células escamosas, que se desprenden continuamente por fricción y que son reemplazadas por células del estrato germinativo. Estas células se conocen como queratinocitos, porque el protoplasma es rico en queratina, que contribuye a las propiedades protectoras de la piel. Los queratinocitos se originan a partir de divisiones mitóticas de células madre de la capa basal. La célula hija formada migra hacia la superficie celular y durante su recorrido, sufre un proceso de diferenciación morfológica y bioquímica conocido como queratinización. Finalmente, se desprenden de la superficie celular en aproximadamente 30 días (1).

Además de los queratinocitos (~80-90% de la población celular epidérmica total), en la epidermis se localizan otras células residentes:

- Las células de Langerhans (~5-8%) y células dendríticas libres (~1-2%), responsables de la respuesta inmunitaria epidérmica;
- Los melanocitos (~5%), células dendríticas localizadas principalmente en la capa basal, encargadas de la producción de pigmentos polifenólicos conocidos como melaninas;
- Las células de Merkel (~3-5%), células neuroendocrinas que actúan como receptores sensoriales cutáneos.

2. MELANOCITOS.

2.1. Melanocitos. Origen y función: síntesis de melaninas.

Los melanocitos se originan a partir de células pluripotentes de la cresta neural del embrión denominados melanoblastos, mediante un proceso de proliferación y diferenciación (2). Una vez formados, los melanocitos migran a la capa basal de la epidermis (lámina basal), al bulbo piloso y al ojo, donde producen melanina, el principal pigmento de la piel. La correcta migración, distribución y funcionamiento de melanoblastos/melanocitos determina el fenotipo visible de la piel. Los melanocitos se distribuyen regularmente en la lámina basal siguiendo un patrón característico. Cada melanocito de la epidermis está funcionalmente conectado a los fibroblastos subyacentes de la dermis y a los queratinocitos de la epidermis suprayacente. La distribución de los melanocitos entre los queratinocitos basales ocurre en una proporción de un melanocito por cada 4-10 queratinocitos basales.

Las melaninas son pigmentos fotoprotectores de naturaleza polifenólica, derivados de la oxidación del aminoácido tirosina y de la polimerización posterior de sus productos oxidados. Las melaninas protegen la piel de la radiación solar UV, absorbiéndola y actuando como un filtro (3). Los melanocitos pueden producir dos tipos de pigmentos melánicos:

- Las *eumelaninas*, de color marrón o negro y fuertemente fotoprotectoras;
- Las *feomelaninas*, de color rojizo, mucho menos fotoprotectoras.

La biosíntesis de melaninas transcurre a través de una ruta enzimática compleja conocida como vía de Raper-Mason, que se esquematiza en la Figura 2. Los pigmentos melánicos son sintetizados a partir del aminoácido L-tirosina y se almacenan en unos orgánulos esféricos u ovoides llamados melanosomas. Los dos tipos de melanina difieren no sólo en color, sino también en el tamaño, forma y modo de empaquetamiento en los melanosomas que las contienen.

La enzima clave en la ruta de síntesis de melaninas es tirosinasa, TYR, que cataliza dos reacciones sucesivas: la hidroxilación de tirosina a DOPA, y la oxidación de DOPA a dopaquinona (DQ). En condiciones de bajo contenido en compuestos con grupos sulfhidrilo, la vía melanogénica conduce a la producción de eumelaninas. En estas condiciones, DQ cicla espontáneamente para formar el metabolito intermedio dopacromo, que espontáneamente pierde el grupo carboxílico generando 5,6-dihidroxiindol (DHI). Este compuesto se oxida y polimeriza dando lugar a polímeros insolubles de alto peso molecular de color marrón oscuro casi negro, conocidos como DHI-melaninas. Sin embargo, en presencia de la enzima dopacromo tautomerasa (DCT, también conocida como Tyrp2), dopacromo tautomeriza sin perder su grupo carboxílico dando lugar al ácido DHI-2-carboxílico (DHICA), que puede oxidarse y polimerizar, dando lugar a un subtipo de eumelaninas, conocidas como DHICA-melaninas, caracterizadas por un color marrón claro, moderada solubilidad y tamaño intermedio (4). En cambio, en presencia de grupos tiólicos de bajo peso molecular con grupos sulfhidrilo libres, como cisteína o glutatión, se produce una reacción de conjugación entre la DQ (formada a partir de tirosina por TYR) con dichos compuestos tiólicos, generando 3- ó 5-cisteinil DOPAs. Estos compuestos se oxidan y polimerizan, dando lugar a melaninas de color amarillo-rojizo conocidas como feomelaninas (Figura 2) (5;6). Este tipo de melanina es menos fotoprotectora que la eumelanina, y por interacción con la radiación UV puede producir peróxido de hidrógeno, superóxido y radicales hidroxilo, que conducen a un estrés oxidativo que puede causar daño en el ADN.

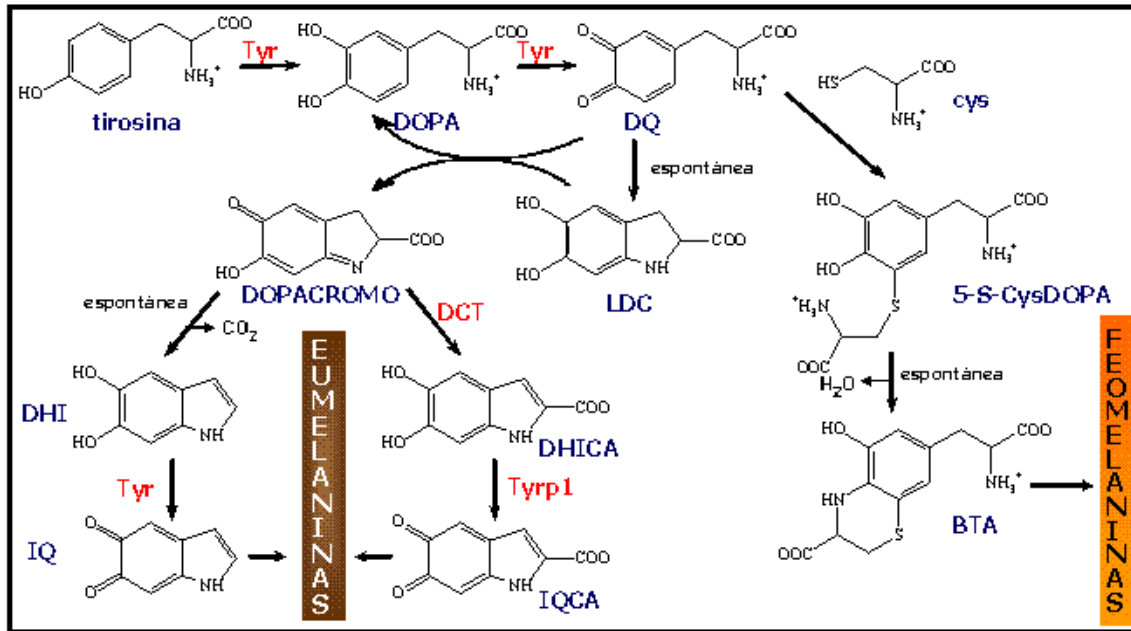


Figura 2. Vía de síntesis de eu- y feomelaninas.

Los melanocitos sintetizan tanto eumelaninas como feomelaninas, con una relación entre las dos que viene determinada por un balance entre la expresión de las distintas enzimas melanogénicas y la disponibilidad en la célula de tirosina y agentes reductores con grupos sulfhidrido (7). La proporción en la que se encuentran determina la pigmentación visible.

Los melanosomas, orgánulos citoplásmicos en los que tiene lugar la biosíntesis del pigmento, sufren un proceso de maduración y, finalmente, cuando están cargados

de melanina son transportados a lo largo de las dendritas del melanocito y transferidos a los queratinocitos adyacentes. Una vez en los queratinocitos, los melanosomas se distribuyen y, en respuesta a la radiación UV, se sitúan alrededor del núcleo para formar unas estructuras que lo protegen (Figura 3).

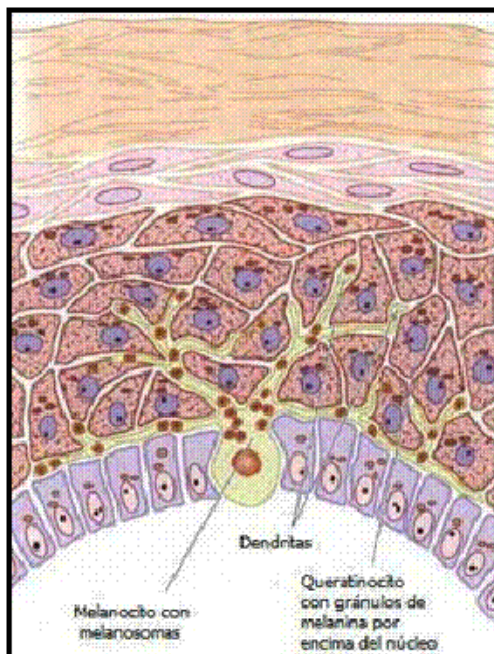


Figura 3. Relaciones estructurales y funcionales entre los melanocitos y los queratinocitos en la lámina basal de la epidermis.

2.2. Formación de melanosomas.

En condiciones fisiológicas, la síntesis de melanina está restringida a los melanosomas, y sus elementos enzimáticos y estructurales se organizan y ensamblan separadamente en un proceso que recuerda a la formación del lisosoma (8). En general, la estructura de los melanosomas depende del tipo de melanina producida. Los eumelanosomas son elípticos y contienen una matriz fibrilar, mientras que la forma de los feomelanosomas es variable con un contorno redondeado y contiene una matriz vesiculoglobular. Los melanosomas se desarrollan en cuatro etapas. La etapa I es una etapa temprana que corresponde a un estado sin organización evidente de la matriz (premelanosoma). En la etapa II, ya se ha organizado la matriz, pero aún no hay formación de melanina (eumelanosomas); en los feomelanosomas la melanina ya se ha formado en esta etapa. En la etapa III, hay deposición de melanina. En la etapa IV, los melanosomas están completamente llenos de melanina, es decir, están melanizados, son densos y han perdido la actividad tirosinasa (9) (Figura 4).

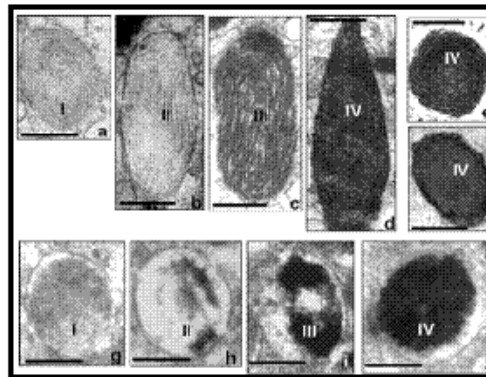


Figura 4. Microscopía electrónica del desarrollo de eumelanosomas (a-f) y de feomelanosomas (g-j) (9).

3. REGULACIÓN DE LA MELANOGÉNESIS.

La pigmentación melánica depende de la proliferación y diferenciación de los melanocitos. En los mamíferos estos procesos están regulados por numerosos genes (10), así como por controles epigenéticos mediados por otros tipos celulares adyacentes, especialmente los queratinocitos (11). Estas células contribuyen también de forma decisiva a la proliferación, supervivencia y diferenciación de los melanocitos a través de la secreción de moléculas de señalización intracelular.

La proliferación del melanocito está regulada positivamente por factores de crecimiento mitogénicos, como el factor de crecimiento básico de fibroblastos (bFGF)

(12) y otros agentes como ésteres de forbol. En los melanocitos, el AMPc colabora de forma sinérgica con estos factores para inducir la proliferación celular (13).

Por otra parte, la regulación de la diferenciación del melanocito está mediada por distintos agentes físicos y químicos, que utilizan múltiples vías de señalización, siendo la radiación UV el principal inductor fisiológico externo de la melanogénesis.

3.1. Efectos de la radiación UV sobre la pigmentación.

La radiación UV es el principal factor inductor de la pigmentación inducida o facultativa de la piel humana. La radiación UV provoca el oscurecimiento rápido de la piel (reacción de bronceado), que ocurre inmediatamente tras la exposición a la radiación. Este bronceado no se produce por una síntesis de melanina, sino por la oxidación y polimerización de intermedios melanogénicos preexistentes y la redistribución de los melanosomas (14). Por otra parte, días después de la exposición a la radiación UV se produce una respuesta de bronceado tardía y más duradera, que implica la activación de los melanocitos. Esta respuesta es compleja, ya que tiene al menos dos componentes: por una parte, un efecto directo sobre los melanocitos y, por otra parte, una activación de la liberación de moléculas de señalización por los queratinocitos, que provocan una respuesta melanocítica secundaria (15). La combinación de estos efectos conduce a un aumento en la expresión y la actividad en los melanocitos de Microphthalmia (MITF), principal regulador transcripcional de genes de proteínas melanogénicas como Pmel17, MART-1, TYR, TRP1 y DCT, que finalmente provoca un aumento en el contenido de melanina (16).

En cuanto a las respuestas directas de los melanocitos, la radiación UV provoca la aparición de fotoproductos de ADN y la generación de especies reactivas de oxígeno (ROS), que provoca el daño oxidativo de ADN, así como la peroxidación lipídica y proteica. La activación de p38 provoca una estimulación de la transcripción de genes melanogénicos (17;18). Por otra parte, en las células irradiadas y sobre todo en los queratinocitos, se produce una acumulación de p53, y una inducción del inhibidor de la quinasa dependiente de ciclina p21 (Figura 5). Como consecuencia, el ciclo celular entra en parada, y las células más dañadas entran en apoptosis, mientras que en las que han sufrido un daño moderado se activan mecanismos de reparación del ADN (19). Otro efecto importante de la acumulación de p53 es la inducción de genes controlados por este factor de transcripción, tanto en los queratinocitos como en los melanocitos. Uno de ellos es el de POMC, el precursor de las melanocortinas α -MSH (hormona estimulante del melanocito α) y ACTH (hormona adenocorticotropa). La producción y liberación de estas hormonas se activa por tanto en los queratinocitos,

conduciendo a la activación paracrina de los melanocitos (17;20). Esta activación es tanto más potente cuanto que la acumulación de p53 en melanocitos produce un incremento de la expresión del gen que codifica para el receptor MC1R reconocido por las melanocortinas y mediador de sus efectos. Por otra parte, en los queratinocitos, la radiación UV también estimula la liberación de endotelina 1 (ET-1) y de bFGF, que actúan sobre sus receptores en el melanocito. El efecto conjunto de estos factores es complejo e incluye la inducción transcripcional de MITF, su activación por fosforilación, la inducción de los genes melanogénicos, y la activación de las vías de supervivencia como la de Akt, y de proliferación como la de los receptores del bFGF (19;21). Así, la radiación UV media una respuesta de gran complejidad que afecta a la supervivencia, proliferación y diferenciación de los melanocitos.

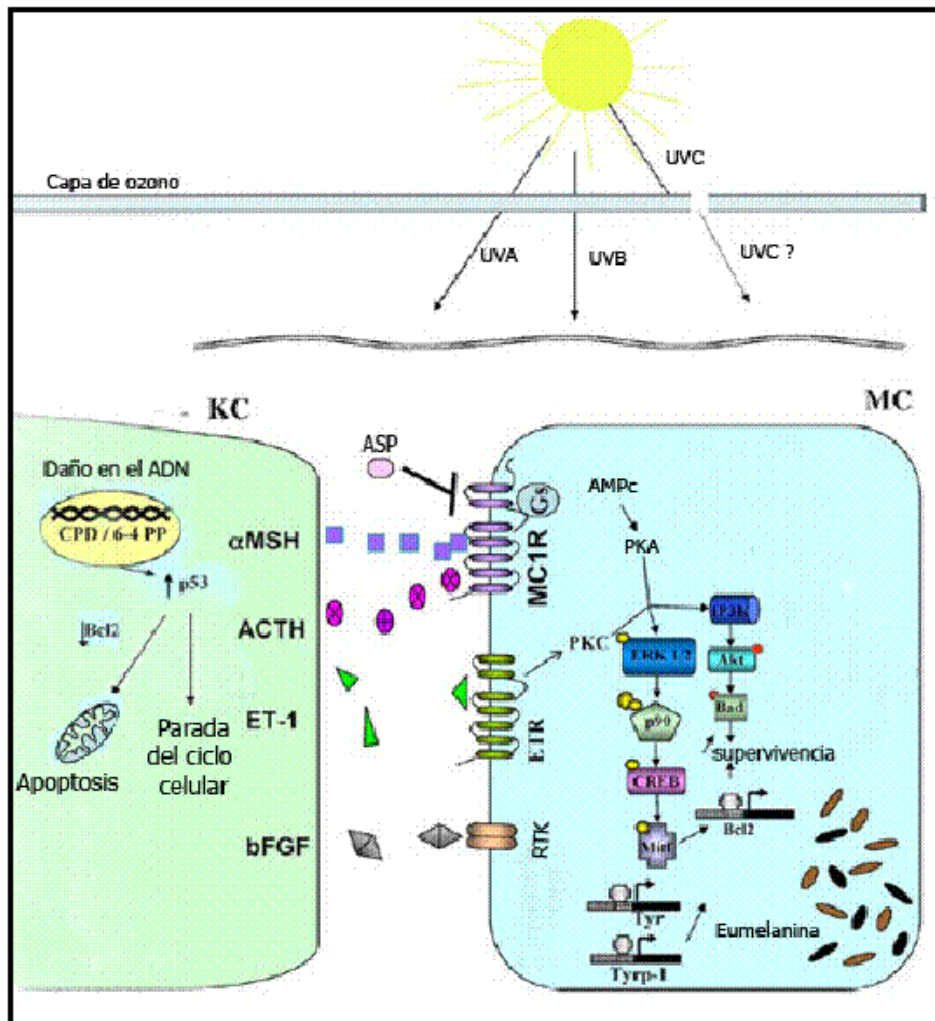


Figura 5. Esquema sobre los efectos de la radiación UV en los melanocitos y queratinocitos de la piel humana. Se muestra únicamente una parte de los efectos. La inducción de p53 no es exclusiva de los queratinocitos y se produce también en los melanocitos.

3.2. Las melanocortinas.

El principal agente para- y endocrino inductor de la diferenciación del melanocito es α -MSH, perteneciente a la familia de las melanocortinas.

Las melanocortinas (MCs) forman una familia de péptidos neuroendocrinos, originalmente caracterizados como reguladores de la pigmentación cutánea y de la producción de cortisol. Las MCs se expresan en la pituitaria y en localizaciones periféricas, incluyendo la piel. La disfunción de la señalización por MCs se ha relacionado con cuadros dispares como hiperfagia y obesidad severa prematura, deficiencia familiar de glucocorticoides, vasculitis, trastornos fibróticos de la epidermis, y distintos tipos de cáncer cutáneo, incluido el melanoma (21).

Las MCs naturales son las hormonas estimulantes del melanocito, α -, β - y γ -MSH, y la hormona ACTH, todas derivadas de un precursor común de 235 aminoácidos, la pro-opiomelanocortina (POMC) (9) (Figura 6).

Estructuralmente, los péptidos MSH (α -, β - y γ - MSH) comparten con ACTH la secuencia aminoacídica -Tyr-x-Met-x-His-Phe-Arg-Trp- que contiene el tetrapéptido His-Phe-Arg-Trp (HFRW), crítico para la actividad melanotrópica.

Los queratinocitos y los melanocitos de la epidermis humana expresan POMC y todas las enzimas necesarias para su procesamiento, por lo que la síntesis ectópica y la liberación de las hormonas ACTH, α - y β - MSH y β -endorfina, también tienen lugar en la piel (21).

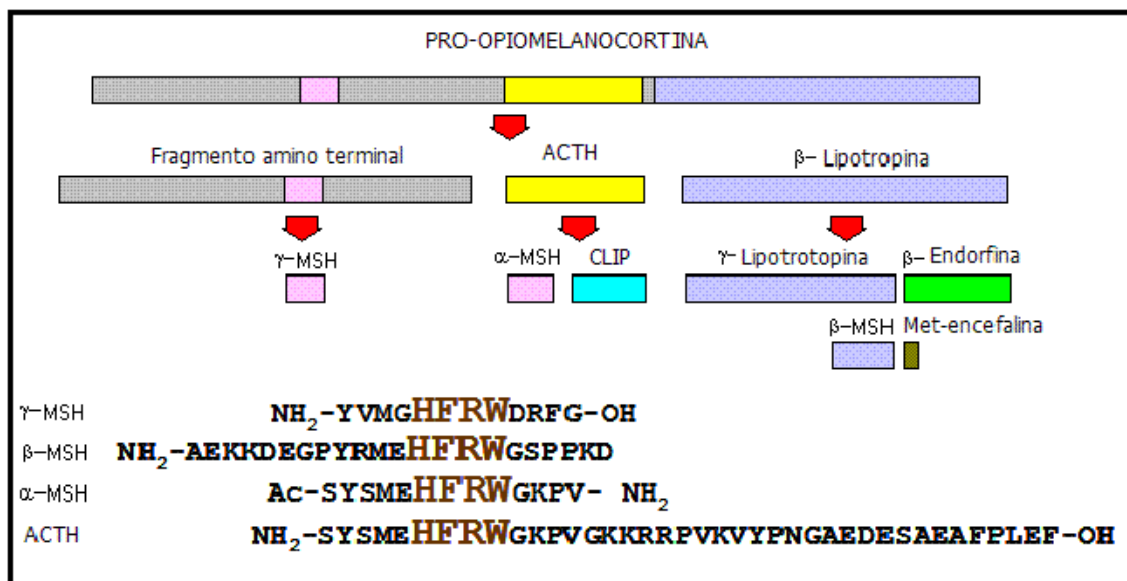


Figura 6. Procesamiento proteolítico de la POMC. Las enzimas proconvertasas, PC1 y PC2, realizan el procesamiento proteolítico de la POMC hasta los péptidos biológicamente activos. PC1 libera ACTH y β -lipotropina, mientras que PC2 libera β -endorfinas y α -MSH.

El péptido de 13 aminoácidos α -MSH es el producto de POMC con mayor relevancia fisiológica para los melanocitos y junto con ACTH, el de mayor actividad melanogénica. Estas MCs ejercen su acción a través de los receptores acoplados a proteínas G (GPCRs), que estimulan la cascada de señalización del AMPc (22;23).

4. RECEPTORES ACOPLADOS A PROTEÍNAS G.

Los GPCRs constituyen una superfamilia formada por más de 1000 miembros, lo que supone más del 1% de los genomas de mamíferos. Los miembros de esta familia incluyen receptores para muchas hormonas, neurotransmisores, iones de calcio, además de receptores sensoriales para varios odorantes, sabor dulce y amargo, e incluso fotones. Los GPCRs regulan la actividad de enzimas metabólicas y vías de señalización, canales iónicos y transportadores de membrana, y la función de las maquinarias transcripcional y secretora. Están implicados en numerosas funciones vitales como neurotransmisión, regulación de la secreción de glándulas endocrinas y exocrinas, exocitosis, foto y quimiorrecepción, quimiotaxis, control de la presión sanguínea, función plaquetaria, embriogénesis, angiogénesis, regeneración tisular y control del crecimiento celular normal o aberrante. Además, existen muchos GPCRs huérfanos, de los que se desconoce su ligando o su función. Estos receptores son la diana de más del 50% de los agentes terapéuticos actuales del mercado (24;25).

4.1. Características estructurales y clasificación.

La información acerca de la estructura de los GPCRs se basó inicialmente en las estructuras de alta resolución de la forma inactiva de la rodopsina (26), ya que es posible obtener grandes cantidades altamente enriquecidas de esta proteína a partir de retina bovina. Además, la rodopsina es un GPCR extraordinariamente estable, que retiene su función bajo condiciones que desnaturalizan otros GPCRs. Posteriormente, se han cristalizado algunos otros GPCRs como el β 2-adrenérgico o el de adenosina, lo que ha mejorado nuestra comprensión de la estructura general de la familia (27;28).

Todos los GPCRs comparten una estructura básica común formada por siete hélices transmembrana (TM), tres bucles extracelulares, tres bucles intracelulares, un extremo N-terminal extracelular y un extremo C-terminal intracelular. Cada uno de los segmentos TM está compuesto generalmente por 20-27 aminoácidos mayoritariamente hidrofóbicos, que forman hélices α , por lo que también se conoce a los GPCRs como receptores siete hélices transmembrana. Los segmentos N-terminal (7-595 aminoácidos), los bucles hidrofílicos (5-230 aminoácidos) y los segmentos C-

terminal (12-359 aminoácidos) son muy variables en cuanto al tamaño y la secuencia de aminoácidos. El mayor grado de homología entre los GPCRs se observa dentro de los segmentos TM, mientras que las estructuras más variables son los extremos amino y carboxilo terminal, y el bucle intracelular que une los segmentos TM5 y TM6.

Los GPCRs se clasifican en tres familias, A, B y C, en base a la similitud de su secuencia. Los miembros de cada familia comparten más de un 25% de identidad en las regiones TM, así como residuos y motivos muy conservados (Figura 7).

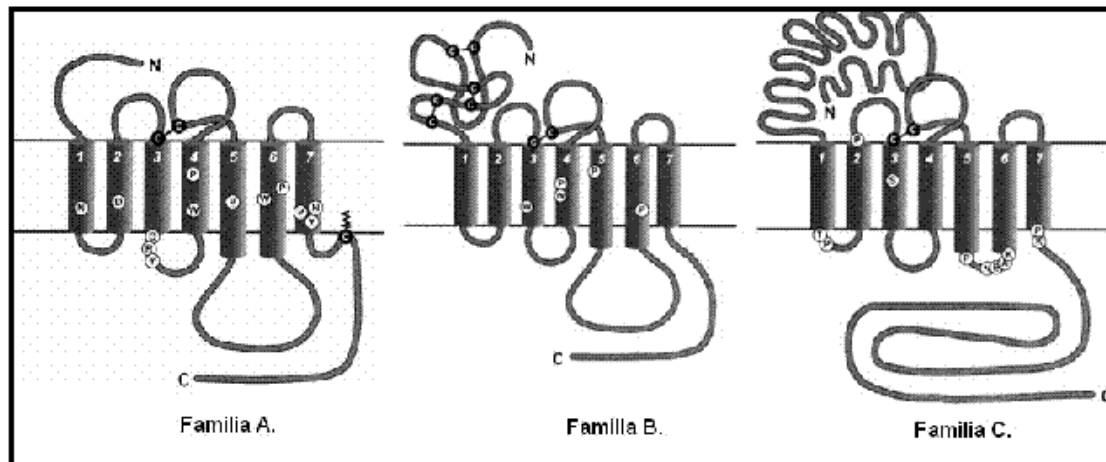


Figura 7. Estructura de las familias de GPCRs.

- La familia A es el grupo más extenso e incluye los fotorreceptores (rodopsina), los receptores adrenérgicos y otros muchos tipos de receptores, como el subgrupo de receptores olfativos y el de receptores de melanocortinas.
- La familia B contiene sólo 25 miembros e incluye los receptores para la familia de hormonas peptídicas gastrointestinales, la hormona liberadora de corticotropina, calcitonina y la hormona paratiroidea.
- La familia C es relativamente pequeña y contiene la familia de receptores metabotrópicos de glutamato, el receptor de GABA_B, el receptor sensible a calcio y los receptores del gusto.

La dimerización de los GPCRs es un fenómeno común que modula propiedades clave de los receptores, como la unión del ligado, la eficiencia de acoplamiento, la desensibilización y el tráfico desde el retículo endoplásmico (RE) hasta la membrana celular, y a través de rutas endocíticas (29;30).

La dimerización de los GPCRs puede ser mediada por puentes disulfuro y/o interacciones no covalentes entre los dominios extracelulares, los C-terminales y los segmentos TM (31). Se han propuesto dos hipótesis para el mecanismo de dimerización de los GPCRs. En los *dímeros de contacto*, los dos monómeros se pliegan independientemente como unidades individuales, y establecen un número

limitado de contactos como consecuencia de su empaquetamiento lateral en el plano de la membrana. Algunas de estas interacciones pueden ser puentes disulfuro covalentes, o bien, interacciones de tipo "coiled-coil" entre los dominios C-terminales de los monómeros. Por otra parte, en los *dímeros de intercambio de dominios*, los monómeros intercambian dominios conectados por un bucle bisagra, de forma que la unidad funcional está formada por un dominio de cada monómero. Existen ejemplos que se asocian a ambos modelos, como es el caso del receptor GABA_B, cuya heterodimerización implica interacciones entre dominios "coiled-coil" del extremo C-terminal (32), el receptor metabotrópico de glutamato, mGlu5, que dimeriza mediante la formación de puentes disulfuro covalentes (33), el receptor de histamina H1, cuya dimerización ocurre a través del intercambio de dominios (34) y el MC1R, que dimeriza mediante la formación de puentes disulfuro, así como mediante interacciones no covalentes posiblemente por intercambio de dominios (35;36).

4.2. Señalización a través de la proteína G.

La mayoría de los GPCRs se activan por la unión de agonistas a la cara extracelular de los segmentos TM o al entorno N-terminal extracelular, que induce un cambio de conformación. Este cambio conformacional permite el acoplamiento del receptor a una proteína G heterotrimérica, compuesta por tres subunidades distintas, α , β y γ , formándose el complejo ligando-receptor-proteína G. Esta interacción se produce a través de los bucles intracelulares. Los distintos GPCRs varían en el grado de actividad constitutiva o independiente de agonista para acoplarse a la proteína G, de forma que se han encontrado receptores mutantes con una actividad constitutiva aumentada en numerosas enfermedades (37;38). La proteína G libera el GDP unido a la subunidad $G\alpha$ y lo sustituye por una molécula de GTP. Este intercambio conduce a la disociación de la subunidad $G\alpha$, el dímero $G\beta\gamma$ y el receptor. Como consecuencia, las subunidades $G\alpha$ y $G\beta\gamma$ estimulan moléculas efectoras como las adenilato y guanilato ciclasas, fosfodiesterasas, fosfolipasa A₂, fosfolipasa C; por lo que activan o inhiben la producción de distintos segundos mensajeros tales como AMPc, GMPc, diacilglicerol, inositoltrifosfato, ácido araquidónico y ácido fosfatídico (39).

Finalmente, la subunidad $G\alpha$ posee actividad enzimática e hidroliza el GTP a GDP, permitiendo su reasociación con $G\beta\gamma$ (Figura 8).

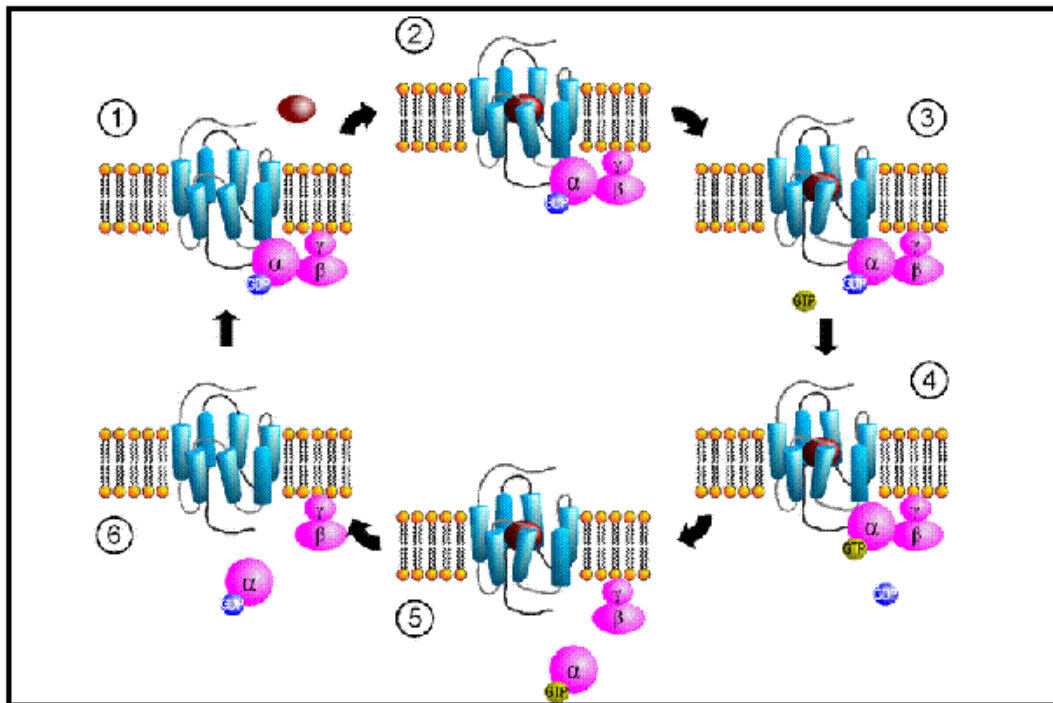


Figura 8. Mecanismo de acoplamiento de los GPCRs a la proteína G.

Las proteínas G se clasifican según el tipo de subunidad α , en seis familias (40):

- G_s : proteínas que están acopladas a la estimulación de la adenilato ciclasa.
- G_i : proteínas acopladas a la inhibición de la adenilato ciclasa y a la activación de canales de potasio (GIRK).
- G_q : proteínas acopladas a la activación de fosfolipasa C_p .
- G_{12} : proteínas acopladas a la activación del factor de intercambio de nucleótidos de guanina Rho.
- G_{olf} : proteínas acopladas a la estimulación de la adenilato ciclasa, en células olfativas.
- G_t : proteínas acopladas a la fosfodiesterasa de $GMPC$, en células fotorreceptoras.

Por tanto, el tipo de proteína G que se asocie con cada receptor determina la vía o cascada de transducción de la señal que se dispare en respuesta a una determinada señal extracelular. Más adelante se detalla la vía de señalización inducida por $AMPC$, porque es la vía canónica activada en los melanocitos por unión de α -MSH a su receptor $MC1R$.

4.3. Glicosilación de los GPCRs.

Así como la mayoría de las proteínas de membrana que transitan a través del RE, los GPCRs suelen estar N-glicosilados. Esta modificación post-transduccional implica un complejo proceso que transcurre en varias etapas que tienen lugar en el RE y a lo largo del aparato de Golgi.

La primera etapa de la N-glicosilación es la unión covalente del precursor $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$. Esta cadena se transfiere a partir del transportador lipídico dolicol-pirofosfato a la proteína naciente en un residuo de asparragina en el lumen del RE, dentro de la secuencia consenso Asn-X-Ser/Thr. Después de esta transferencia, las siguientes reacciones de procesamiento incluyen la eliminación de residuos de glucosa y de al menos un residuo de manosa en el RE, que conducen a la formación de una cadena rica en manosa. El polipéptido parcialmente procesado es transportado al aparato de Golgi, donde se eliminan varios monosacáridos y se añaden de manera secuencial N-acetilglucosamina, fucosa, galactosa y ácido siálico. La glicoproteína recién sintetizada sale del aparato de Golgi y es transportada a su destino final (Figura 9).

El procesamiento del glicano en el RE parece estar conservado en todas las células y juega un papel importante en el plegamiento proteico (41;42), en el control de calidad a través del ciclo de calnexina (43), en la oligomerización (44) y en el tráfico lisosomal (45). La presencia del carbohidrato de las glicoproteínas también es importante en otros procesos celulares, como la estabilidad, la degradación asociada al RE (ERAD) como consecuencia de la eliminación de residuos de manosa (46), la actividad enzimática, la protección frente a la degradación mediada por proteasas (47), funciones estructurales adicionales, la unión de ligandos con alta afinidad, la expresión en la superficie celular y/o el acoplamiento de los GPCRs (48-52). Por otro lado, las reacciones que tienen lugar en el aparato de Golgi explican la diversidad estructural de los N-oligosacáridos en las glicoproteínas maduras.

La presencia, el tamaño y la naturaleza del carbohidrato en una proteína se pueden analizar mediante la digestión con glicosidasas específicas. La endoglicosidasa H (EndoH), digiere selectivamente cadenas de N-glicanos ricas en manosas y cadenas de N-glicanos de tipo híbrido (Figura 9); pero no digiere oligosacáridos complejos, lo que permite analizar la maduración del carbohidrato. La N-glicosidasa F (PNGasa F), hidroliza todos los tipos de cadenas de N-glicanos de glicopéptidos y glicoproteínas, por lo que elimina toda la fracción glucídica.

La mayoría de los GPCRs contienen uno o más sitios potenciales de N-glicosilación dentro de la secuencia consenso Asn-X-Ser/Thr, donde X representa

cualquier aminoácido excepto una prolina, localizado en el extremo N-terminal y/o en los bucles extracelulares. Los efectos de la glicosilación en los GPCRs son específicos de cada receptor, lo que requiere realizar estudios sobre cada uno de ellos.

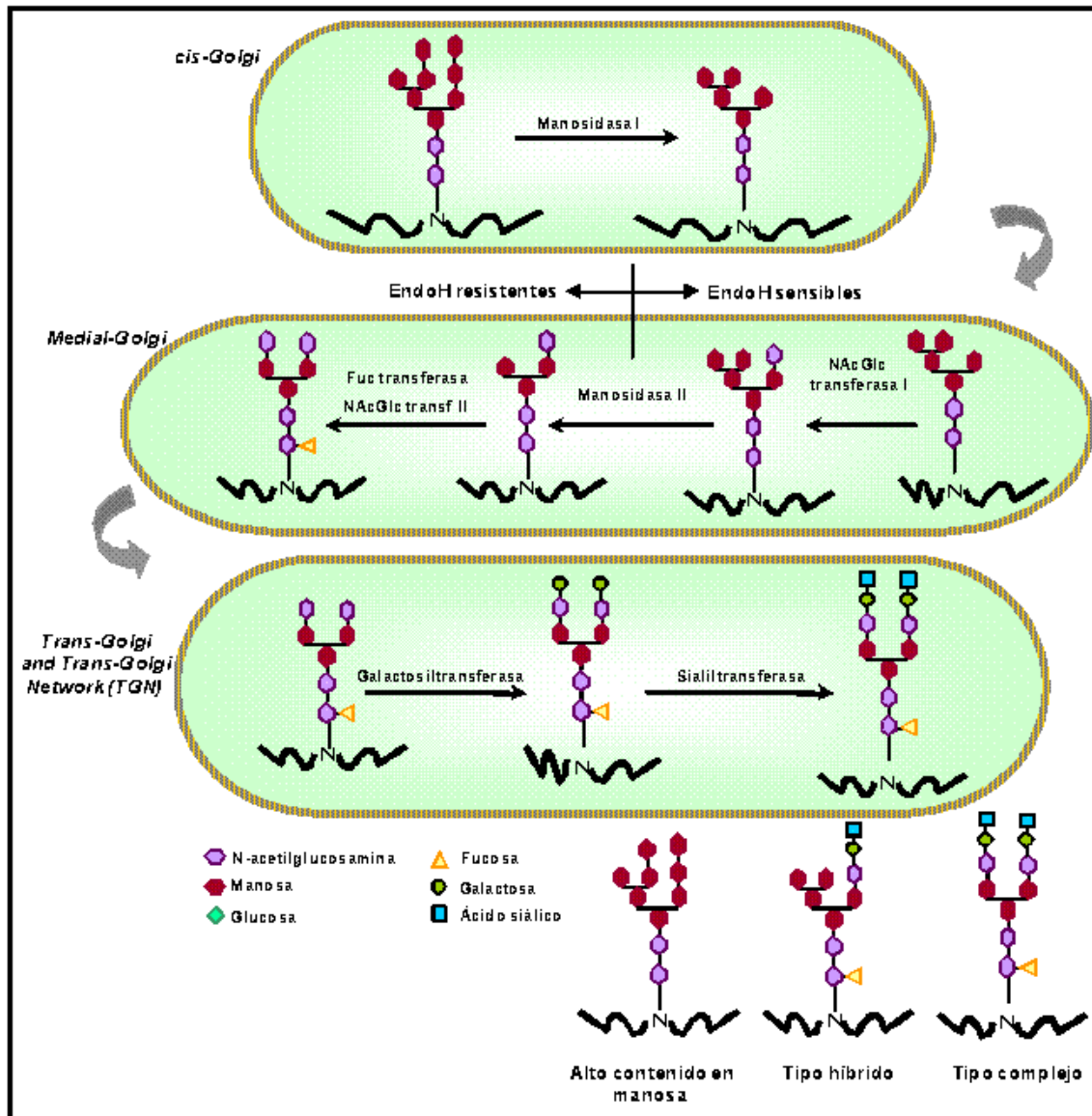


Figura 9. Procesamiento de los oligosacáridos con unión tipo N en el aparato de Golgi.

5. MC1R: RECEPTOR DE MELANOCORTINAS 1.

5.1. Subfamilia de receptores de melanocortinas.

Existen cinco receptores de MCs (MCRs), denominados MC1R a MC5R, que forman una subfamilia de la clase A de la superfamilia de los GPCRs (53). Puesto que las MCs están implicadas en la regulación de procesos fisiológicos clave, en la

actualidad se considera a los MCRs dianas farmacológicas potenciales de primer orden (54).

Los MCRs se acoplan a la adenilato ciclasa a través de la proteína Gs, produciendo la transformación de ATP en AMPc y su acumulación dentro de la célula diana. Existe una alta homología de secuencia entre los MCRs, con un 60% entre MC4R y MC5R, aproximadamente un 45% entre MC3R y MC1R y un 38% de identidad entre MC2R y MC4R (Figura 10). Los MCRs difieren entre sí en su distribución tisular y en su afinidad por las MCs naturales y sintéticas (55) (Tabla 1) y sus antagonistas, la proteína de señalización agouti (ASP) y la proteína relacionada con el gen *Agouti* (AGRP).

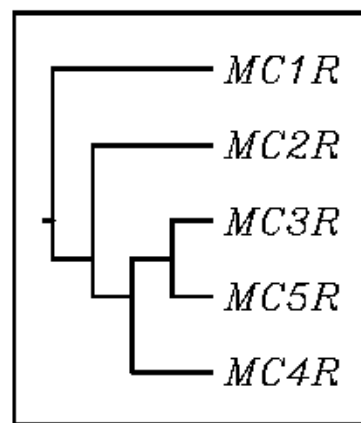


Figura 10. Dendrograma de los MCRs humanos.

- El MC1R fue el primer miembro de la familia de MCRs clonado. Es el receptor para la α -MSH en melanocitos y el que presenta la mayor afinidad por esta MC. El MC1R humano, además, tiene una alta afinidad por la ACTH, menor afinidad por β -MSH y la más baja afinidad por γ -MSH.
- Después de la clonación del MC1R, se clonó el MC2R. Este receptor tiene la característica particular de que sólo se activa en respuesta a ACTH, por lo que se le denomina también receptor de ACTH. Se expresa en la glándula adrenal, donde regula la producción de mineralocorticoides y glucocorticoides.
- El MC3R tiene una afinidad similar para α -, β -, y γ -MSH, y para ACTH. Se expresa en muchas áreas del sistema nervioso central y en varios tejidos periféricos incluyendo el tracto intestinal y la placenta. MC3R parece intervenir en la modulación de funciones del sistema nervioso autónomo, de la inflamación y de la ingesta de alimentos, entre otras.
- El MC4R se expresa predominantemente en el sistema nervioso central. El orden de afinidad por MCs es: α -MSH = ACTH > β -MSH >>> γ -MSH. Entre sus

funciones destacan la regulación de la ingesta de alimentos y la homeostasis de energía.

- El MC5R fue el último en ser clonado. Es similar al MC1R y al MC4R en su capacidad para responder a todas las MCs, excepto a γ -MSH. Se expresa en glándulas exocrinas y en la piel y su principal función parece ser la regulación de las secreciones glandulares.

MCR	Agonistas	Antagonistas	Tejido de expresión mayoritaria
MC1R	α MSH = ACTH > β MSH > γ MSH	ASP	Melanocitos, sistema inmune, queratinocitos
MC2R	ACTH	---	Corteza adrenal
MC3R	α MSH = β MSH = γ MSH = ACTH	AGRP	Sistema nervioso central Macrófagos
MC4R	α MSH = ACTH > β MSH > γ MSH	AGRP, ASP	Sistema nervioso central
MC5R	α MSH > ACTH > β MSH > γ MSH	AGRP	Glándulas exocrinas, linfocitos

Tabla 1. Sistema de los MCRs. Distribución tisular de los MCRs y propiedades farmacológicas.

5.2. Características generales del MC1R.

El MC1R humano es un GPCR relativamente pequeño, con 317 aminoácidos y una homología de secuencia del 60% con el resto de los MCRs. Se expresa en melanocitos y en células de melanoma.

Se ha identificado una isoforma generada por procesamiento alternativo del ARNm (56). Esta isoforma difiere de la forma mayoritaria de 317 aminoácidos en que contiene un fragmento adicional de 65 aminoácidos en el extremo C-terminal, por lo que tiene una longitud de 382 aminoácidos. Es similar a la isoforma mayoritaria en su afinidad por las diversas MCS.

De todos los genes implicados en el control de la pigmentación del pelo y de la piel, el MC1R es el principal determinante de las variaciones normales de tipo de piel y el color del pelo en el hombre. Este receptor regula la cantidad y el tipo de pigmento producido por los melanocitos y es el principal determinante del fototipo de la piel y de la sensibilidad al daño inducido por luz UV (57;58).

El sistema de las MCs, sobre todo α -MSH y ACTH, constituye la principal red neuroendocrina cutánea. En la epidermis humana los MCRs presentes son el MC1R y

el MC5R. MC5R se expresa casi exclusivamente en sebocitos, donde media una respuesta sebotrónica a α -MSH (21). El MC1R se expresa preferentemente en los melanocitos, siendo el mediador exclusivo de las respuestas a MCs de este tipo celular. Sus principales efectos consisten en la activación de la proliferación y diferenciación de los melanocitos, que se manifiesta por un incremento selectivo en la síntesis de eumelaninas (59). Por ello, el MC1R juega un papel central en la fotoprotección, a través de la estimulación selectiva de la eumelanogénesis.

5.3. Estructura del MC1R.

El MC1R es una proteína integral de membrana con las características típicas de los GPCR: un extremo N-terminal, 7 fragmentos TM y un extremo C-terminal extracelular (Figura 11).

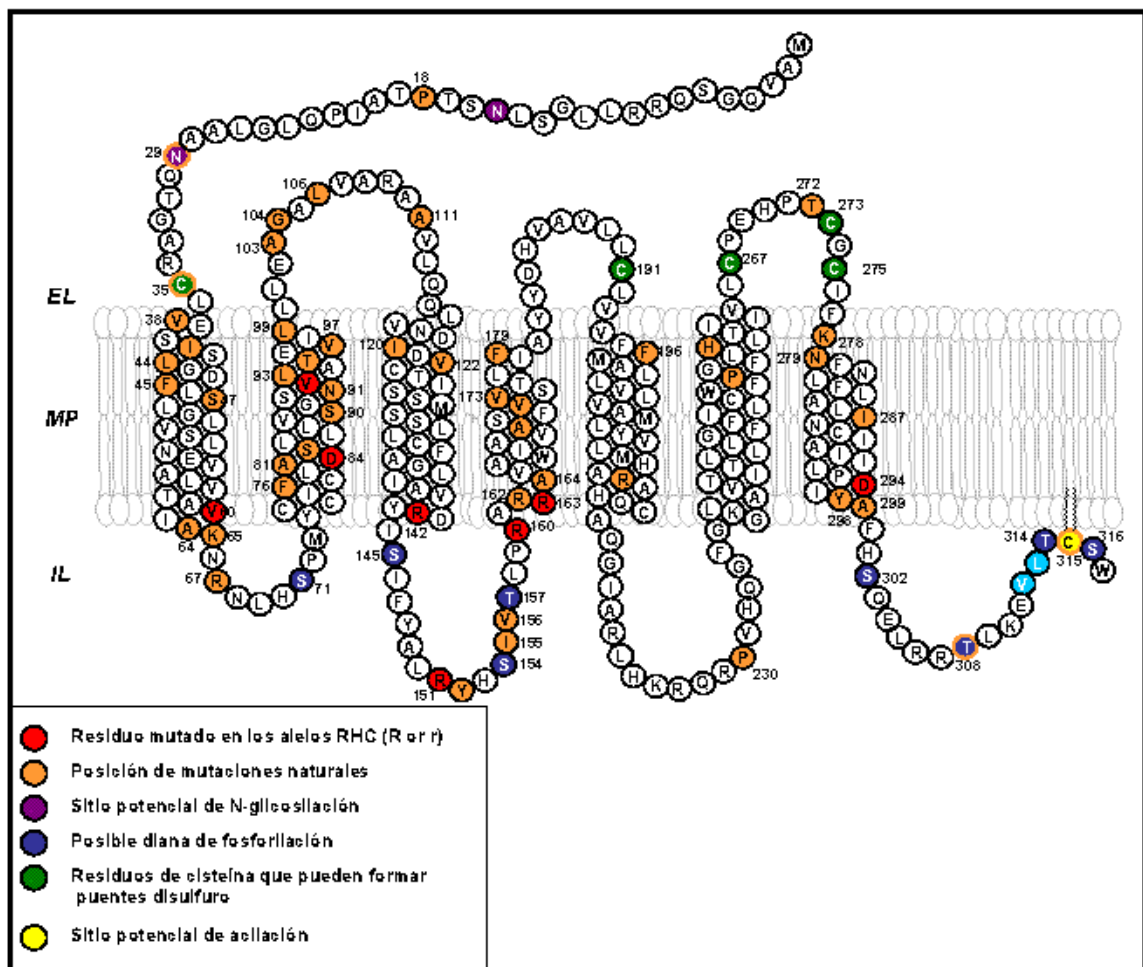


Figura 11: Estructura del MC1R humano según el modelo de Rhingolm et al (60). La secuencia de aminoácidos corresponde al MC1R silvestre (Número de acceso GenBank AF326275). EL, extracelular; MP, membrana plasmática; IL, intracelular.

5.3.1. Extremo N-terminal.

La mayoría de las proteínas de membrana plasmática contienen un péptido señal en el extremo N-terminal que guía la proteína nascente a la membrana del RE y es rápidamente eliminado por un corte proteolítico. Sin embargo, el extremo N-terminal del MC1R no encaja con el consenso descrito para el péptido señal y la eliminación de los 27 primeros aminoácidos no tiene efectos en la unión al ligando o en los niveles de expresión (61). Además, un epítipo Flag unido al extremo amino terminal del MC1R, no es eliminado durante el procesamiento de la proteína en células HEK293T (62). Ello sugiere que el MC1R pertenece a la clase minoritaria de proteínas de membrana que usan el primer fragmento TM como señal de tráfico hacia la membrana plasmática (63).

El MC1R maduro contiene dos posibles secuencias de N-glicosilación en el extremo N-terminal, ¹⁵NTSP¹⁸ y ²⁹NQTG³². Además, el MC1R sufre N-glicosilación como se observa por el desplazamiento de la movilidad electroforética de la proteína tras el tratamiento con endoglicosidasas (64). Sin embargo, no se conoce si ambas secuencias están glicosiladas, aunque la presencia de un residuo Pro en el lado carboxílico de la Gln15, disminuye su potencial de N-glicosilación. Aún no se ha analizado el posible papel de la N-glicosilación del MC1R.

Por otro lado, un residuo de Cys cercano al primer fragmento TM, está conservado en todos los MCRs y es crítico para su función, ya que su mutación a Gly o Ala provoca la pérdida de función del receptor y su retención intracelular en el RE (65). Este comportamiento se debe a que el residuo Cys35 está implicado en la formación de un puente disulfuro intermolecular que contribuye a la dimerización del receptor junto con los puentes disulfuro formados por los residuos Cys267, Cys273 y Cys275 (36).

5.3.2 Los bucles extracelulares.

Los bucles extracelulares del MC1R son pequeños (60;66;67), particularmente el segundo, que es muy corto. Esta característica se ha relacionado con la alta actividad constitutiva que muestran varios miembros de la subfamilia de los MCRs (68) y en particular el MC1R (22). El tercer bucle extracelular de los MCRs está inusualmente conservado y es rico en residuos de Pro y Cys, lo que sugiere que tiene una función altamente especializada (68). Por otra parte, la mutación de los residuos Cys267 o Cys275 a Gly o Ala, provoca la pérdida de función del receptor (65), debido a su participación en puentes disulfuro críticos para el mantenimiento de la estructura

nativa (36). El residuo Cys271 en el MC4R, que equivale al residuo Cys267 en el MC1R, está unido mediante un puente disulfuro al residuo Cys279, que equivale a la Cys275 del MC1R, y se ha descrito una mutación natural C271R, asociada con obesidad mórbida (69). Estos hallazgos, junto a la perfecta conservación de los residuos de Cys presentes en el tercer bucle extracelular en todos los MCRs, sugieren un papel general de los puentes disulfuro en la función de los MCRs.

5.3.3. Los bucles intracelulares.

Los bucles intracelulares (ils) de los GPCRs proporcionan la zona de unión a las proteínas heterotriméricas G y contienen dianas de fosforilación implicadas en la regulación de la señalización, internalización y ciclado de los receptores (70). Dentro del il2 del MC1R humano se han identificado 6 variantes naturales, de las cuales al menos 4 corresponden a formas de pérdida parcial o total de función, lo que indica la importancia de este dominio en la función del MC1R (22). Además, el il2 contiene sitios diana de fosforilación de proteína quinasa A (PKA) conservados y de proteína quinasa C (PKC), aunque aún no se ha determinado si se encuentran fosforilados en condiciones fisiológicas.

5.3.4. Los fragmentos TM y la zona de unión al agonista.

Los fragmentos TM se sitúan aproximadamente perpendiculares al plano de la membrana, en una disposición en sentido contrario a las agujas del reloj. Para los GPCRs de la Clase A, el sitio de unión del ligando es un bolsillo formado por la contribución de varios TM, que se localiza debajo de la interfase entre la membrana plasmática y el medio extracelular. Se han desarrollado diversos modelos tridimensionales para explicar la estructura de los complejos ligando-receptor. Para el MC1R, se ha propuesto que residuos cargados negativamente presentes en el TM2 y TM3 interaccionan con el aminoácido Arg contenido en la secuencia His-Phe-Arg-Trp de las MCs naturales. Además, residuos aromáticos situados cerca del lado extracelular de los fragmentos TM4, 5 y 6, también contribuirían a la unión del agonista mediante su interacción con los residuos aromáticos del farmacóforo (70;71).

5.3.5. El extremo citosólico C-terminal.

El extremo citosólico del MC1R es corto, contiene 19 aminoácidos de los cuales 6 están presentes en todos los MCRs.

Una delección de los últimos cinco aminoácidos del MC1R, que incluye el tripéptido terminal CSW presente en todos los MCRs secuenciados hasta el momento, es suficiente para anular su función (62). Este mutante delecionado es retenido en un compartimento intracelular y se expresa en muy baja densidad en la membrana plasmática. Por tanto, la expresión del MC1R en la superficie celular depende de la integridad de su extremo C-terminal.

Por otro lado, la mutación C315A también afecta a la función del MC1R (62). El mutante C315A muestra un menor número de sitios de unión en la superficie celular en relación al MC1R silvestre. Por lo tanto, la acilación podría ser un determinante importante de la densidad del receptor en la membrana, bien por mejorar el tráfico anterógrado o inhibir la internalización, bien por modificar la estabilidad de la proteína en la membrana plasmática (72). Además, el extremo C-terminal del MC1R contiene residuos de Ser y Thr fosforilables que participan en la regulación del estado funcional del receptor. La importancia reguladora de estos residuos se discutirá más adelante.

5.4. Vías de señalización del MC1R.

El MC1R, activado por unión del agonista α -MSH, señala a través de la activación de la proteína Gs heterotrimérica, que a su vez estimula la adenilato ciclasa. El resultado es un aumento en los niveles intracelulares de AMPc. El AMPc se une a los dos sitios de las subunidades reguladoras de la proteína quinasa A, PKA, permitiendo que las subunidades catalíticas se liberen y se activen. A su vez, PKA fosforila canales iónicos, enzimas y proteínas reguladoras. Además, PKA se trasloca al núcleo donde fosforila la familia de factores de transcripción CREB (proteína de unión a elementos sensibles a AMPc). Una vez fosforiladas, las proteínas CREB activan la expresión de genes específicos que tienen las secuencias consenso CRE (elemento sensible a AMPc) en sus promotores (Figura 12).

El AMPc es, además, responsable de la mayoría de las acciones melanogénicas de α -MSH (73). Éstas incluyen la activación de la enzima TYR y un cambio en la síntesis de feomelaninas a la producción de las eumelaninas, más oscuras y más fotoprotectoras que las feomelaninas (74). La activación de TYR ocurre principalmente a nivel transcripcional y está mediada por la inducción de Microphthalmia (MITF), un factor de transcripción de la familia hélice-bucle-hélice, cuya expresión se activa en respuesta a las proteínas CREB.

MITF se une a la caja M, una región conservada que se encuentra en los promotores de los genes *TYR*, *TYRP1* y *DCT*, y estimula la regulación transcripcional de estas proteínas y la maduración de los melanosomas (75). El resultado final es un

aumento de la actividad de TYR, la enzima limitante en la síntesis de melanina, con el consiguiente aumento de la pigmentación de la piel.

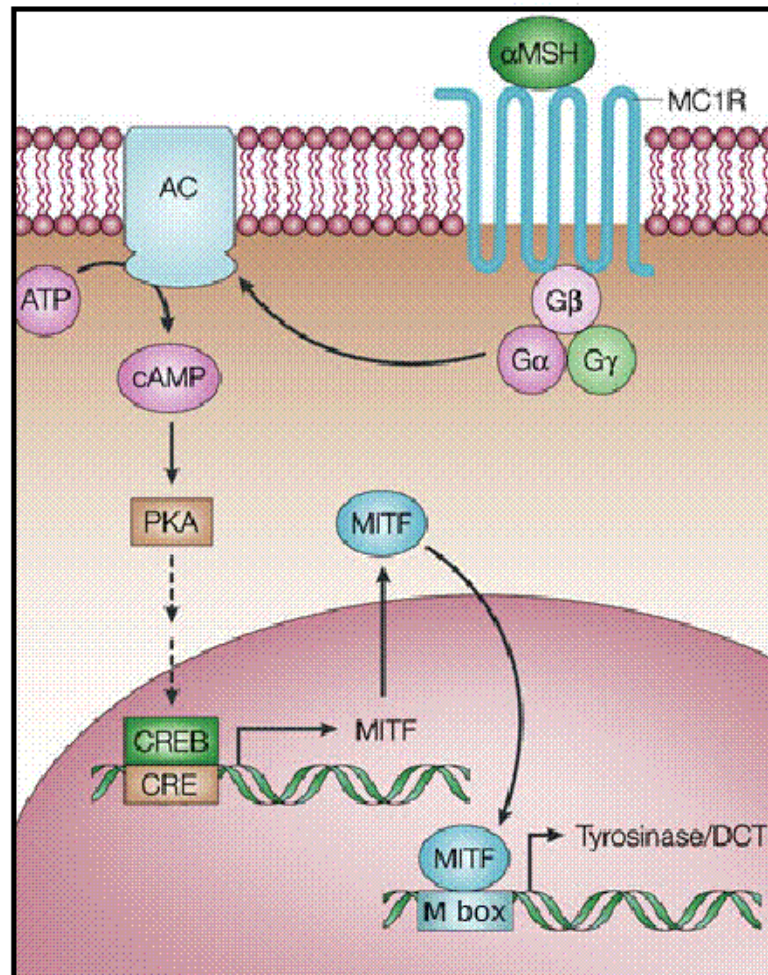


Figura 12. El mecanismo de regulación de la melanogénesis inducida por AMPc implica al factor de transcripción Microphthalmia como transductor de la señal.

Existen algunos datos que permiten suponer que el MC1R utiliza otras vías de señalización además de la cascada del AMPc, pudiendo activar la cascada de las proteínas quinasas activadas por mitógenos (MAPK) ERK1 y ERK2 (73) o de la quinasa de estrés p38 (76). Los mecanismos de activación de estas vías son muy poco conocidos, aunque en melanocitos de ratón se ha propuesto que son dependientes del AMPc. No existen estudios al respecto para el MC1R humano. Además, aunque el efecto de las mutaciones del MC1R relacionadas con cáncer de piel sobre la señalización vía AMPc se conoce relativamente bien, no ocurre lo mismo con posibles efectos sobre su capacidad de la activación de las quinasas mencionadas. También se desconocen las posibles relaciones de entrecruzamiento de las distintas vías de señalización activadas por MC1R. Por último, se sabe

relativamente poco de la regulación de la señalización por MC1R, y los datos disponibles se refieren únicamente a la cascada del AMPc.

5.5. Actividad constitutiva del MC1R.

El MC1R posee actividad constitutiva independiente de agonista (77). Nuestro grupo ha demostrado que en células HEK que sobreexpresan MC1R, las concentraciones basales de AMPc se elevan hasta el 40% de los niveles máximos obtenidos en presencia de agonistas.

En receptores constitutivamente activos, los agonistas inversos compiten con los ligandos activadores por los sitios de unión, y estabilizan la forma inactiva del receptor, disminuyendo la señalización independiente de agonista. Además de los ligandos activadores naturales, se han descrito dos antagonistas para los miembros de la familia de los MCRs, ASP y AGRP. Ambos antagonistas participan en el control de la señalización por MCs (78). La actividad antagonista de ambas moléculas reside en sus dominios C-terminales ricos en cisteínas (79). ASP posee una elevada afinidad para el MC1R, para el que se considera un agonista inverso (22), al menos en el modelo murino.

6. REGULACIÓN DE LA FUNCIÓN DEL MC1R.

La actividad del MC1R está estrechamente regulada. El genotipo del *MC1R* proporciona un primer nivel de regulación, ya que el gen humano es altamente polimórfico con más de 100 variantes alélicas, la mayoría de las cuales corresponden a la sustitución de un solo aminoácido (22;80).

Además de este nivel de regulación, la actividad del MC1R se modula por varios mecanismos que incluyen cambios en la expresión génica, estabilidad del ARNm y eficiencia de traducción o en la velocidad de procesamiento post-traduccional de la proteína y en su tráfico a través de la vía secretora.

Por último, una vez en la membrana plasmática, la actividad del MC1R está controlada por la unión de las MCs, lo que puede conducir a su desensibilización homóloga y a su internalización en vesículas endocíticas, así como por péptidos competidores o agonistas inversos como ASP y AGRP. Algunos de estos tipos de regulación se describen a continuación.

6.1. Variantes alélicas del MC1R.

En la especie humana se han identificado más de 100 variantes naturales del *MC1R*. La tabla 2 incluye una recapitulación de las variantes naturales conocidas hasta el momento (80).

Valverde et al. (81) identificaron por primera vez la relación entre un polimorfismo del *MC1R* y el pelo rojo, la piel clara y la escasa capacidad de broncearse, conocido como fenotipo RHC ("pelo de color rojo").

Las variantes del *MC1R* más fuertemente asociadas con el fenotipo RHC incluyen D84E, R142H, R151C, R160W y D294H, que se han designado como alelos 'R' de alta penetrancia. Estas variantes alélicas se asocian además con un aumento en el riesgo de melanoma y de cánceres de piel de tipo no melanoma (64). Las variantes alélicas V60L, V92M y R163Q tienen una asociación más débil con el fenotipo RHC y se designan como alelos 'r'.

Las variantes R R142H, R151C, R160W y D294H junto con el alelo r V60L, están presentes en el 30% de la población del norte de Europa y corresponden, en conjunto, a más del 60% de todos los casos de pelo rojo (82).

Los alelos R R142H, R151C, R160W y D294H corresponden a formas con disminución de función, pero el grado de disfunción y sus bases moleculares aún no están completamente establecidos. Se ha demostrado una capacidad disminuida pero detectable para estimular la producción de AMPc tras la unión del agonista en el siguiente orden: WT (receptor silvestre) >> R160W ≈ R151C > R142H ≈ D294H. Esto sugería que la disfunción de estos alelos RHC estaba principalmente relacionada con un fallo en la activación de la proteína Gs. Estudios recientes han demostrado una retención intracelular con una reducida expresión en superficie celular para D84E, R151C y R160W, pero no para D294H, por lo que un procesamiento aberrante puede contribuir a la pérdida parcial de función asociada con ciertos alelos RHC (35;83;84).

Hasta el momento, se han descrito algunos análisis funcionales de variantes naturales de baja frecuencia, pero no hay ningún estudio convincente de su asociación con el fenotipo, precisamente a causa de su escasa incidencia. Entre éstos se encuentra el mutante R162P, que tiene un interés particular, ya que corresponde a la primera secuencia descrita como *MC1R* silvestre, aunque estudios posteriores encontraron un residuo Arg en la posición 162 del receptor (56;85). La Pro distorsiona las estructuras helicoidales y, por tanto, una sustitución R/P en la interfase entre il2 y TM3 puede ser relevante funcionalmente. La expresión en células heterólogas demostró que R162P es una variante con pérdida de función completa en la vía del AMPc (86). Además, el receptor R162P se encuentra retenido en un compartimento

intracelular y su expresión en la membrana celular es indetectable. Otra variante natural poco frecuente, L93R, encontrada en una línea celular de melanoma, tampoco responde a NDP-MSH (64). En sistemas heterólogos, el mutante L93R es incapaz de unir agonista y producir AMPc, aunque sus niveles de ARNm son similares a los del MC1R silvestre. La proteína L93R se retiene en un compartimento intracelular, lo que indica un procesamiento aberrante y pérdida de expresión en superficie como causa probable de su pérdida de función (87).

Nuestro grupo, en colaboración con otros laboratorios, ha caracterizado funcionalmente diversas variantes alélicas del MC1R, algunas que no habían sido descritas aún (V51A y C289R) y otras cuya función no se había estudiado todavía (V38M, S41F, M128T y N281S). La mutación N281S corresponde a un polimorfismo silencioso funcionalmente, mientras que las otras mutaciones están asociadas con diversos grados de pérdida de función, desde una disminución moderada en el acoplamiento al AMPc (V38M y V51A) hasta la casi completa ausencia de acoplamiento funcional (S41F, M128T y C289R). Los mutantes M128T y C289R alcanzan la superficie celular en su tráfico anterógrado, pero son incapaces de unir hormona. Los mutantes V38M, S41F y V51A se encuentran retenidos en el RE y su expresión en la superficie celular es muy baja (80).

Por otra parte, experimentos realizados en nuestro laboratorio (35;36) mostraron que el MC1R sufre dimerización constitutiva. La dimerización ocurre en una etapa temprana en la vía biosintética secretora, probablemente en el RE y podría ser necesaria para la llegada del receptor a la membrana plasmática, como ocurre en otros GPCRs (29). Además, mutantes naturales del MC1R son capaces de homodimerizar y heterodimerizar con el MC1R silvestre. La heterodimerización puede causar alteraciones funcionales y, de hecho, se ha demostrado que la cotransfección del MC1R silvestre y un mutante con pérdida parcial de función, da lugar a formas de afinidad alterada y menor eficacia de acoplamiento a la vía del AMPc (35). Por lo tanto, la heterodimerización del MC1R con las formas mutadas puede tener consecuencias funcionales al modular propiedades farmacológicas clave como la afinidad por el agonista o la eficacia de acoplamiento a las proteínas G.

En resumen, el haplotipo MC1R determina características importantes de la pigmentación y constituye un primer nivel de regulación de la actividad del MC1R.

Tabla 2: Variantes naturales del MC1R.

Variante	Mutación	Referencia	Variante	Mutación	Referencia
P18A	CCC→GCC	(Bast aens, 2001)	M128T	ATG→ACG	(Fernandez, 2007; Perez-O va, 2009)
T19	ACA→ATA	(Land, 2005)	R142H (R)	CGC→CAC	(Beaumont, 2007; Sch oth, 1999)
Q23X	CAG→TAG	(McKenze, 2003)	R142C	CGC→TGC	(Matchard, 2004)
N29 ns	nt86_87 nsA	(Beaumont, 2008; Harding, 2000)	P147de	439_441de TTC	(Nakayama, 2006)
C35Y	TGC→TAC	(Fargno, 2003; Zanna, 2008)	R151C (R)	CGC→TGC	(Beaumont, 2005; Sch oth, 1999)
V38M	GTG→ATG	(Fargno, 2003; Perez-O va, 2009)	R151G	CGC→GGC	SNP nked to Gene, GenBank
40T	ATC→ACC	(Jimenez-Cervantes, 2001)	Y152stop	TAC→TAA	(John, 2002)
S41F	TCT→TTT	(Fernandez, 2007; Perez-O va, 2009)	155T (R)	ATC→ACC	(Beaumont, 2007; Box, 1997)
S41 ns	nt123_124 ns T	(Fargno, 2006)	V156L	GTG→CTG	SNP nked to Gene, GenBank
L44V	CTC→GTC	(Fargno, 2003)	T157	No d spon b e	(Nakayama, 2006)
F45L	TTC→CTC	(Pastor no, 2004)	T157A	ACC→GCC	(Scherer, 2008)
S47	AGC→ATC	(John, 2003)	P159T	CCG→ACG	(Nakayama, 2006)
V51A	GTG→GCG	(Fernandez, 2007; Perez-O va, 2009)	P159L	CCG→CTG	(Scherer, 2008)
V60L (f)	GTG→TTG	(Beaumont, 2007; Sch oth, 1999)	R160W (R)	CGG→TGG	(Beaumont, 2005; Sch oth, 1999)
V60G	GTG→GGG	(Land, 2005)	R160Q	CGG→CAG	(Pastor no, 2004)
A64S	GCC→TCC	(Va verde, 1995)	R162P	CGG→CCG	(Garc a-Borron, 2005; Jimenez-Cervantes, 2001)
K65N	AAG→AAT	(Box, 1997)	R163Q (f)	CGA→CAA	(Beaumont, 2007; Box, 1997)
R67Q	CGG→CAG	(Nakayama, 2006; Rana, 1999)	R163X	CGA→TGA	(Scherer, 2008)
R67W	CGG→TGG	(Bran ck, 2007)	A164R	GCC→CGC	(Chhajan, 1992)
R67de	nt200de G	(Land, 2005)	A166G	GCC→GGC	(Nakayama, 2006)
F76Y	TTC→TAC	(Va verde, 1995)	A166 ns	nt496_497 nsG	(Scherer, 2008)
L80P	CTG→CCG	(Gerstenb th, 2007)	A171D	GCC→GAC	(John, 2002)
A81P	GCC→CCC	(Bast aens, 2001)	A171S	GCC→TAC	SNP nked to Gene, GenBank
S83P	TCG→CCG	(John, 2002)	V173de	nt520_523de GT C	(Bast aens, 2001)
S83L	TCG→TTG	(Leonard, 2003)	V174	GTC→ATC	(Bast aens, 2001)
D84E (R)	GAC→GAA	(Beaumont, 2007; Va verde, 1995)	F179 ns	nt537_538 nsC	(Beaumont, 2008; Sturm, 1998)
G89R	GGG→CGG	(McKenze, 2003)	D184H	GAC→CAC	(Land, 2005)
G89A	GGG→AGG	SNP nked to Gene, GenBank	V186M	GTG→ATG	(Scherer, 2008)
G89V	GGG→GTG	(Scherer, 2008)	F196L	TTC→CTC	(John, 2002)
S90T	AGC→ACG	(Chhajan, 1992)	V205M	GTG→ATG	(Scherer, 2008)
N91D	AAC→GAC	(Va verde, 1995)	R213W	CGG→TGG	(Pastor no, 2004)
V92L	GTG→TTG	(Box, 1997)	A218T	GCC→ACC	(Land, 2005)
V92M (f)	GTG→ATG	(Beaumont, 2005; Koppua, 1997; Va verde, 1995)	A222T	GCC→ACC	(Pastor no, 2004)
L93R	CTG→CGG	(Garc a-Borron, 2005; Sanchez, 2002)	R223Q	CGG→CAG	(Land, 2005)
T95M	ACG→ATG	(Va verde, 1995)	P230L	CCG→CTG	(Bast aens, 2001)
V97	GTC→ATC	(Va verde, 1995)	G248V	GGC→GTC	(Land, 2005)
L99	CTC→ATC	(John, 2003)	P256S	CCC→TCC	(John, 2002)
L99V	CTC→GTC	(Scherer, 2008)	H260P	CAT→CCT	(Bast aens, 2001)
A103V	GCC→GTC	(Va verde, 1995)	V265	GTC→ATC	(Pastor no, 2004)
G104S	GGT→AGT	(Bast aens, 2001)	T272M	ACG→ATG	(Chhajan, 1996)
L106Q	CTG→CAG	(Va verde, 1995)	G274S	GGC→AGC	(Scherer, 2008)
R109W	CGG→TGG	(Bran ck, 2007; Bran ck, 2007)	K278E	AAG→GAG	(Bast aens, 2001)
A111V	GCG→GTG	(Pastor no, 2004)	N279S	AAC→GGC	(Bast aens, 2001)
V112M	GTG→ATG	(Land, 2005)	N279K	AAC→AAA	(Bast aens, 2001)
N118K	AAT→AAA/G	(L boutet, 2006)	A299V	GCC→GTC	(Peng, 2001)
120T	ATT→ACT	(Fargno, 2003; Nakayama, 2006)	R306H	CGC→CAC	(L boutet, 2006)
D121E	GAC→GAG	(Scherer, 2008)	T308M	ACG→ATG	(Pastor no, 2004; Sanchez-Laorden, 2007)
V122M	GTG→ATG	(Jimenez-Cervantes, 2001)	C315R	TGC→CGC	(Pastor no, 2004; Sanchez-Mas, 2005)

6.2. Tráfico de MC1R a través de la vía secretora.

Como hemos comentado, algunos mutantes naturales del MC1R presentan defectos en su tráfico anterógrado. El tráfico de los GPCRs comienza en el RE donde son sintetizados, plegados y ensamblados. Los receptores adecuadamente plegados son reclutados y empaquetados en vesículas derivadas del RE cubiertas de COPII. Estas vesículas migran del RE hasta el complejo intermedio RE-Golgi (ERGIC), el aparato de Golgi y la red del Trans-Golgi (TGN). Durante su migración, los receptores sufren modificaciones post-transduccionales hasta alcanzar el estado maduro, y ser finalmente transportados a la membrana plasmática. Una vez en la superficie celular, la estimulación con sus ligandos puede conducir a la internalización de los GPCRs, mediante un mecanismo que implica la fosforilación de los GPCRs por quinasas de receptores acoplados a proteínas G (GRKs), la unión de los receptores fosforilados a las arrestinas y la internalización en vesículas endocíticas, cuyo destino final puede ser la degradación en lisosomas o el reciclaje a la membrana plasmática. El balance entre el tráfico del GPCR a la membrana plasmática y la internalización y degradación determina el nivel de expresión en la superficie celular (Figura 13) (88).

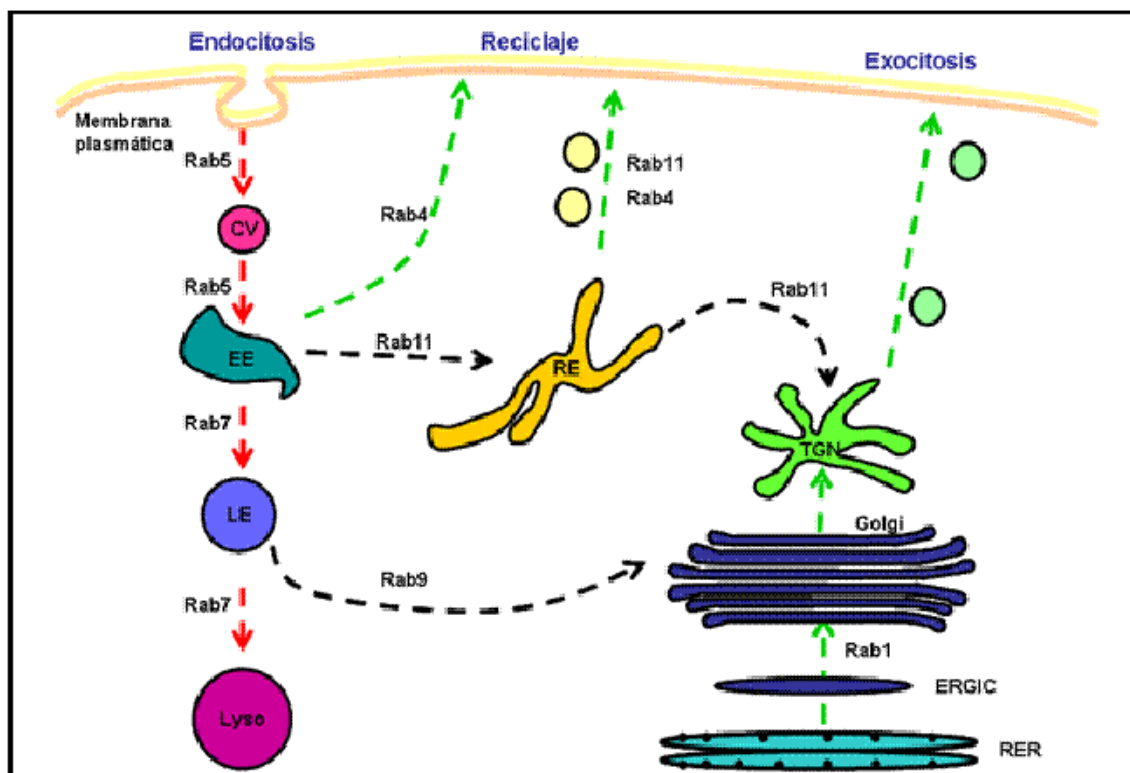


Figura 13. Esquema de las vías que regulan la expresión en membrana plasmática de los GPCRs.

El tráfico de las proteínas de membrana de la vía secretora está regulado por rigurosos mecanismos de control de calidad (QC). Las proteínas incorrectamente

plegadas son retenidas en el RE y, frecuentemente, son retrotraslocadas al citosol y degradadas por el proteasoma. Hasta el momento, se han descrito dos tipos de mecanismos de QC. Un sistema general implica las chaperonas residentes en el RE que controlan características conformacionales como la correcta formación de enlaces disulfuro o la exposición de zonas hidrofóbicas (89;90). Este mecanismo de control reconoce las conformaciones no nativas de las proteínas e impide su tráfico anterógrado hacia la superficie celular. Las chaperonas del RE, proteínas accesorias y proteínas modificadoras de la actividad del receptor (RAMPs) se unen a superficies hidrofóbicas y glicanos inmaduros de proteínas incompletamente plegadas. Las chaperonas del RE mejor caracterizadas que interactúan con los GPCRs son calnexina, calreticulina y BiP (91). Cuando la proteína ha alcanzado su conformación nativa es liberada de la unión a la chaperona para incorporarse a las vesículas de transporte del RE. Si la proteína no se pliega correctamente, las chaperonas permanecen unidas a la proteína e impiden su salida del RE. En este caso, las proteínas mal plegadas suelen ser retrotraslocadas al citosol, ubiquitinadas y degradadas por el proceso de degradación asociada al RE (ERAD) (92).

El segundo y más específico sistema de QC consiste en el reconocimiento de motivos específicos que interactúan con proteínas que ayudan al correcto plegamiento de la proteína o que conducen las proteínas bien plegadas a través del RE y del Golgi (93;94). Así, proteínas incorrectamente plegadas o incluso proteínas bien plegadas pueden ser retenidas en el RE debido a la presencia de motivos de retención (88;93;95).

Tras la salida del RE, los GPCRs son transportados a través del ERGIC, el aparato de Golgi y el TGN. Las proteínas Rab GTPasas son una superfamilia de GTPasas relacionadas con RAS, implicadas en la regulación del tráfico intracelular. Cada proteína Rab tiene un patrón de localización subcelular que se relaciona con los compartimentos entre los que coordina el transporte. Rab1 es una de las GTPasas mejor estudiadas y caracterizadas en el tráfico de la vía secretora. Se localiza específicamente en el RE y en el aparato de Golgi y regula el transporte anterógrado desde el RE hasta y a través del Golgi (95;96). En el tráfico retrógrado, Rab5 regula la internalización de los GPCRs desde la membrana plasmática en vesículas endocíticas denominadas endosomas tempranos (EE) y Rab7 contribuye a dirigir los GPCRs hacia endosomas tardíos (LE) y lisosomas para su degradación. Por otro lado, Rab4 y Rab11 están implicadas principalmente en el reciclaje del receptor internalizado desde EE hasta la membrana plasmática (Figura 13) (97).

Las variantes alélicas del MC1R con pérdida de función R151C y R160W son formas deficientes en su tráfico anterógrado, ya que se encuentran retenidas en el RE

y el aparato de Golgi, respectivamente (84). Además, la retención intracelular de estas variantes es debida probablemente a su incapacidad para sufrir una fosforilación en el residuo Thr157 y la acumulación del mutante R160W en el Golgi estaría causada por la ruptura de un motivo basado en residuos de arginina que funciona como señal de devolución al RE (94).

Además, otras dos mutaciones en residuos próximos a Arg151 y Arg160 tienen efectos fenotípicos consistentes con los cambios presentes en R151C y R160W. La variante artificial T157A se encuentra retenida en el RE, mientras que el mutante natural R162P muestra una acumulación en la cara proximal del Golgi similar a R160W. Estos datos, así como el hecho de que el motivo $^{157}\text{TLPR}^{160}$ es diana de PKC y se fosforila *in vivo*, sugiere un modelo de regulación en la vía secretora, en el que la ruptura de la secuencia consenso $^{157}\text{TLPR}^{160}$ impediría la fosforilación del residuo Thr157, lo que conduciría a una retención intracelular (84). La secuencia $^{157}\text{TLPRARR}^{163}$ (Figura 14) con la diana de fosforilación y el motivo dibásico aparece perfectamente conservada en el hombre, ratón, gorila y en muchos otros vertebrados. Además, la Thr157 es invariable en muchas especies así como en los cinco MCRs humanos, por lo que su estado de fosforilación/defosforilación podría proporcionar un mecanismo general de regulación del tráfico anterógrado de los miembros de esta subfamilia de GPCRs.

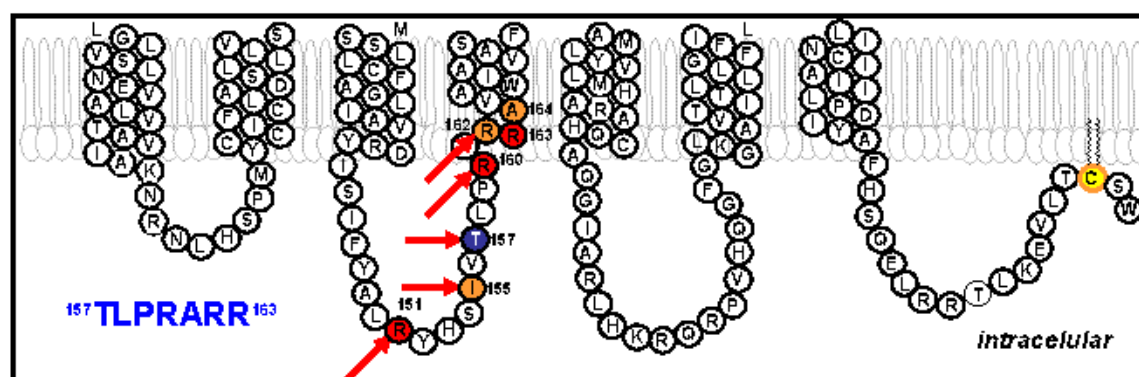


Figura 14. Estructura del bucle intracelular 2 del MC1R humano. En rojo, se señalan los residuos mutados en los alelos RHC, en naranja, la posición de otras mutaciones naturales y en azul, las posibles dianas de fosforilación.

6.3. Desensibilización e internalización del MC1R.

La desensibilización es un mecanismo de regulación de la señalización muy extendido entre los receptores de membrana. La desensibilización homóloga se define como una inhibición transitoria de la respuesta del receptor a sus agonistas y está mediada por una familia de Ser/Thr quinasas conocidas como GRKs, que reconocen el

receptor activado (40;98). El receptor fosforilado interacciona con proteínas de la familia de las arrestinas (ARR), lo que desacopla el receptor de la proteína G y provoca su internalización a través de vesículas de clatrina (Figura 15). Las ARR y otras proteínas de la maquinaria de internalización actúan como adaptadores capaces de aproximar otros componentes, lo que a menudo conduce a la activación de otras vías de señalización como la vía de las MAPKs (99;100).

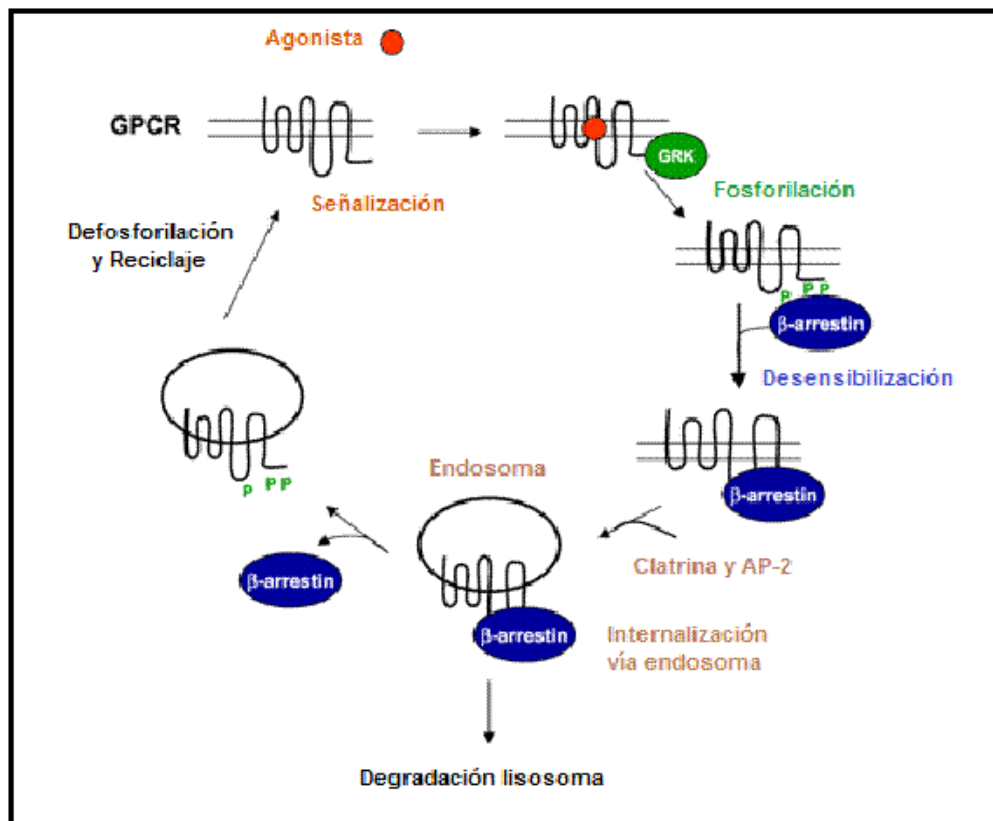


Figura 15. Mecanismo de desensibilización mediada por GRK, internalización dependiente de clatrina, resensibilización y degradación de un GPCR.

El MC1R experimenta desensibilización homóloga tras la exposición a agonistas en células de melanoma en cultivo (101) y en sistemas heterólogos. La desensibilización del MC1R está mediada probablemente por GRK2 o GRK6. GRK6 parece más potente que GRK2 ya que es capaz de inhibir la señalización constitutiva e inducida por agonista del MC1R; mientras que GRK2 sólo inhibe la función dependiente de agonista. Las dianas de fosforilación en el MC1R de GRK2 y GRK6 que regulan la internalización y desensibilización del mismo son los residuos Thr308 y Ser316, localizados en el extremo C-terminal citosólico, de manera que el doble mutante T308A/S316A tiene una tasa de internalización muy reducida (102).

Resultados no publicados de nuestro grupo sugieren que una vez fosforilado, las ARR se unen al MC1R, impidiendo la activación de la proteína G y la señalización.

Las células de melanoma humano expresan las siguientes formas de ARR: ARRB1-201, ARRB2-201 y una nueva forma de ARRB2, designada ARRB2-001 (manuscrito en preparación). Parece ser que esta nueva variante es capaz de unirse al MC1R en condiciones basales, incluso en ausencia de agonista.

Además de interactuar con el receptor fosforilado, las ARR también se asocian a moléculas de clatrina y a una proteína adaptadora (AP2), lo que induce la formación de vesículas de clatrina enriquecidas en el receptor. Tras la acción de la actividad GTPasa de la dinamina, estas vesículas se internalizan o endocitan (103).

En el caso de las variantes alélicas RHC, el número de receptores en membrana plasmática para R151C y R160W es 5 veces más bajo que para el MC1R silvestre, mientras que el D294H expresa un número de receptores en membrana 3 veces mayor que el MC1R silvestre (102). El índice de internalización para el D294H es similar al que se obtiene con el doble mutante T308A/S316A, que tiene bloqueadas las dianas de fosforilación por GRKs y es resistente a internalización. Ello sugiere que su expresión alterada en superficie se pueda deber a su incapacidad para internalizarse. Las variantes R151C y R160W poseen un índice de internalización más alto que el MC1R silvestre, lo que podría contribuir a su baja expresión en superficie, además de su retención en compartimentos intracelulares como consecuencia de su procesamiento aberrante.

7. LA SUPERFAMILIA DE LAS QUINASAS ACTIVADAS POR MITÓGENOS.

7.1. Características funcionales de las MAPKs y clasificación.

Múltiples vías de señalización intracelulares median los efectos proliferativos de los GPCRs. Dentro de estas vías se encuentra la cascada de señalización de las MAPKs. Los miembros de esta familia, una vez activados por fosforilación, se traslocan al núcleo donde fosforilan factores de transcripción, regulando la expresión de genes que tienen un papel importante en el crecimiento celular normal y aberrante (104). Así, las MAPK son capaces de transformar estímulos extracelulares en señales intracelulares, que controlan la expresión génica y, finalmente, regulan la progresión del ciclo celular a través de la fase G1, el desarrollo embrionario, el movimiento celular y la apoptosis, así como la diferenciación celular (105-107).

La vía de transducción de señales que conduce a la activación de las MAPK es uno de los mecanismos más extendidos de regulación de la proliferación y diferenciación de células eucariotas. Todas las células eucariotas tienen varias vías

MAPK, cada una de las cuales se activa por una serie de estímulos, a través de diversas familias de receptores: hormonas y factores de crecimiento, que actúan a través de receptores tirosina quinasa, citoquinas y hormonas, que actúan mediante GPCRs, la familia de citoquinas inflamatorias del factor de necrosis tumoral (TNF), que poseen receptores específicos. Además, las MAPKs se activan en respuesta a situaciones de estrés como choque osmótico, radiación ionizante y daño isquémico.

La superfamilia de MAPK en mamíferos está formada por cuatro familias principales: las proteínas quinasas reguladas por señales extracelulares (ERKs), que incluye ERK1 y ERK2 (también conocidas como p44^{mapk} y p42^{mapk}, respectivamente); las quinasas c-Jun-N-terminal o proteínas quinasas activadas por el estrés (JNK/SAPK); la familia de quinasas p38, el homólogo del gen HOG1 de *Saccharomyces cerevisiae*; y la MAPK grande (BMK, ERK5) (108). Cada una de estas MAPKs incluye a su vez varias isoformas.

Cada familia de MAPK está formada por tres quinasas conservadas que actúan secuencialmente: una MAP quinasa, un activador de la MAP quinasa (MAP quinasa quinasa o MEK), y una MAP quinasa quinasa quinasa (MEK quinasa, MAP3K) (Figura 16).

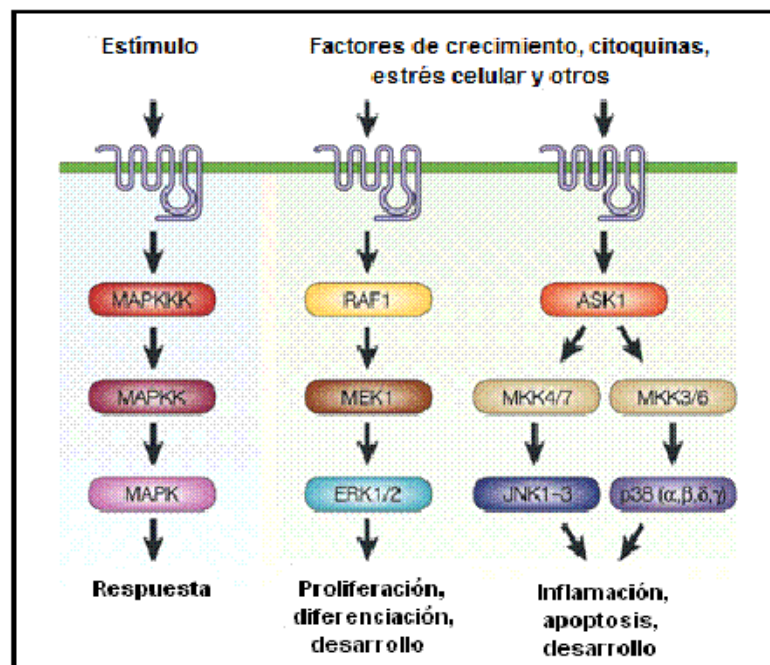


Figura 16. Esquema de tres de las vías de MAPKs en células de organismos superiores (ERKs, JNKs y p38).

Las MAP3K, que son Ser/Thr quinasas, se activan a menudo a través de fosforilación y/o como resultado de la interacción con una proteína pequeña de unión a GTP de la familia Ras/Rho en respuesta a estímulos extracelulares (109). La activación de la MAP3K conduce a la fosforilación y activación de la MAPKK, que a su

vez estimula la actividad MAPK a través de la fosforilación dual en residuos de Tyr y Thr dentro de un motivo conservado de Thr-X-Tyr en el bucle de activación del subdominio quinasa (donde X es Glu, Pro o Gly para ERK, JNK y p38, respectivamente).

Una vez activas, las MAPKs fosforilan sustratos diana en residuos de Ser o Thr seguidos de Pro. Además, la especificidad de sustrato depende a menudo de la presencia de motivos de interacción específicos localizados en los sustratos fisiológicos. La especificidad de la cascada MAPK también está mediada por la interacción con proteínas adaptadoras que organizan las vías en módulos específicos a través de la unión simultánea de varios componentes (110).

Algunos de los sustratos fisiológicos de las MAPKs son proteínas nucleares como factores de transcripción. Las MAPKs también catalizan la fosforilación y la activación de varias proteínas quinasas conocidas como proteínas quinasas activadas por MAPK (MKs). La familia MK comprende las quinasas S6 ribosomal (RSKs), las quinasas activadas por mitógenos y estrés (MSKs), las quinasas de interacción con MAPK (MNKs), las proteínas quinasas activadas por MAPKs 1 y 2 (MK2 y 3) y la proteína quinasa 5 activada por MAPK (MK5). En conjunto, estas quinasas median una gran cantidad de funciones biológicas en respuesta a mitógenos y a estímulos de estrés. Sin embargo, la falta de inhibidores específicos ha dificultado la identificación de sus funciones biológicas exactas.

7.2. Vía de las ERKs.

La vía ERK1/2 de mamíferos, también conocida como cascada clásica de MAPKs, está formada por las MAP3Ks A-RAF, B-RAF y C-RAF (también conocida como RAF-1), las MAPK quinasas MEK1 y MEK2, y las MAPKs ERK1 y ERK2. ERK1 y ERK2 tienen una identidad de aminoácidos del 83% y se expresan en todos los tejidos (111) (Figura 17).

La vía ERK1/2 es uno de los principales reguladores de la proliferación celular. Existen varios inhibidores de esta vía que se usan como agentes contra el cáncer. Dos de los compuestos más usados en células en cultivo, U0126 y PD98059, son inhibidores no competitivos de MEK1/2/5 que impiden la estimulación de ERK1/2/5.

ERK1/2 son capaces de fosforilar sustratos variados en residuos de Ser y Thr que se encuentran próximos a una o varias Pro. Dentro de estos sustratos se encuentran proteínas de membrana (CD120a, Syk y calnexina), proteínas del citoesqueleto (neurofilamentos y paxilina), proteínas citosólicas como fosfolipasa A₂

(PLA₂) y varias subfamilias de quinasas activadas por MAPK, MKs (MSKs, MNKs y RSKs).

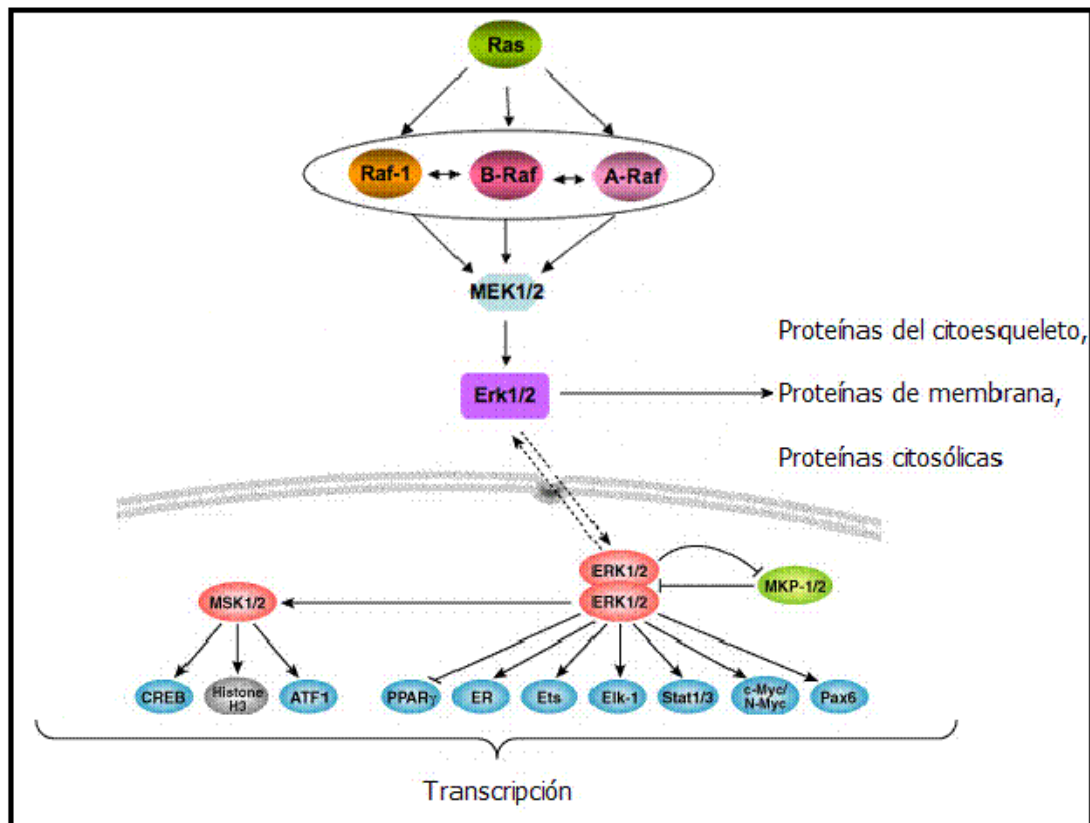


Figura 17. Esquema de los componentes de la vía de ERK1/2 y sus respuestas celulares.

En células quiescentes, las ERKs se encuentran en el citoplasma. Esta localización está mediada por su unión específica a las MAPKKs, MEK1 y MEK2, que se localizan en el citoplasma debido a la existencia de una secuencia NES (señal de salida del núcleo) en su dominio amino terminal (112).

La fosforilación de ERK inducida por el estímulo extracelular conduce a la disociación de ERK de MEK. Una vez activa, ERK disociada se trasloca al núcleo, donde activa por fosforilación diversas dianas nucleares, entre ellas, los factores de transcripción c-Myc, Elk1 y ATF2. ERK1 actúa preferentemente sobre Elk1, factor de transcripción que contiene el dominio "Ets" y que está implicado en la transcripción de genes que promueven la progresión del ciclo celular, mientras que ERK2 muestra preferencia por c-Myc (107).

Además, ERK1/2 son capaces de fosforilar el factor de transcripción Microphthalmia (MITF) en la Ser73 (113). Esta fosforilación aumenta la actividad transcripcional de MITF y, sin embargo, sirve como señal que conduce a su ubiquitinación y degradación en el proteasoma. Este proceso es dependiente de la enzima conjugada con ubiquitina hUBC9. Por tanto, la disminución en los niveles de

MITF conduce a una regulación negativa de la expresión de las enzimas melanogénicas y a una inhibición de la melanogénesis (114).

Tras la traslocación al núcleo, ERK1/2 se inactivan por defosforilación y deben relocalizarse en el citoplasma. Esta relocalización parece depender de un mecanismo de transporte activo basado en la secuencia NES presente en MEK1 y MEK2. MEK entra transitoriamente en el núcleo, se une a la ERK inactiva y la transporta desde el núcleo al citoplasma (112).

La cascada de señalización ERK1/2 se activa por factores de crecimiento, suero y ésteres de forbol y menos frecuentemente por ligandos de los GPCRs, citoquinas, estrés osmótico, y desorganización de los microtúbulos. El mecanismo clásico y mejor conocido de activación de la vía de las ERK es, sin duda, el mediado por los factores de crecimiento. Los factores de crecimiento son pequeñas proteínas o péptidos de señalización intracelular que desencadenan un mecanismo elaborado que se inicia por la dimerización de sus receptores específicos, proteínas con actividad tirosina quinasa (RTK), su autofosforilación y el reclutamiento secuencial a la membrana de las proteínas adaptadoras Shc y Grb2, el factor de intercambio de GTP Sos, la proteína G pequeña con actividad GTPasa RAS y la proteína quinasa C-RAF (RAF-1). Algunos ejemplos de RTK son el receptor de insulina, el receptor del factor de crecimiento epidérmico (EGFR), el receptor del factor de crecimiento de fibroblastos (FGFR) o el receptor del factor de crecimiento de células madre (c-KIT), del que hablaremos más adelante. C-RAF (MAP3K) se activa a nivel de la membrana plasmática para iniciar una cascada de activación y fosforilación de MEK (MAPKK) y ERK (115) (Figura 18).

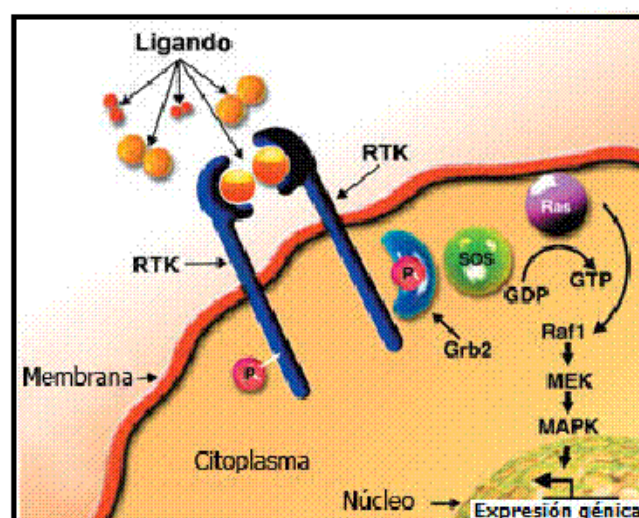


Figura 18. Vía RAS-RAF-MEK-ERK.

Sos es un factor de intercambio de nucleótidos de guanina unidos a RAS citoplásmico (RAS-GEF) que se asocia constitutivamente con la proteína adaptadora

Grb2 y que puede activar a RAS (116). Cuando Grb2 interactúa con un receptor de membrana fosforilado en residuos de Tyr, posiciona Sos en la membrana plasmática donde promueve la activación de RAS.

Las proteínas RAS (H-RAS, K-RAS y N-RAS) son proteínas pequeñas de unión de nucleótidos de guanina, que se encuentran asociadas a la superficie interior de la membrana plasmática (117). Se encuentran inactivas en el estado de unión a GDP y se activan cuando intercambian GDP por GTP. Tras la formación del complejo Grb2/Sos con RAS, se activa el intercambio del nucleótido de RAS, generando la forma RAS-GTP, que permite la transducción de la señal, ya que es capaz de interactuar con una gran variedad de proteínas efectoras, que incluye la familia de proteínas quinasas RAF.

Las proteínas RAF son Ser/Thr quinasas citosólicas. Existen tres isoformas de RAF en mamíferos, A-RAF, B-RAF y C-RAF; todas ellas pueden activar MEK pero cada una realiza funciones distintas. La forma activa RAS-GTP se asocia con el dominio de unión de RAS (RBD) situado en la región reguladora N-terminal de la quinasa. Esto promueve un cambio en la conformación y el reclutamiento de RAF a la membrana, conduciendo a su fosforilación; lo que permite estimular su actividad Ser/Thr quinasa (109). La hidrólisis del GTP unido a RAS por una actividad intrínseca GTPasa relaja la conformación y finaliza la señal.

La regulación de RAS y RAF es crucial para el mantenimiento adecuado de la proliferación celular, puesto que mutaciones activadoras en estos genes conducen a la oncogénesis. De hecho, RAS está mutada en el 30% de los cánceres humanos, mientras que B-RAF está mutada en el 60% de los melanomas malignos (118).

RAF activado se une y fosforila las quinasas con especificidad dual MEK-1 y MEK-2 (MAPKKs), que a su vez fosforilan ERK1/2 dentro del motivo conservado Thr-Glu-Tyr (TEY) en el bucle de activación del subdominio quinasa.

Además de la activación por factores de crecimiento, las MAPKs también se estimulan por otros agentes extracelulares como factores de estrés, hormonas y citoquinas. Muchos de estos ligandos transmiten señales vía GPCRs.

7.3. Mecanismos de activación de las ERK iniciados por GPCRs.

Bajo ciertas condiciones, las MAPKs ERK1/2 pueden activarse a través de componentes de las vías de señalización iniciadas por GPCRs. Los principales mecanismos descritos hasta la fecha se describen a continuación.

7.3.1. Mecanismos dependientes de segundo mensajero.

Las subunidades $G\alpha$ activan la producción de segundos mensajeros que, a su vez, estimulan la actividad de proteínas quinasas que pueden regular la actividad de los componentes de la cascada de las ERK. Los detalles del mecanismo de entrecruzamiento de las dos vías dependen del tipo de proteína G inicialmente activado por el GPCR. Las señales que estimulan receptores acoplados a G_q conllevan la activación de PKC, que puede fosforilar y activar C-RAF (119). En cuanto a los receptores acoplados a la proteína G_s , el AMPc actúa como segundo mensajero para activar PKA y el Ras-factor de intercambio de GTP sensible a AMPc (RAS-GEF), Epac, de una manera independiente de PKA. Cada uno de los componentes puede inducir la activación de la GTPasa pequeña de membrana (smGP) RAP1 que, dependiendo del sistema celular y del nivel de expresión de las isoformas RAF, puede activar o inhibir la cascada de las ERK1/2 (Figura 19). La isoforma C-RAF se expresa ubicuamente, mientras que el nivel de expresión de A-RAF y B-RAF varía según el sistema celular. B-RAF se expresa mayoritariamente en células de origen neural como los melanocitos. Además, la actividad basal de B-RAF es mayor que la actividad basal de C-RAF, y puede ser activada completamente por la unión de RAS-GTP y de RAP1-GTP. En cambio, C-RAF sólo puede ser activada por RAS-GTP.

En unos pocos tipos celulares en los que la isoforma B-RAF se expresa en altos niveles, como células endocrinas y nerviosas como las células de feocromocitoma de rata PC12 (células usadas como modelo de diferenciación de células nerviosas), los melanocitos y células de melanoma y las células tiroideas, el AMPc activa la vía de las ERK. El mecanismo de activación de ERK1/2 inducido por AMPc no se conoce con exactitud. Parece ser que en las células PC12, la activación de ERK1/2 estaría mediada por B-RAF y, además, parece que RAS, RAP1, Src, PKA y 14-3-3 pueden estar implicados en la vía de señalización. En el caso de los melanocitos, se ha sugerido que la vía de activación de ERK por AMPc debe ser similar a la de las células PC12, ya que ambos tipos celulares son de origen neural. La activación de ERK en melanocitos parece implicar muchos factores, aunque el mecanismo exacto no se conoce. Para melanocitos de ratón, se ha propuesto que el AMPc puede activar RAS; aunque el mecanismo de activación de RAS no se conoce y parece que en él no estarían implicados ni Epac, ni PKA, ni el activador clásico de RAS Sos (120).

En la mayoría de células, el AMPc inhibe la vía de señalización de las ERK. En células en las que se expresa sólo o principalmente C-RAF (RAF-1), como fibroblastos

o células hematopoyéticas, la inhibición se piensa que puede ocurrir por distintos mecanismos (Figura 19). Uno de los modelos propuestos sugiere que la smGP RAP1 es activada mediante un proceso dependiente de PKA, secuestrando C-RAF e impidiendo su activación (121). Un modelo alternativo sugiere que PKA inhibe C-RAF por fosforilación directa en el residuo Ser621 (122). El tercer modelo propuesto sugiere también la fosforilación directa de C-RAF por PKA. En este caso, PKA fosforila tres sitios dentro del dominio N-terminal de C-RAF (Ser43, Ser233 y Ser259). Estos tres sitios actúan independientemente bloqueando la activación de C-RAF, lo que asegura que C-RAF está inactivada cuando los niveles de AMPc son elevados. Los tres residuos fosforilados bloquean la interacción de C-RAF con RAS. El residuo Ser43 fosforilado lo hace directamente mediante impedimento estérico, y los residuos Ser233 y Ser259 por reclutamiento de la proteína 14-3-3 en el extremo N-terminal de C-RAF (123;124).

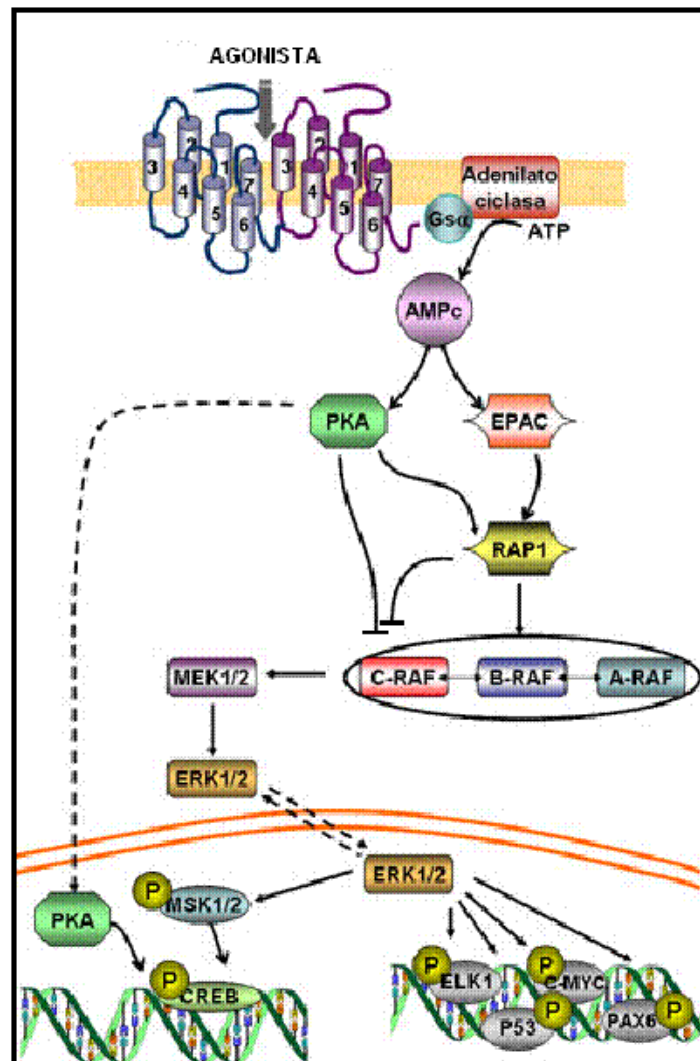


Figura 19. Mecanismo de señalización de ERK mediado por la activación de GPCRs acoplados a Gs.

7.3.2. Activación por el dímero $\beta\gamma$ de la proteína G.

El dímero $\beta\gamma$, tras su disociación de la subunidad α de la proteína G, puede interactuar con la vía de las ERK por diversos mecanismos: por activación de RTKs que reclutan Grb2 y Sos (125); por la activación dependiente de fosforilación de Ras-factor liberador de nucleótidos de guanina (GRF), que conduce a la activación de RAS; por la activación de la proteína quinasa Ser/Thr KSR-1, que inhibe la activación de ERK; y por interacción y activación de PI3K γ . Se han descrito otros mecanismos, como la activación de la quinasa Src, cuyo papel se comentará más adelante, o de PKC (119). Por lo tanto, los posibles efectos del dímero $\beta\gamma$ son complejos y dependerían del tipo celular considerado.

7.3.3. Regulación por proteínas adaptadoras.

Además de su función en la desensibilización e internalización de los GPCRs, se ha demostrado que las ARR actúan como proteínas adaptadoras o de andamiaje que pueden facilitar la activación de la vía ERK1/2 (Figura 20). Tras la fosforilación de los GPCRs por las quinasas de receptores acoplados a proteínas G (GRKs), las ARR se asocian al receptor fosforilado y reclutan los componentes de la cascada ERK1/2: RAF, MEK y ERK. Además, el complejo se internaliza mediante vesículas recubiertas de clatrina. Esta asociación facilita la activación de las ERKs y su retención en el citosol. Por tanto, tras la activación del receptor, las ARR promueven su internalización y la co-localización con los componentes de la cascada ERK1/2 en grandes vesículas endocíticas denominadas "señalosomas" (100;126).

Estos complejos cumplen varias funciones. Por un lado, sirven para aumentar la eficiencia de la señalización entre las sucesivas quinasas en la cascada de fosforilación; aseguran la fidelidad de la señalización evitando el entrecruzamiento con las otras vías de MAPKs y permiten aproximar las MAPKs a ubicaciones subcelulares específicas. Por otra parte, debido a la localización citosólica de las ARR, la formación de complejos estables con ERK1/2 activadas conduce a su retención citosólica y reduce su señalización nuclear limitando el efecto sobre la proliferación celular (24;126;127). Así, la activación de las ERK mediada por ARR tendría menor efecto mitogénico y se relacionaría más bien con un control de la diferenciación celular.

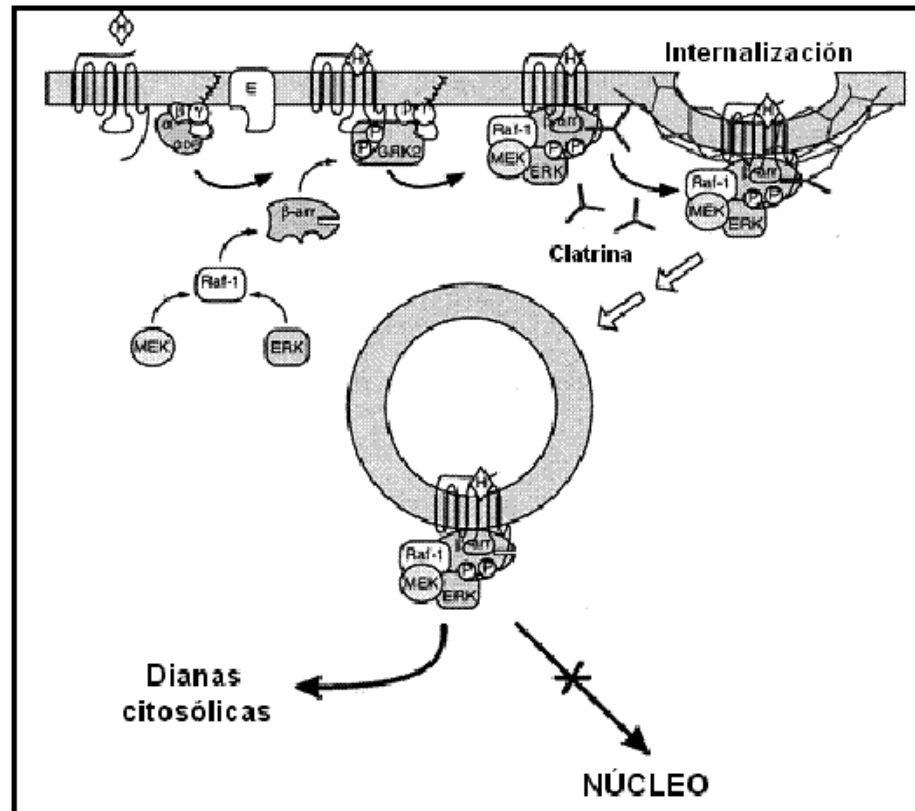


Figura 20. Modelo para la activación de ERK mediado por arrestina.

7.3.4. Transactivación.

Otro posible mecanismo de activación de la vía ERK1/2 se conoce con el nombre de transactivación. El concepto de transactivación se utiliza para designar un fenómeno por el cual un receptor es activado por un ligando de un receptor heterólogo, que posiblemente pertenece a una clase distinta de receptor con respecto al mecanismo de transducción de la señal. Clásicamente, la transactivación se refiere a la activación de un RTK por el ligando de un GPCR, lo que constituye una importante vía de acoplamiento de los GPCRs a la cascada de señalización de las ERKs.

Desde el descubrimiento inicial de varios agonistas de GPCRs capaces de transactivar al EGFR (128), este mecanismo se ha generalizado para otros muchos RTKs, como los receptores de neurotrofinas, del factor de crecimiento derivado de plaquetas (PDGF) y del factor de crecimiento de fibroblastos (FGF), lo que conduce a una variedad de efectos celulares, que incluyen proliferación, diferenciación, migración y supervivencia (129;130).

Hasta el momento, se han identificado dos modelos de transactivación de RTKs por GPCRs: dependientes o independientes del agonista del RTK.

7.3.4.1. Transactivación de RTKs por mecanismos independientes de sus ligandos.

En este caso, el GPCR activado por su propio agonista consigue estimular la actividad tirosina quinasa del RTK sin la participación del ligando de este último. Ello puede conseguirse por varias vías. En algunos casos, se produce una asociación física entre el GPCR y el RTK que va a resultar transactivado a través de la formación de un complejo multiproteico u otras formas de compartimentalización funcional, como el ensamblaje en microdominios de membrana (131). En otros casos, la transactivación se produce por la fosforilación del RTK por componentes que actuarían aguas abajo del GPCR, como tirosina quinasas de la familia de proteínas Src, PKC y PI3K. Por ejemplo, se ha propuesto que las tirosina quinasas Src y Pyk median la activación del EGFR tras la estimulación de algunos GPCR por interacción directa y fosforilación del RTK (132).

La familia de quinasas Src (SFKs) es una subclase de proteínas tirosina quinasas asociadas a membrana, implicadas en una variedad de procesos de transducción de señales, que conducen a diversas respuestas celulares, tales como adhesión, quimiotaxis, supervivencia, proliferación y tráfico proteico (133;134). La familia Src está formada por nueve miembros, de los cuales tres se expresan de forma ubicua, c-Src, c-Yes, y Fyn, mientras que los otros seis, Lck, Hck, Fgr, Lyn y Blk, tienen una expresión más restringida a ciertos tejidos, principalmente en células hematopoyéticas (135).

Estas tirosina quinasas son proteínas de 52-62 kDa. Los miembros de la familia comparten una estructura conservada que consiste en un dominio "único" en la región N-terminal de 50-70 residuos, de alta variabilidad entre los miembros de la familia, pero que siempre contiene señales de modificación lipídica que conduce a miristoilación y, en algunos casos, a palmitoilación, que sirven para anclar las quinasas a la membrana plasmática. A continuación, se encuentra el dominio SH3, que se asocia de forma específica con dominios ricos en residuos de Pro relacionados con la secuencia consenso PXXP. El dominio SH3 está seguido por el dominio SH2, que proporciona interacción con motivos de Tyr fosforilados, presentando la máxima afinidad por el dominio pYEEI. La última región es el dominio quinasa o SH1, responsable de la actividad enzimática.

Como la mayoría de las proteínas quinasas, los miembros de la familia Src requieren la fosforilación dentro de un segmento del dominio quinasa denominado bucle de activación para su completa actividad quinasa. Una característica importante de esta familia es la presencia de dos residuos de Tyr que regulan la actividad de la

enzima de manera opuesta. El residuo de Tyr416 se encuentra fosforilado en el estado activo, mientras que el residuo de Tyr527 está fosforilado en el estado inactivo. En la conformación inactiva, el dominio SH3 interacciona con la región entre el dominio SH2 y el dominio quinasa, rica en residuos de Pro. Esta interacción ayuda a posicionar el bucle de activación en su posición inactiva. Además, el dominio SH2 interacciona con el residuo de Tyr fosforilado del extremo C-terminal (Tyr527), cuya fosforilación es llevada a cabo por la quinasa específica de Src (Csk) o por su homóloga Chk (136). La combinación de ambas interacciones estabiliza la configuración inactiva de la enzima.

La pérdida de fosforilación del residuo Tyr527 conduce a la activación de la actividad catalítica de Src. La autofosforilación del residuo Tyr416 dentro del dominio catalítico también es importante para la regulación de la actividad quinasa. Por tanto, existen múltiples vías para activar la familia Src. Estas vías incluyen el desplazamiento de las interacciones intramoleculares de los dominios SH2 o SH3 por ligandos de alta afinidad, o la modificación de ciertos residuos: defosforilación de pTyr527 por una tirosina fosfatasa o fosforilación de Tyr416. Los distintos miembros de la familia Src pueden ser más sensibles a la regulación por un mecanismo particular que por otro. Además, parece que existen distintos niveles de actividad dentro de la familia Src: i) la forma completamente cerrada inactiva que presenta las interacciones de los dominios SH3/SH2, la cola C-terminal fosforilada y el bucle de activación no fosforilado, ii) una forma parcialmente activa en la que las interacciones SH3 y/o SH2 están desplazadas pero el bucle de activación no está fosforilado y, iii) una forma completamente activa que presenta el bucle de activación fosforilado pero que puede o no presentar las interacciones intramoleculares de los dominios SH3/SH2 (Figura 21).

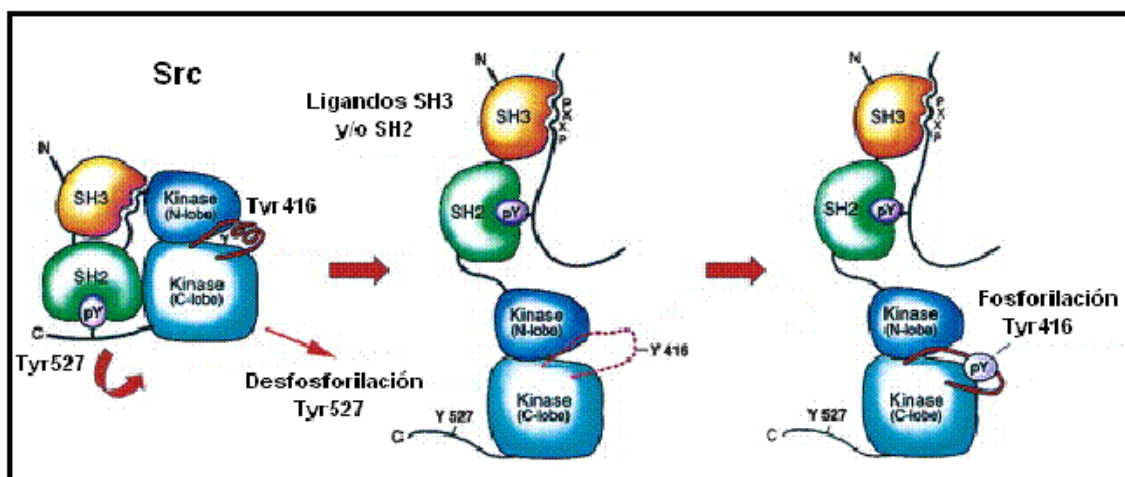


Figura 21. Mecanismos implicados en la activación de las quinasas de la familia Src.

La relación entre las SFKs y los RTKs es compleja y bidireccional. Por un lado, se ha demostrado que la familia Src está implicada en la señalización de muchos RTKs, como el receptor del PDGF (PDGFR), EGFR, FGFR, c-KIT y otros (137). El mecanismo de activación de las Src por los RTKs es variado. Las SFK pueden asociarse con el RTK a través de la interacción de su dominio SH2 con residuos de tirosina fosforilados del receptor activado. Esta asociación libera la interacción intramolecular entre el dominio SH2 y la cola C-terminal, permitiendo que la molécula adopte la conformación catalíticamente activa. Sin embargo, otros estudios sugieren mecanismos de activación distintos. En otros casos, se ha implicado a la Tyr fosfatasa Shp2 en la activación de las quinasas de la familia Src en respuesta a PDGF, EGF y FGF. En el caso de c-KIT, se ha demostrado que la unión de SCF a su receptor conduce a un rápido aumento de la actividad quinasa de las Src. Investigaciones realizadas sugieren que las SFKs se asocian con los residuos fosforilados Tyr568 y Tyr570 de c-KIT.

Por otro lado, las SFKs pueden modular la actividad y la señalización de los RTKs, particularmente de EGFR, PDGFR, c-KIT y de IGFR, mediante fosforilación directa del RTK. Se ha demostrado que las Tyr quinasas Src pueden fosforilar de manera directa y selectivamente la Tyr900 de c-KIT (138). Esta tirosina, que no se fosforila por la actividad quinasa intrínseca de c-KIT, sirve como sitio de unión a CrkII. Además, este residuo de tirosina se corresponde con el residuo Tyr934 de PDGF β -R que también es fosforilado por SFKs, lo que sugiere que estos dos receptores pueden compartir componentes de señalización de la fosforilación dependiente de SFKs.

Como se ha mencionado anteriormente, las SFKs, como Src, Fyn o Yes, pueden ser activadas por ligandos de GPCRs mediante mecanismos heterogéneos. Las SFKs parecen asociarse con y ser activadas por GPCRs, bien a través de la interacción directa con dominios intracelulares del receptor ricos en residuos de Pro, que sirven de sitios de anclaje para el dominio SH3 de Src (139) o a través de residuos de Tyr fosforilados del receptor, que sirven como sitios de anclaje para los dominios SH2 de Src (140); o bien a través de la unión con proteínas asociadas a GPCRs como las subunidades de la proteína heterotrimérica G o las ARR (127;141).

Otro mecanismo de transactivación de RTKs independiente del ligando implica la inactivación de la familia de proteínas Tyr-fosfatasas (PTPs), que controlan la actividad de RTKs y su señalización (142). El peróxido de hidrógeno (H₂O₂) y otras especies reactivas de oxígeno inactivan las PTPs por oxidación de la cisteína catalítica en el sitio activo de estas enzimas. La estimulación de muchos GPCRs conduce a la generación de H₂O₂ (143), lo que puede inactivar las PTPs que controlan

negativamente los RTKs y, por tanto, conducen a la transactivación del RTK (Figura 22).

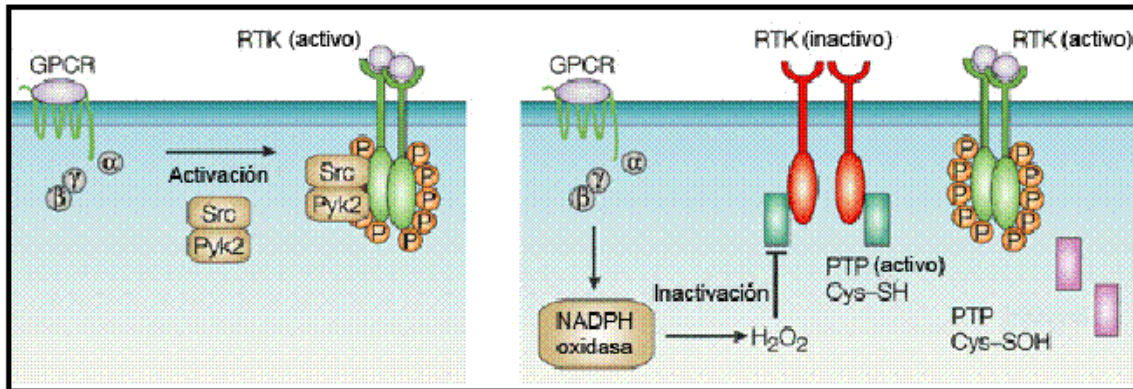


Figura 22. Mecanismos de transactivación de RTKs independientes de ligando.

7.3.4.2. Transactivación de RTKs por mecanismos dependientes de su ligando.

En el segundo modelo de transactivación, dependiente de ligando, la estimulación del GPCR induce la activación de una metaloproteasa de matriz (MMP) o de un componente de la familia de desintegrinas y metaloproteasas (ADAM), que produce el corte proteolítico de un precursor transmembrana del ligando del RTK que, a continuación, actúa uniéndose al dominio extracelular de unión del ligando del RTK, activándolo (Figura 23) (130;144). Este mecanismo se ha demostrado para la activación del EGFR por algunos GPCRs. También podría operar en otros RTK cuyos ligandos están asociados a membranas, como el receptor c-KIT.

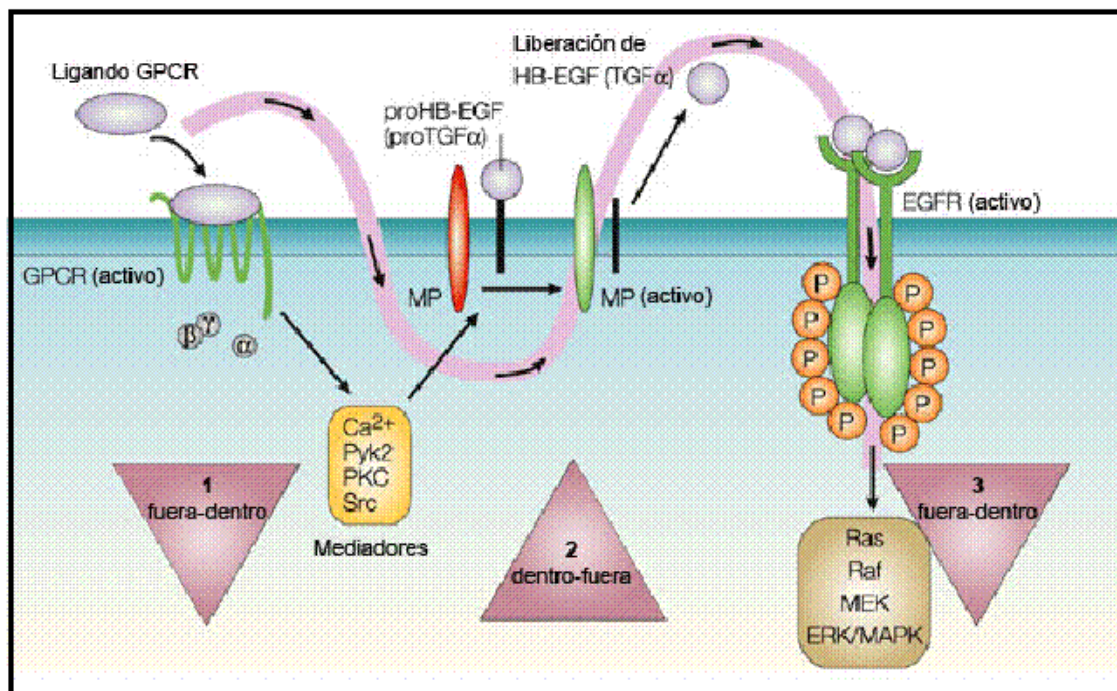


Figura 23. Mecanismo de transactivación de RTKs dependiente de ligando.

7.4. Receptor tirosina quinasa c-KIT.

c-KIT es el receptor con actividad tirosina quinasa del ligando de células madre, SCF ó KL, y juega un papel crítico en la fisiología del melanocito, induciendo melanogénesis, proliferación, migración y supervivencia. c-KIT pertenece a la subclase III de la superfamilia de RTKs. La organización estructural de esta subclase de RTKs se caracteriza por la presencia de una región extracelular con cinco dominios de tipo inmunoglobulina (Ig), un dominio transmembrana y un dominio intracelular, que posee actividad tirosina quinasa.

c-KIT se expresa en mastocitos y células progenitoras hematopoyéticas, mientras que la mayoría de las células hematopoyéticas diferenciadas carecen de expresión de c-KIT. Además, se expresa en melanocitos cutáneos y coroidales. Las mutaciones pérdida de función en c-KIT se asocian con el desorden conocido como piebaldismo. Estas mutaciones conducen a sordera, megacolon y pigmentación defectuosa en pelo y piel. La pérdida de expresión de c-KIT parece estar asociada con la progresión de ciertas formas de melanoma. Aproximadamente el 70% de las lesiones metastáticas y de las líneas celulares de melanoma humano no expresan niveles detectables de c-KIT (145). Sin embargo, hay estudios que han identificado mutaciones activadoras de c-KIT presentes en pacientes con melanoma, como la mutación L576P (146), lo que sugiere que la activación constitutiva de c-KIT regularía positivamente la mitogénesis.

La unión de SCF al dominio extracelular del receptor desencadena una cascada de sucesos intracelulares que comienzan con la dimerización del receptor, seguida de la estimulación de su actividad intrínseca tirosina quinasa y consecuente autofosforilación. Tras la estimulación de la actividad quinasa, se fosforilan residuos de Tyr, que funcionan como sitios de anclaje para moléculas de transducción de señales con dominios SH2 (Figura 24).

La proteína adaptadora Grb2 puede asociarse directamente con Tyr703 y Tyr936 fosforiladas de c-KIT. Además, Grb2 se puede asociar con la Tyr fosfatasa SHP-2 o con la proteína adaptadora ShcA tras la estimulación con SCF. Por otra parte, las quinasas SFK también se unen a c-KIT a través de motivos de fosfotirosina. Algunos estudios han demostrado que la activación de las SFKs, inducida por SCF, es importante para la activación de la cascada RAS-ERK mediada por c-KIT (147). Sin embargo, otros estudios encontraron que la inhibición de SFK no tiene efecto sobre la actividad de ERK, por lo que el papel de las SFK en la señalización de c-KIT permanece por aclarar (148).

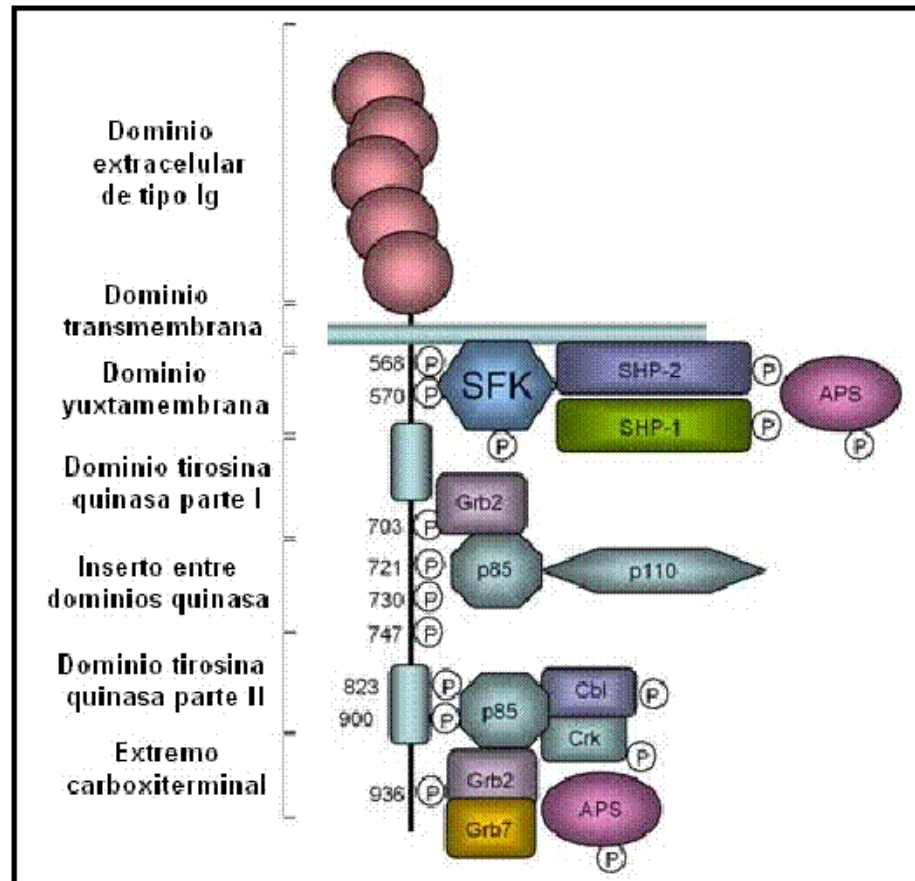


Figura 24. Estructura del receptor c-KIT y moléculas de transducción de señales que se unen al receptor activado.

El ligando de c-KIT, SCF, se expresa como una proteína de membrana altamente glicosilada. La forma biológicamente activa de SCF es un dímero asociado no covalentemente. Su procesamiento alternativo conduce a dos isoformas, que difieren en la ausencia o presencia del exón 6, que se caracteriza por la presencia de un sitio específico de corte proteolítico (149). La isoforma que contiene el sitio de corte sufre proteólisis, generándose una forma soluble (18-kDa), mientras que la isoforma que carece del sitio de corte proteolítico permanece asociada a la membrana (31-kDa). Las formas soluble y asociada a membrana de SCF presentan efectos distintos en la autofosforilación de c-KIT. La estimulación con la isoforma soluble conduce a una activación rápida y transitoria, la autofosforilación de c-KIT, así como su rápida degradación. Por otro lado, la estimulación con la isoforma asociada a membrana conduce a una activación más sostenida (150).

8. MELANOMAS.

8.1. Melanomas: etiología y subtipos.

Existen dos tipos generales de cánceres de piel: El cáncer de piel de tipo no-melanoma (generalmente de células basales y de células escamosas) y el cáncer de piel de tipo melanoma.

El melanoma es un tumor que se origina en los melanocitos, localizados en la lámina basal de la epidermis y en el ojo (151). El único factor ambiental de riesgo conocido es la exposición a la luz UV y en personas con la piel clara el riesgo está aumentado (152). El melanoma es el quinto cáncer más común en hombres y el tercero más común en mujeres por debajo de los 35 años.

Los melanomas constituyen la etapa final de una serie de lesiones a nivel de los melanocitos. Una de las teorías más aceptadas propone que los precursores de los melanomas son las lesiones de la pigmentación conocidas como nevi. La primera etapa sería la transformación de un melanocito normal en un nevus benigno y, luego, en un nevus displásico (153). A continuación, se formaría un melanoma que crece lateralmente en la epidermis, denominado fase de crecimiento radial (RGP). Este melanoma puede ser tratado por cirugía, pero si no se extirpa, puede progresar a la fase de crecimiento vertical (VGP), que se asocia con la invasión de la dermis y la adquisición de capacidad metastásica (Figura 25). Estos melanomas son muy agresivos y pueden extenderse a órganos periféricos como el cerebro y el corazón, y no tienen tratamiento efectivo.

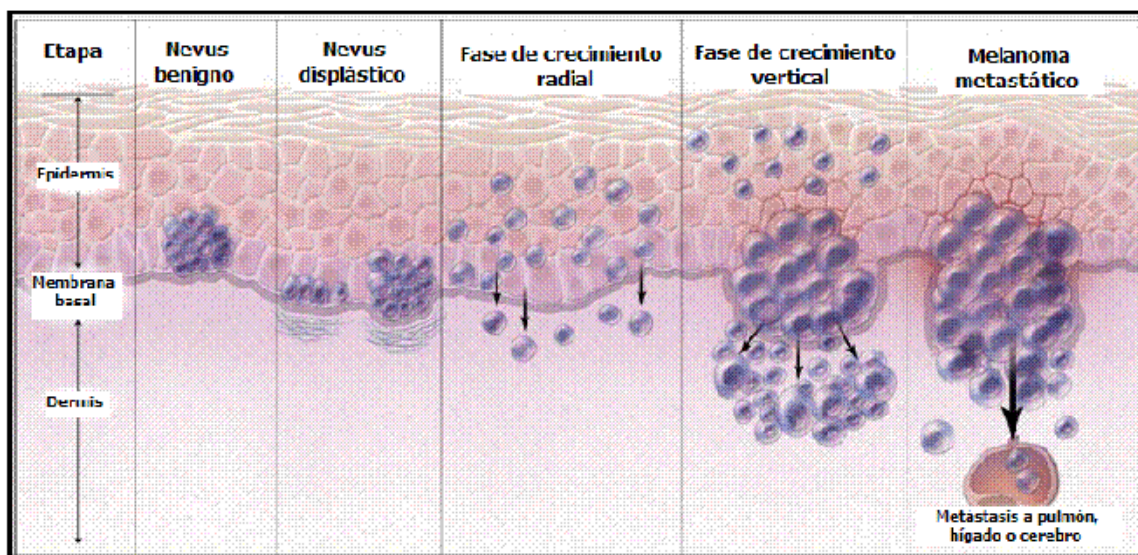


Figura 25. Etapas en la progresión de un melanoma.

La exposición extrema a luz UV (quemaduras de sol) produce daños en el ADN y puede conducir a la muerte de los queratinocitos por apoptosis, pero los melanocitos tienden a sobrevivir debido a su robusta respuesta de reparación frente a daño en el ADN. El cáncer de piel de tipo melanoma se asocia con regiones de la piel sometidas a exposiciones de sol intermitentes pero de alto nivel (154).

Las características morfológicas e histológicas del tumor definen cuatro subtipos principales de melanomas (154):

- Melanoma de extensión superficial: Representa el 70% de todos los melanomas cutáneos y aparece frecuentemente sobre un nevus previo.
- Melanoma nodular: Es el segundo tipo más frecuente (10-30% de todos los melanomas) y forma el grupo clínico más agresivo y con mayor capacidad metastática.
- Melanoma lentiginoso maligno: Se produce en zonas con exposición solar crónica.
- Melanoma lentiginoso acral: Se localiza en zonas no expuestas a la luz solar (palma de las manos, planta de los pies y la región periungueal).

El melanoma es una enfermedad multifactorial. En su etiología, tanto en los casos familiares como en los esporádicos, influyen factores ambientales o exógenos y factores endógenos (155).

8.2. Melanoma familiar: genes de alta penetrancia.

El melanoma familiar es poco frecuente, constituye entre el 8 y el 12% de los casos. Sin embargo, estudios de ligamiento han permitido identificar dos genes implicados en el melanoma familiar que confieren susceptibilidad con alta penetrancia: *CDKN2A/ARF* y *CDK4*. Ambos genes son importantes en el control de la división celular.

El locus *CDKN2A/ARF* codifica para dos proteínas diferentes: p16-INK4A, que interviene en la regulación del ciclo celular a través de la proteína del retinoblastoma (Rb) y p14-ARF, importante en la vía de p53, por lo que ambas tienen un papel fundamental en la regulación de la progresión a través del ciclo celular y la senescencia. La proteína p16-INK4A inhibe la activación de CDK4 y CDK6 por la ciclina D1, impidiendo la fosforilación de Rb1. Rb1 no fosforilada secuestra al factor de transcripción E2F, lo que impide que pueda inducir la progresión de la fase G1 a la fase S del ciclo celular (Figura 26). Por otro lado, la proteína p14-ARF regula la actividad de p53 inhibiendo MDM2, una ubiquitina ligasa que conduce a p53 a la degradación por el proteasoma. Altos niveles de p14-ARF estabilizan p53, permitiendo

que induzca p21, un inhibidor del ciclo celular que bloquea la fosforilación de Rb1 mediada por CDK2/ciclina E (Figura 26). Las mutaciones relacionadas con melanoma familiar pueden afectar a p16-INK4A, p14-ARF o a ambos. Se encuentran mutaciones en el locus *CDKN2A/ARF* en el 25-40% de todas las familias con melanoma (156). La penetrancia de dichas mutaciones está fuertemente condicionada por influencias ambientales, depende de la población, la localización geográfica y la presencia de otros genes modificadores (157).

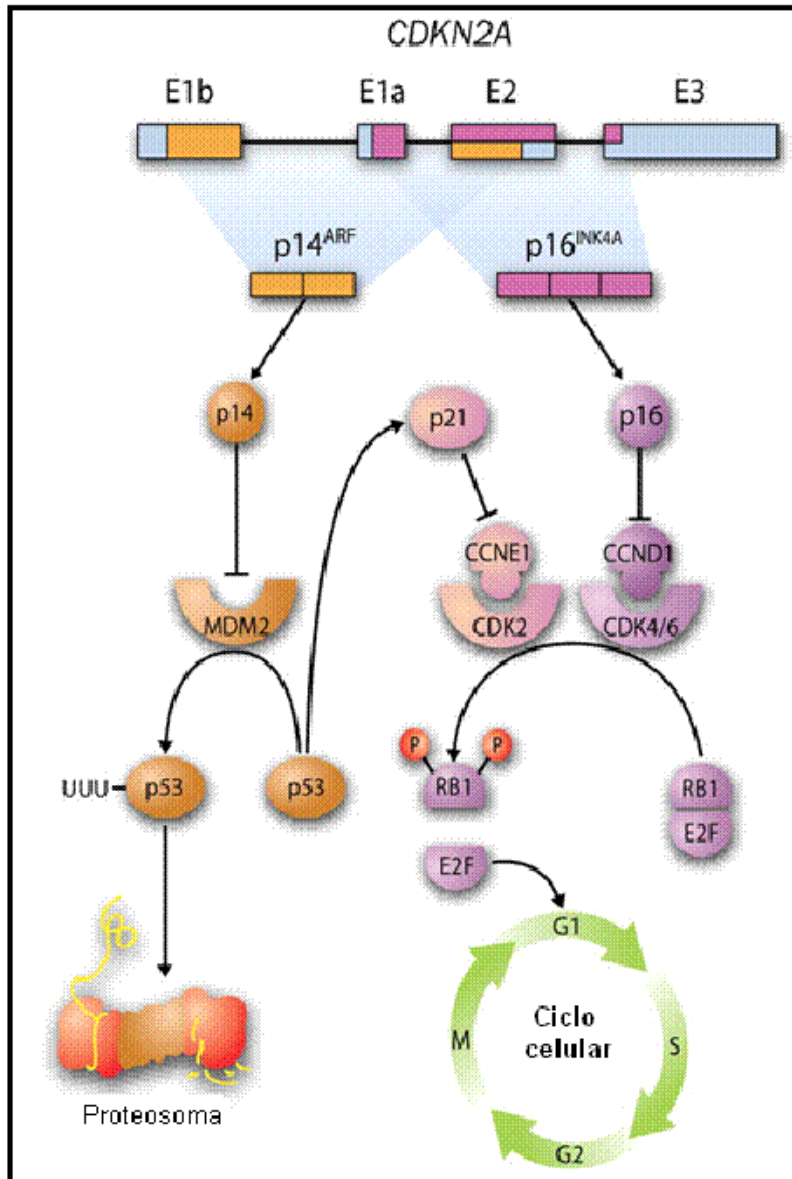


Figura 26. El locus *CDKN2A* y el control del ciclo celular.

El locus *CDK4* codifica la quinasa 4 dependiente de ciclina. Las mutaciones en *CDK4* son muy poco frecuentes, ya que se han identificado muy pocas familias que las presentan. Dichas mutaciones se localizan frecuentemente en la zona de unión a INK4A-p16, resultando en una proteína que no se inhibe por p16-INK4A. Las

mutaciones activadoras en *CDK4* son oncogénicas ya que esta quinasa regula negativamente a la proteína Rb (158-160).

También se han asociado con el melanoma familiar variaciones en otros genes, particularmente en el *MC1R*. Como se ha mencionado anteriormente, el *MC1R* es un gen de susceptibilidad de penetrancia moderada. En estudios epidemiológicos de grandes grupos de población se han encontrado varios alelos como R151C, R160W y D294H que se asocian a un riesgo incrementado de todas las formas de cáncer de piel. Esta asociación es dependiente de la carga alélica, de manera que el riesgo es mayor para portadores de dos alelos mutados (58).

MC1R puede además interaccionar con otros genes. Se ha demostrado que variantes del *MC1R* tienen un efecto modificador en la penetrancia de mutaciones en el locus *CDKN2A*, en familias con antecedentes de melanoma y, aquellas personas portadoras de mutaciones en *CDKN2A* y de variantes en *MC1R* desarrollan melanoma a una edad anterior que aquellos que no poseen dichos polimorfismos (161).

8.3. Melanoma esporádico.

En el melanoma esporádico no son frecuentes las mutaciones en *CDK4* y *CDKN2A* y, sin embargo, son muy comunes las mutaciones en genes que codifican proteínas de la vía de señalización de las ERK. La vía de señalización de ERK está hiperactivada en más del 90% de los melanomas humanos (154). Mientras su actividad es baja en los nevi, está elevada en la fase RGP y en las etapas siguientes del melanoma, lo que sugiere que tiene una importante función en el progreso de la enfermedad.

Los cambios más comunes que conducen a una hiperactivación de esta vía son mutaciones en *B-RAF*, que conducen a una activación constitutiva. Mutaciones en *B-RAF* aparecen hasta en el 70% de melanomas, mientras que aún no se han descrito mutaciones en *A-RAF* y *C-RAF* asociadas a melanoma (162). En los nevi benignos, *B-RAF* se encuentra también frecuentemente mutado, aunque estas lesiones raras veces se transforman en malignas. La mayoría de las más de 20 mutaciones en *B-RAF* son poco frecuentes, debido a que una de ellas, la sustitución de la Val en posición 600 por Glu, es la predominante (163). Además, el residuo Val600 se encuentra mutado a otros aminoácidos, por lo que es el más importante en melanoma y sugiere que las mutaciones oncogénicas activadoras de *B-RAF* promueven la malignización. *B-RAF* mutante es esencial para el crecimiento y mantenimiento del melanoma y estimula la supervivencia y proliferación mediada por MAPK (118;164;165).

La señalización constitutiva de ERK inducida por B-RAF oncogénico puede contribuir a la secreción autocrina de factores de crecimiento como bFGF, la hormona α -MSH, EGF y SCF. Su importancia en melanoma se acentúa conforme progresa la enfermedad y, además, tienen la capacidad de retroalimentar la vía de señalización de RAS. Por último, esto conduce a la pérdida de dependencia de los melanocitos de los factores de crecimiento derivados de queratinocitos, lo que permite a los melanocitos su invasión y supervivencia en la dermis. Por otro lado, formas mutantes de B-RAF son capaces de activar NF- κ B (166), un conocido factor antiapoptótico. Esto conduce a la supresión de la apoptosis, lo que puede contribuir a la quimiorresistencia del melanoma. Además, B-RAF regula la migración celular. En melanoma, B-RAF puede facilitar la invasión regulando la expresión de proteínas como la metaloproteasa de matriz-2 (MMP-2) y β -integrina. Las MMPs degradan la matriz extracelular y, de esta manera, afectan a la invasión y metástasis de las células cancerígenas.

Además de mutaciones en *BRAF*, también son frecuentes mutaciones en *NRAS*, entre el 15 al 30% de los melanomas, mientras que mutaciones en las otras isoformas de RAS (*HRAS* y *KRAS*) son poco frecuentes (162). Al igual que las mutaciones en *BRAF*, mutaciones en *NRAS* producen una activación constitutiva de esta vía de señalización similar cuantitativamente a la asociada a mutaciones en B-RAF. En ausencia del inhibidor del ciclo celular INK4A-p16, RAS oncogénico puede inducir melanoma y parece tener un papel crítico en el mantenimiento del tumor (167). Además, melanomas primarios que aparecen en la piel sin exposición crónica al sol tienen frecuentemente mutaciones en *BRAF* o *NRAS*, mientras que estas mutaciones son poco comunes en tumores de la piel con daño solar crónico o en melanoma acral o de las mucosas, lo que sugiere caminos distintos para la génesis de estos subtipos de melanomas.

Mutaciones simultáneas en N-RAS y B-RAF son muy poco frecuentes en el melanoma, como corresponde a proteínas de la misma vía de señalización. La adquisición de mutaciones en *B-RAF* y *N-RAS* no es la única estrategia que usan las células de melanoma para asegurarse que la vía de las MAPK esté constitutivamente activa. Además de estas alteraciones genéticas, los melanomas frecuentemente no expresan genes que codifican proteínas que regulan negativamente la fosforilación de ERK (168).

8.4. Relación entre el MC1R y el cáncer de piel.

El melanoma es una de las neoplasias con un incremento de incidencia más notable. La incidencia y mortalidad en individuos de raza blanca crecen con más

rapidez que en cualquier otra forma de cáncer, a excepción del de pulmón en mujeres de algunos países, duplicándose cada 10 a 20 años. En España la incidencia actual supone un aumento del 400% en relación con la década pasada. Estudios de población han demostrado que algunas variantes alélicas del *MC1R* se correlacionan positivamente con el riesgo de melanoma y otros carcinomas cutáneos. En los cánceres de piel esporádicos esta asociación ha sido demostrada para alelos del tipo RHC, que doblan el riesgo de melanoma y que se asocian también con un fenotipo de pigmentación feomelánica y elevada sensibilidad a la radiación solar (58). Este fenotipo se corresponde con los fototipos I y II, frecuentes en el Norte de Europa y el Reino Unido. Por contraposición, los individuos con piel oscura, pelo negro, facilidad para broncearse tras exposición al sol y resistencia a las quemaduras solares (fototipos III y IV) son casi siempre portadores del alelo *MC1R* silvestre. Así, *MC1R* es el principal determinante genético del fototipo humano, con un comportamiento casi mendeliano (57;169). Por último, cabe destacar que además de su efecto "per se" como gen de susceptibilidad de baja penetrancia, el *MC1R* es un modificador del riesgo asociado a genes de alta penetrancia, como ya se comentó más arriba. Por tanto, la relación entre *MC1R* y melanoma es compleja y aún poco comprendida.

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OBJETIVOS.

Como se ha mencionado anteriormente, la vía de α -MSH/MC1R es esencial en la homeostasis cutánea. Además, las MCs son moléculas que participan en la regulación de procesos tan importantes y variados como la ingesta de alimentos, la homeostasis energética, el comportamiento sexual, algunas respuestas inflamatorias, la regeneración nerviosa y la función de las glándulas exocrinas, además de la pigmentación melánica y la función de la corteza adrenal. Estas acciones están mediadas por una familia de cinco receptores muy homólogos, para los que el MC1R constituye un buen modelo. Por ello, la caracterización a nivel molecular de la señalización por MC1R puede proporcionar nuevos paradigmas de regulación de la señalización por receptores de MCs, con posibles implicaciones para nuevas terapias de trastornos cutáneos y sistémicos. Sin embargo, este tipo de estudios se ven complicados por el carácter altamente polimórfico del MC1R humano, en el que son frecuentes las variantes alélicas de función desconocida y a menudo con tráfico intracelular alterado. No se conocen las características del acoplamiento funcional de muchas de estas variantes, ni tampoco se comprende el papel de algunos aspectos esenciales del procesamiento del receptor como su glicosilación, como determinantes de su nivel de actividad.

Por otra parte, la relación entre algunas formas alélicas del MC1R con señalización alterada y la susceptibilidad al melanoma está demostrada, pero aún no se comprende la(s) base(s) molecular(es) de la misma. El incremento en la incidencia del melanoma maligno en los últimos años y su estrecha relación con los factores individuales (como el genotipo MC1R) y ambientales (como la exposición a la radiación UV), así como la importancia de un diagnóstico precoz, son datos que justifican la enorme importancia de la investigación de este tumor, tanto a nivel básico como clínico. Por tanto, un mejor conocimiento biológico de esta neoplasia, permitirá avanzar más rápido en el control del melanoma cutáneo. En este contexto, uno de los aspectos cruciales de la biología del melanocito es la señalización por el sistema α -MSH/MC1R y su relación con vías de transducción de señales inductoras de la diferenciación (y por tanto de la pigmentación y de la fotoprotección) y de la proliferación.

A la vista de estos antecedentes y en el contexto de la relación de MC1R con cáncer de piel, proponemos los siguientes objetivos concretos:

- 1- Análisis del patrón de glicosilación del MC1R silvestre y algunas de sus principales variantes naturales asociadas a fenotipos cutáneos y a riesgo incrementado de cáncer de piel.
- 2- Caracterización de la función del carbohidrato en el MC1R.
- 3- Efecto de las mutaciones más frecuentes relacionadas con melanoma maligno en la señalización del MC1R por la vía de las ERK. Se analizarán las formas R151C, R160W y D294H, entre otras.
- 4- Estudio del mecanismo de activación de las ERK por el MC1R humano.
- 5- Estudio de las posibles relaciones de entrecruzamiento de las distintas vías de señalización mediadas por MC1R.

MATERIALES Y MÉTODOS.

1. REACTIVOS Y MATERIALES.

El sistema ECL Plus Western Blotting Detection System, [¹²⁵I]-NDP-MSH (2000Ci/mmol), Cyclic AMP [³H]-Biotrak Assay System son de Amersham Pharmacia/Ge Healthcare (Little Chalfont, Inglaterra).

Tris, el ácido etilendiaminotetraacético (EDTA), y el dodecil sulfato sódico (SDS) son de AppliChem GMBH (Darmstad, Alemania).

Los reactivos necesarios para la realización de la electroforesis de proteínas y transferencia Western: acrilamida, bisacrilamida, persulfato amónico, TEMED, Tween 20, Extra Thick Blot Paper Protean XL Size, así como los marcadores de peso molecular de proteínas fueron suministrados por Bio-Rad (Richmond, CA, USA).

El medio de montaje de inmunofluorescencia es de DAKO Corporation (Carpinteria, USA).

Los estándares de peso molecular de ADN, las enzimas EcoTaq DNA polimerasa, la Pfu DNA polimerasa y las endonucleasas de restricción son de Fermentas (Barcelona, España).

La Lipofectamina 2000, las células químicamente competentes Library Efficiency DH5 α , la enzima T4 DNA ligasa, los anticuerpos marcados con fluoróforos Alexa 488-AntiRabbit conjugate y Alexa 568-AntiMouse conjugate, el vector de expresión pcDNA3.1 y el kit de síntesis de cDNA SuperScript First-Strand Synthesis System for RT-PCR son de Invitrogen Corporation (Carlsbad, CA, USA).

El etanol y el isopropanol son de Merck (Darmstadt, Alemania).

Immobilion-Ny+ Blotting Membrane es de Millipore (Billerica, MA, USA).

La agarosa, el medio LB agar y SOB son de Pronadisa (Madrid, España).

El Quiaquick Gel Extraction Kit es de Quiagen (Hilden, Alemania).

Las enzimas PNGasa F y Endoglicosidasa H, y todos los cebadores son de Roche Applied Sciences (Mannheim, Alemania).

El Igepal CA-630, la albúmina de suero bovino (BSA), el fluoruro de fenilmetilsulfonilo (PMSF), el ácido bicinconínico, la ampicilina, la kanamicina, el p-formaldehído, el β -mercaptoetanol, Mouse Monoclonal Anti Clone HA7, Anti Flag M2 monoclonal antibody, Anti Flag Rabbit polyclonal, GenElute HP Plasmid Maxiprep Kit, GenElute HP Plasmid Miniprep Kit y la iodoacetamida son de Sigma-Aldrich Co. (St Louis, MO, USA).

El kit QuickChange Site-directed mutagénesis es de Stratagene (La Jolla, CA, USA).

El material de plástico utilizado en los cultivos celulares es de TPP (Trasadingen, Suiza).

El kit DNA Clean & Concentrator™ es de Zymo Research (Orange, CA, USA).

Los productos que no aparecen descritos en esta sección, se especifican en los apartados correspondientes.

2. LINEAS CELULARES, CULTIVOS CELULARES Y PREPARACIÓN DE EXTRACTOS.

Los medios de cultivo empleados fueron: Opti-MEM y DMEM, sin glutamina. Los medios de cultivo, el suero bovino fetal (SBF), la glutamina, la tripsina/EDTA y los antibióticos penicilina y estreptomycin fueron suministrados por Gibco BRL-Life Technologies (Gaithersburg, USA). El antibiótico geneticina G418 fue suministrado por Calbiochem (Damstadt, Alemania).

Se utilizaron distintas líneas celulares de melanoma humano establecidas en el laboratorio del Prof. G. Ghanem, LOCE, Universidad Libre de Bruselas, Bélgica. Estas células se cultivaron en medio HAMF-10 (Invitrogen-Gibco, Paisley, UK), suplementado con 10% de SBF y antibióticos 100 U/ml de penicilina (MP Biomedicals, Asse-Relegem, Belgium), 100 µg/ml de sulfato de kanamicina y 100 µg/ml de estreptomycin (Invitrogen-Gibco).

Las transfecciones transitorias se realizaron con células de feocromocitoma de rata, PC12, con células embrionarias humanas, HEK293T y con la línea de melanoma HBL.

Las células HBL, PC12 y HEK293T se cultivaron con medio MEM modificado por Dulbecco, DMEM, suplementado con un 10% de SBF, 2 mM de glutamina, 100 U/ml de penicilina y 100 µg/ml de sulfato de estreptomycin.

Los cultivos primarios de melanocitos normales humanos se obtuvieron a partir del prepucio de neonatos en el laboratorio de la Prof. Z. Abdel-Malek, Universidad de Cincinnati, USA. Las células fueron cultivadas en medio MCDB 153 (Sigma-Aldrich Co.), suplementado con 5% de SBF, 13 ng/ml de extracto de pituitaria bovina (BPE), 8 nM de 12-miristato 13-acetato de forbol (PMA), 5 µg/ml de insulina, 1 µg/ml de transferrina, 1 µg/ml de α-tocoferol y 0.6 ng/ml del factor de

crecimiento de fibroblastos humano (bFGF). El BPE fue eliminado del medio de cultivo 2 ó 3 días antes y durante la duración de cada experimento.

Los diferentes tipos de cultivos celulares fueron mantenidos en un incubador ThermoQuest (Forma Scientific, Marietta, OH, USA) a una temperatura constante de 37° C en una atmósfera saturada de humedad y con un 5% de CO₂. El mantenimiento de dichas líneas celulares se llevó a cabo sembrando 5 x 10⁵ células por botella de 75 cm² en 10 ml de medio de cultivo adecuado. Las células se dejaron crecer 3-4 días hasta un 85% de confluencia. A continuación, se lavaron con PBS (NaCl 137 mM, KCl 2.7 mM, Na₂HPO₄ 10 mM, KH₂PO₄ 1 mM, pH 7.2), y se recogieron por centrifugación 5 min a 1200 xg, tras el tratamiento con tripsina/EDTA (0.5% tripsina, 0.2% EDTA en PBS) en el caso de las líneas celulares de melanoma, melanocitos normales humanos y las células PC12.

Para la extracción proteica, se siguieron dos procedimientos distintos en función de las proteínas que se deseaban detectar.

1. Para la extracción y posterior análisis por transferencia Western de proteínas fosforiladas, las células se lavaron con PBS y se resuspendieron con tampón de solubilización celular I, que contiene PMSF 100 ng/ml, 1% de Igepal y 1% de inhibidores de fosfatasas (imidazol 200 mM, NaF 100 mM, molibdato de sodio 115 mM, o-vanadato de sodio 100 mM y tartrato de sodio dihidratado 400 mM) (Calbiochem) en PBS. A continuación, se centrifugaron a 12000 rpm durante 20 minutos a 4° C en una centrifuga Eppendorf 5415 R. Los sobrenadantes se recogieron para determinar el contenido total de proteína mediante el kit del ácido bicinconínico, utilizando BSA como patrón.
2. Para la extracción y posterior detección de proteínas en presencia del MC1R, las células se lavaron con PBS frío por centrifugación a 2000 rpm, a 4° C durante 10 min. Una vez aspirado el sobrenadante, se solubilizaron en tampón de solubilización celular II (50 mM Tris-HCl pH 8, 1% Igepal, 1 mM EDTA, 0.1 mM PMSF), suplementado con iodoacetamida 10 mM. La iodoacetamida carboximetila los grupos sulfhidrilo de los residuos de cisteína e impide la formación de agregados que alteren el proceso de separación electroforética. Tras la resuspensión, las muestras se incubaron con agitación vigorosa en oscuridad, a 4° C durante 1h. A continuación, se centrifugaron en una centrifuga Eppendorf (30 min a 4° C). Se recogieron los sobrenadantes y se procedió a la cuantificación de la proteína total como se ha descrito anteriormente.

3. ACTIVADORES E INHIBIDORES.

Los activadores e inhibidores usados en los distintos experimentos son los siguientes (Figura 1):

- La hormona fisiológica **α -MSH** y su análogo sintético, **NDP-MSH** (Calbiochem) a una concentración de 10^{-7} M para los experimentos de cinéticas y a concentraciones crecientes de hormona desde 10^{-12} M hasta 10^{-7} M para los experimentos de dosis-respuesta, partiendo en ambos casos de un stock 10^{-3} M en HCl 0.01 N.
- El factor de crecimiento nervioso β humano, **β -NGF** (Calbiochem), se usó a una concentración final de 100 ng/ml.
- El péptido recombinante humano, **SCF** (GenScript), se utilizó a una concentración final de 10 ng/ml.
- El péptido humano endotelina-1, **ET-1** (GenScript), potente ligando de los receptores de endotelina ET_A y ET_B , usado a una concentración final de 10^{-8} M.
- El inhibidor de la MAPK quinasa MEK, **PD98059** (Sigma Chemical Company), a una concentración final de 50 μ M.
- El activador de proteína quinasa C, 12-ministato 13-acetato de forbol, conocido como **PMA** o TPA (Sigma Chemical Company), a una concentración final de 0.1 ng/ μ l.
- El inhibidor selectivo de proteína quinasa C, **Ro-31-8425** (Calbiochem), a una concentración final de 25 nM.
- El activador de la adenilato ciclasa, forskolina (**FSK**) (Calbiochem), a una concentración final de 10^{-5} M, a partir de un stock 10^{-2} M.
- El inhibidor de la adenilato ciclasa, 2',5'-dideoxyadenosine (**DDA**) (Chemical Company), se resuspendió a una concentración de 200 mM en DMSO y, posteriormente, se diluyó para tener una concentración final de 2.5mM.
- El inhibidor de fosfodiesterasas, 3-isobutil-1-metilxantina (**IBMX**) (Sigma Chemical Company), a una concentración de 10^{-4} M.
- El inhibidor de los canales de Ca^{2+} de tipo L, **nifedipine**, (Sigma Chemical Company), a una concentración final de 10 μ g/ml.

- El activador de los canales de Ca^{2+} , **(±)-Bay K8644** (Sigma Chemical Company), se empleó a una concentración de $1\ \mu\text{M}$.
- El activador específico de la proteína de intercambio activada por AMPc (Epac o cAMP-GEF), **8-pCPT-2'-O-Me-cAMP** (Biolog), a una concentración final de $50\ \mu\text{M}$.
- El activador de PKA, **6-Bnz-cAMP** (Biolog), se utilizó a una concentración de $50\ \mu\text{M}$.
- El inhibidor competitivo de PKA de tipo I y II, **Rp-cAMPS** (Biolog), a una concentración final de $20\ \mu\text{M}$.
- El inhibidor selectivo de las $\alpha(1\rightarrow2)$ -manosidasas, 1-deoxymannojirimycin hydrochloride (**1-DMJ**) (Sigma Chemical Company), se empleó a una concentración de $5\ \mu\text{M}$.
- El inhibidor de la N-glicosilación de proteínas que bloquea la transferencia de N-acetilglucosamina 1-fosfato al dolicol monofosfato, **tunicamicina** (Sigma Chemical Company), se utilizó a una concentración final de $5\ \mu\text{g/ml}$.
- El inhibidor de los RTKs, **AG1478** (Calbiochem), a una concentración de $50\ \mu\text{M}$.
- Los inhibidores de la actividad del RTK c-KIT, **ISCK03**, **GTP-14564** (Calbiochem) y **Sunitinib** (Tocris Bioscience), usados a una concentración final de $1\ \mu\text{M}$, $1\ \mu\text{M}$ y $10\ \text{nM}$, respectivamente.
- El inhibidor de los RTK de la clase III, **GTP-14564** (Calbiochem), a una concentración final de $1\ \mu\text{M}$.
- El inhibidor selectivo del RTK EGFR, **PD153035** (Calbiochem), usado a una concentración final de $0.1\ \mu\text{M}$.
- Los inhibidores selectivos de la familia de proteínas tirosina quinasa Src, **PP2** y **NA-PP-1** (Calbiochem), usados en experimentos de dosis-respuesta a concentraciones crecientes desde $0.01\ \mu\text{M}$ hasta $10\ \mu\text{M}$ y desde $0.1\ \mu\text{M}$ hasta $1\ \mu\text{M}$, respectivamente.
- El inhibidor de la familia de proteínas tirosina quinasa Src, así como inhibidor de varios RTK como c-KIT, **Dasatinib** (Biotang Inc), usado a una concentración de $10\ \text{nM}$.

- El inhibidor de las metaloproteasas (MMPs) 1, 2, 3, 8 y 9, **GM6001** (Millipore), a concentraciones crecientes desde 0.1 μ M hasta 10 μ M.

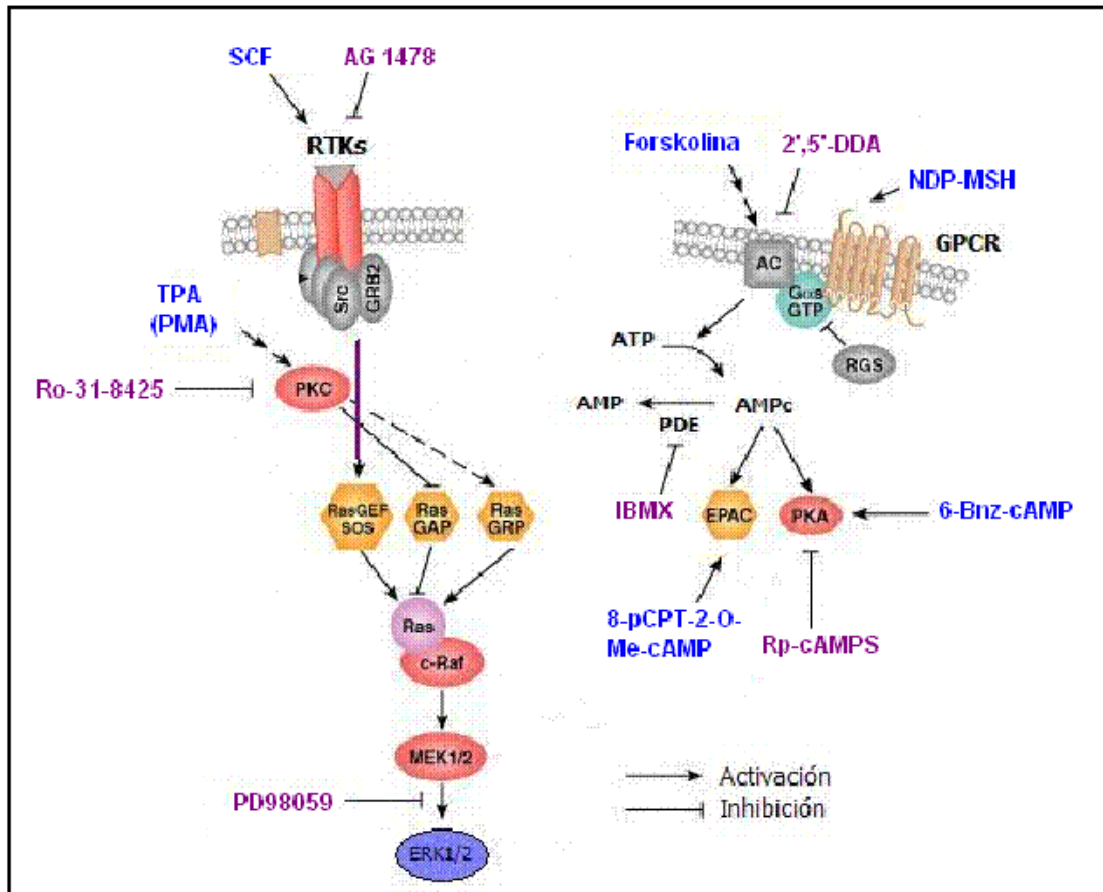


Figura 1. Dianas de algunos de los activadores e inhibidores descritos anteriormente.

4. TRANSFECCIONES TRANSITORIAS.

4.1. Transfección transitoria de ADN plasmídico.

Para las transfecciones transitorias se utilizó el reactivo Lipofectamina™ 2000 (Invitrogen, Carlsbad, CA) a una concentración de 1 mg/ml. En algunos casos, la lipofectamina y el ADN se diluyeron antes de la formación de los complejos lipofectamina-ADN en medio Opti-MEM reducido en suero para aumentar la eficiencia de transfección.

Las células se cultivaron en placas de 6, 12 ó 24 pocillos, según las necesidades. Cuando las células alcanzan una confluencia del 85-90%, se procede a la transfección. Para una placa de 12 pocillos, el procedimiento es el siguiente:

El ADN (0.3 $\mu\text{g/pocillo}$) y la Lipofectamina (1.5 $\mu\text{l/pocillo}$) se diluyen por separado en 50 μl de medio DMEM sin suero (protocolo 1) o en medio Opti-MEM reducido en suero (protocolo 2) durante 10 min a temperatura ambiente. Transcurrido este período, el ADN diluido se mezcla con la Lipofectamina diluida (el volumen final es ahora de 100 $\mu\text{l/pocillo}$) y se incuban durante 20 min como máximo, a temperatura ambiente para permitir que se formen los complejos ADN-Lipofectamina 2000. A continuación, se añaden 100 μl de la mezcla a cada pocillo que contiene 300 μl de medio DMEM sin suero (protocolo 1) ó 300 μl de medio DMEM suplementado con 10% de SBF y glutamina 2 mM (protocolo 2). No se añaden antibióticos durante la transfección. Transcurridas 6h, el medio es sustituido por DMEM suplementado con 10% de SBF, glutamina 2 mM, 100 U/ml de penicilina y 100 $\mu\text{g/ml}$ de sulfato de estreptomicina. Las células se procesan entre 24 y 48 h después de la transfección. Para placas de 6 y 24 pocillos, las cantidades y volúmenes utilizados se corresponden con el doble y la mitad de lo mencionado anteriormente, respectivamente.

4.2. Transfección transitoria de ARN pequeño de interferencia.

Para el silenciamiento de la expresión de genes se realizaron ensayos de interferencia de ARN por transfección transitoria con ARN pequeños de interferencia (siRNA). Los siRNAs son moléculas de ARN de 20-25 nucleótidos de longitud, que silencian genes promoviendo la hidrólisis de ARNm cuyas secuencias son exactamente complementarias. Cada molécula de siRNA es incorporada a un complejo multiproteico conocido como RISC (Complejo de Silenciamiento inducido por ARN), que separa las dos hebras de la molécula de siRNA, quedándose una de ellas incorporada en el complejo. Esta hebra es usada como molde para reconocer a la molécula de ARNm lo que, posteriormente, conduce a su degradación por RISC.

La transfección transitoria de los siRNAs se realizó siguiendo el protocolo 2 descrito anteriormente. El siRNA específico para el gen de interés se resuspendió en agua libre de RNAsa hasta una concentración de 10 μM . A continuación, se diluyó por separado el siRNA (4 $\mu\text{l/pocillo}$) y la Lipofectamina (1.5 $\mu\text{l/pocillo}$) con 50 μl de Opti-MEM reducido en suero y se incubó a temperatura ambiente durante 10 min. El procedimiento continúa según se ha detallado en el protocolo 2, pero las células se procesan 48 h después de la transfección. Como control negativo, se

utilizó una mezcla de secuencias de ARN que no conduce a la degradación específica de ningún ARNm celular conocido.

5. ELECTROFORESIS ANALÍTICA DE PROTEÍNAS.

Una vez recogidos los lisados celulares, se mezcló un volumen del sobrenadante conteniendo 20-30 μg de proteína con tampón de electroforesis de proteína (180 mM Tris-HCl, pH 6.8, 15% glicerol, 9% SDS, 0.075% azul de bromofenol, y 7.5% β -mercaptoetanol). La electroforesis se realizó en un gel de acrilamida en presencia de SDS (SDS-PAGE) mediante el sistema discontinuo de Laemmli con algunas modificaciones, con una cubeta Mini Protean II ó III y una fuente de alimentación 3000 Xi, ambos de BioRad. Se prepararon un gel separador de unos 6 cm de altura y 0.75 cm de grosor de un 8-12% de acrilamida-bisacrilamida, según el tamaño de las proteínas de interés; y un gel hacinador de 1 cm de altura y 0.75 cm de grosor de un 4% de acrilamida-bisacrilamida. La polimerización de los geles está catalizada por los radicales sulfato que se forman a partir de persulfato amónico en presencia de TEMED (N,N,N',N'-tetrametil-etildiamina). Una vez polimerizados los geles, se aplicó en cada calle un volumen de 28 μl de muestra, que corresponden aproximadamente a 25-30 μg de proteína. A continuación, se procedió a la electroforesis usando un tampón de recorrido disociante (Tris 25 mM, Glicina 190 mM, SDS 0.1%, pH 8.3), con una intensidad de corriente de 15 mA/gel durante el avance del frente por el gel hacinador y de 25 mA/gel durante el recorrido por el gel separador.

Tras la electroforesis, la detección específica de las proteínas se realizó mediante la transferencia Western.

6. TRANSFERENCIA WESTERN.

La presencia y cantidad de determinadas proteínas en extractos celulares, se analizó mediante transferencia Western usando anticuerpos específicos (Tabla 1). El gel de poliacrilamida donde se han separado las proteínas, se incubó con un tampón de transferencia (Tris 48 mM, Glicina 39 mM, SDS 0.04%, metanol 20%) durante 5 min. A continuación, se procede a la electrotransferencia de las proteínas a una membrana de PVDF (Immobilon-P) de 0.45 μm de tamaño de poro (Millipore), en una unidad de electrotransferencia semi-seca de Bio-Rad, aplicando un voltaje constante de 22 V.

Cuando la intensidad de corriente desciende desde 0.14 A/gel hasta 0.04 A/gel, aproximadamente 1h, se detiene la electrotransferencia y se bloquea la membrana con 5% de leche desnatada en polvo ó 2-4% de BSA en el tampón correspondiente según el anticuerpo primario que se vaya a utilizar (Tabla 1), en agitación suave (50 rpm) durante 1h. Transcurrido este tiempo, se procede a la incubación con el anticuerpo primario en tampón de bloqueo y se incuba durante toda la noche en agitación suave a 4° C.

Anticuerpo primario	Tampón de bloqueo	Dilución	Anticuerpo secundario	Dilución
α -ERK2 (Santa Cruz)	2% BSA en <u>TGST</u>	1:7500	α -conejo (Millipore)	1:10000
α -pERK1/2 (Santa Cruz)	2% BSA en <u>TGST</u>	1:5000	α -conejo (Millipore)	1:10000
α -cKIT (Cell signaling)	2% BSA en <u>TGST</u>	1:2000	α -conejo (Millipore)	1:10000
α -pckIT (Tyr721) (Santa Cruz)	2% BSA en <u>TGST</u>	1:2000	α -conejo (Millipore)	1:10000
α -pckIT (Tyr703) (Cell signaling)	2% BSA en <u>TGST</u>	1:2000	α -conejo (Millipore)	1:10000
α -pMARCKS (Cell signaling)	2% BSA en <u>TGST</u>	1:2000	α -conejo (Millipore)	1:5000
α -pTYR(PY99) (Santa Cruz)	5% leche en <u>PBS/Tween</u> 0.01%	1:4000	α -ratón (Promega)	1:10000
α -BRaf (Santa Cruz)	2% BSA en <u>TBST</u>	1:2000	α -conejo (Millipore)	1:10000
α -SCF (Santa Cruz)	2% BSA en <u>TGST</u>	1:2000	α -ratón (Promega)	1:10000
α -pSrc (Tyr530) (Santa Cruz)	2% BSA en <u>TGST</u>	1:2000	α -conejo (Millipore)	1:10000
α -Flag-HRP (Sigma-Aldrich)	5% leche en <u>PBS/Tween</u> 0.1%	1:5000	-----	-----
α -Flag (Sigma-Aldrich)	5% leche en <u>PBS/Tween</u> 0.1%	1:10000	α -ratón (Promega)	1:10000
α -HA-HRP (Sigma-Aldrich)	5% leche en <u>PBS/Tween</u> 0.1%	1:1000	-----	-----

Tabla 1. Anticuerpos utilizados en la detección de proteínas mediante transferencia Western. Se especifica para cada anticuerpo la casa comercial, el tampón utilizado en el

bloqueo de la membrana, que coincide con el tampón de unión del anticuerpo eliminando la leche, la dilución utilizada, el anticuerpo secundario (si es necesario) y la dilución empleada.

Seguidamente, se lava la membrana repetidas veces en tampón de lavado, en agitación vigorosa (100 rpm) y, a continuación, en el caso de necesitar anticuerpo secundario, se realiza la incubación durante 1h con éste. Posteriormente, se realiza el lavado de la membrana con tampón de lavado.

Para la detección específica de la proteína de interés, se usa el método de quimioluminiscencia mejorada o aumentada ECL Plus (Amersham Pharmacia) y la emisión se registra en el aparato de adquisición de imágenes Chemi-Smart3000.

Para comprobar que todas las calles del gel de electroforesis han sido cargadas con la misma cantidad de proteínas, se trata la membrana con NaOH 0.5 M durante 10 min en agitación vigorosa (100 rpm) para despegar los anticuerpos primarios y secundarios. A continuación, se realizan varios lavados con TGS/Tween 20 y se bloquea la membrana con TGS/Tween conteniendo BSA al 2% durante 1h en agitación suave (50 rpm). Posteriormente, se incuba la membrana con el anticuerpo α -ERK2 diluido en el tampón de bloqueo (dilución 1:7500) durante toda la noche en agitación a 4° C. El tratamiento continúa lavando la membrana con TGS/Tween 20 e incubando con el anticuerpo secundario α -IgG de conejo conjugado con la peroxidasa de rábano (dilución 1:10000) diluido en TGS/Tween 20 con BSA al 2% durante 1h. Tras varios lavados, se procede al revelado, tal y como se ha indicado anteriormente.

La intensidad de las bandas correspondientes a la proteína de interés, se corrigió por diferencia con el obtenido para el fondo y los resultados de cuantificación se normalizaron dividiendo el valor para la proteína diana entre el obtenido para ERK2.

7. ENSAYO DE UNIÓN HORMONA-RECEPTOR.

7.1. Ensayos de unión y de competición.

El número de sitios de unión para el agonista del MC1R en la membrana plasmática, así como la afinidad de las distintas variantes de interés del receptor por el ligando, se cuantificó mediante ensayos de unión del radioligando [125 I]-NDP-MSH.

Se realizaron ensayos de unión a una concentración fija de hormona radiactiva de 10^{-10} M (0.1 μ Ci) y ensayos de competición. Estos últimos se basan en

la competición para unirse al receptor presente en la membrana plasmática de células en cultivo, del ligando NDP-MSH a diversas concentraciones, frente a una cantidad fija de [125]-NDP-MSH. La radiactividad asociada a las células representa la cantidad de radioligando unido al receptor, que se relaciona inversamente con la cantidad de receptor unido a NDP-MSH no marcado. Así se pueden construir curvas de desplazamiento que indican el valor de la IC_{50} (Concentración de ligando no marcado que conduce a un 50% de inhibición de la unión del radioligando), relacionado directamente con la afinidad del receptor por el ligando. Alternativamente, conociendo la dilución isotópica del trazador radiactivo en cada condición, pueden construirse curvas de saturación, cuyo ajuste proporciona los valores de B_{max} (número total de sitios de unión específicos en la preparación) y de K_d (constante de disociación del complejo ligando-receptor).

Los ensayos se realizaron en placas de 12 pocillos y tras privar a las células de suero al menos durante 3h, se añadió un volumen final de 300 μ l de la dilución isotópica con [125]-NDP-MSH 10^{-10} M y concentraciones crecientes desde 10^{-10} hasta 10^{-6} M de NDP-MSH (competidor no marcado). Tras 1h de incubación, se realizaron 2 lavados con DMEM y posterior recogida de las células con tripsina, en tubos de plástico. La hormona radiactiva unida al receptor, se contó en cpm en un contador gamma (Wizard 1470 Automatic Gamma Counter, Perkin Elmer).

La unión específica para cada concentración de hormona fría utilizada se calculó de la siguiente manera: unión específica = unión total – unión inespecífica. Las cpm correspondientes a la unión inespecífica se obtuvieron a partir de células transfectadas sólo con el plásmido, sin presencia de receptor, e incubadas únicamente con [125]-NDP-MSH o bien células incubadas con el trazador radioactivo en presencia de un gran exceso de ligando nativo (10^{-6} M). Se emplearon pocillos paralelos para estimar la cantidad de proteína por pocillo. Los valores de B_{max} y las constantes de disociación se calcularon por regresión no lineal (hipérbola rectangular) con el programa GraphPad Prism.

7.2. Ensayos de internalización.

Para medir el porcentaje de receptor internalizado en presencia de ligando, se realizaron ensayos de unión de radioligando utilizando una mezcla isotópica de NDP-MSH con una concentración 10^{-9} M y [125]-NDP-MSH con una concentración 10^{-10} M. Tras incubar las células (privadas de suero al menos 3h), con la mezcla durante 90 min, se lavaron 2 veces con DMEM y 2 veces con la solución de lavado ácido a 4 $^{\circ}$ C. Los lavados ácidos liberan el ligando unido al receptor en la membrana plasmática sin lisar las células, por lo que la radiactividad asociada a

estos lavados es indicativa del receptor presente en la superficie celular, no internalizado. Los lavados se recogieron y se midió su radiactividad. Después, se recogieron las células con tripsina, y se contó la radiactividad asociada. El índice de internalización es el porcentaje de radiactividad interna, respecto a las cpm totales (suma de las cpm asociadas a los lavados ácidos y las cpm asociadas al lisado celular).

8. ENSAYO DE ACOPLAMIENTO FUNCIONAL.

La unión del agonista al MC1R produce la activación de la proteína Gs, que a su vez estimula la adenilato ciclasa. El resultado es un aumento en los niveles intracelulares de AMPc. Por tanto, para analizar el acoplamiento funcional del MC1R, se usa el siguiente método de cuantificación de AMPc intracelular.

8.1. Estimulación y extracción de AMPc.

Los ensayos se realizaron en placas de 12 pocillos. Se sembraron pocillos por duplicado y dos pocillos paralelos para determinar la proteína total. Cuando las células se encontraban aproximadamente al 80% de confluencia o 16h después de realizar una transfección transitoria, las células fueron privadas de suero durante un tiempo mínimo de 3h. A continuación, se realizó el tratamiento con el reactivo o inhibidor de interés.

Una vez llevado a cabo el tratamiento, se aspiró el medio de los pocillos y se lavaron con PBS frío. Para liberar el AMPc intracelular al medio, se retiró el PBS de los pocillos y se añadió 200 µl de HCl 0.1 N, previamente calentado a 70° C. Cada pocillo se recogió en tubos eppendorf y éstos fueron liofilizados durante aproximadamente 2h en un liofilizador Speed-Vac Concentrador, SVC100H, Savant. Los precipitados obtenidos se lavaron con 100 µl de agua bidestilada y se secaron durante unos 30 min. Finalmente, cada muestra se disolvió en tampón Tris/EDTA para AMPc en un volumen adecuado según los niveles de AMPc que se esperaba obtener.

8.2. Ensayo de AMPc.

La cantidad de AMPc presente en las muestras se determinó mediante un kit comercial de radioinmunoensayo (Amersham Biosciences), basado en la competencia entre el AMPc no marcado de la muestra y una cantidad fija de [³H]AMPc por una proteína con afinidad por el AMPc. La cantidad de AMPc marcado enlazado por la proteína de unión es inversamente proporcional a la del

AMPc presente en la muestra, que se calcula mediante la extrapolación a partir de una recta de calibrado con concentraciones conocidas de AMPc.

En primer lugar, se enfrían los tubos en hielo y se transfiere a cada uno 50 μ l de muestra. Para la recta de calibrado, se preparan tubos con 0, 2 y 8 pmoles de AMPc, a partir de un stock proporcionado por el kit y para los blancos, se añaden 150 μ l de tampón Tris/EDTA para AMPc. A continuación, se añade a todos los tubos 50 μ l de [H^3]AMPc y 100 μ l de proteína de unión; este último se añade a todos los tubos excepto a los blancos. Todos los tubos se incuban en un baño de agua-hielo durante 2h.

Posteriormente, se añade a cada tubo 100 μ l de carbón activo y se centrifuga a 12000 rpm a 4° C durante 2 min. Así se separa el AMPc unido a la proteína del AMPc libre, puesto que el carbón activo adsorbe el nucleótido libre en el medio. Tras la sedimentación del carbón activo, se transfieren 200 μ l del sobrenadante a viales con 2 ml de líquido de centelleo (Ecoscint, National Diagnostics, Inglaterra). La medida del [H^3]AMPc se realizó mediante un contador de radiación β y los resultados se obtuvieron en dpm. Finalmente, haciendo uso del programa GraphPad Prism y teniendo en cuenta el volumen de tampón usado para disolver las muestras, se calcularon los pmoles de AMPc por μ g de proteína.

9. DETERMINACIÓN DE LA VIDA MEDIA DE PROTEÍNAS.

La determinación de la vida media del receptor MC1R se realizó siguiendo la desaparición de la proteína en células tratadas con el inhibidor de la síntesis proteica, cicloheximida. Las células de interés se incubaron con cicloheximida 10^{-4} M en DMEM completo, durante distintos tiempos desde 0.5 hasta 8h. Las células se lisaron, los extractos celulares se procesaron siguiendo el protocolo 2 descrito anteriormente y la cinética de desaparición de la proteína se analizó por transferencia Western.

10. TRATAMIENTO CON ENDOGLICOSIDASAS.

Para analizar el patrón de glicosilación del receptor MC1R, se llevaron a cabo ensayos de desglicosilación mediante el uso de dos glicosidasas específicas: la endoglicosidasa H (endoH) y la N-glicosidasa F (PNGasa F).

Los extractos proteicos se incubaron a 37° C durante 3h en el tampón de desglicosilación con 2 unidades de PNGasa F o 15 miliunidades de endoH en un

volumen final de 15 μ l. En el caso de las digestiones con PNGasa F, las muestras se incubaron previamente a 95° C durante 5 min para desnaturalizar las proteínas y facilitar la completa desglucosilación.

Tras la incubación a 37° C, se añadió a cada tubo 5 μ l de tampón de muestra (4x), con β -mercaptoetanol. Las muestras se sometieron a SDS-PAGE y la proteína se detectó mediante transferencia Western.

11. EXTRACCIÓN DE ADN GENÓMICO.

Para el aislamiento de ADN genómico de las líneas celulares de melanoma se utilizó el kit DNeasy Blood and Tissue (Qiagen).

En primer lugar, se recogieron las células (un número máximo de 5×10^6 células) mediante tripsinización, se centrifugaron y se resuspendieron en 200 μ l de PBS. Para la lisis celular, se añadieron 20 μ l de la enzima proteinasa K y 200 μ l del tampón que contiene hidrocloreuro de guanidinio. Las muestras se mezclaron mediante agitación vigorosa y se incubaron a 56° C durante 10 min. A continuación, se añadieron 200 μ l de etanol absoluto y se mezclaron hasta obtener una disolución homogénea. La muestra se colocó en un filtro y se centrifugó a más de 6000 xg durante 1 min. Después de realizar sucesivos lavados, el ADN se recuperó mediante centrifugación en 200 μ l de tampón de elución.

Para la cuantificación de la concentración del ADN, se realizó la lectura de la absorbancia a 260 nm mediante un espectofotómetro GeneQuant.

12. EXTRACCIÓN DE ARN.

Para la extracción del ARN total, se utilizó el kit comercial RNAqueous^R (Ambion), partiendo de aproximadamente 10^6 células. Las células cultivadas en botellas de 25 cm² se lavaron con PBS frío y, a continuación, se añadió 1.5 ml de Lysis/Binding Solution, una disolución de sales de guanidinio que permite simultáneamente la lisis celular y la inactivación de las RNAsas endógenas. Se rascó la superficie del frasco con una espátula, hasta obtener un lisado no demasiado viscoso, que se diluyó con etanol al 64% en una proporción de volumen de 1:1 y se homogenizó mediante una micropipeta o bien, haciendo pasar la mezcla del lisado y el etanol a través de una jeringuilla de 25 g hasta obtener una mezcla homogénea. Esta mezcla se colocó sobre el filtro proporcionado por el kit y se

centrifugó a 15000 xg durante 1 min. Tras descartar el sobrenadante, se realizaron lavados sucesivos del filtro a 15000 xg durante 1 min y, finalmente, se recuperó el ARN mediante tampón de elución precalentado a 95° C.

Para la cuantificación de la concentración del ARN, se realizó la lectura de la absorbancia a 260 nm mediante un espectrofotómetro GeneQuant. La pureza del ARN se determinó mediante la relación entre las absorbancias a 260 y 280 nm.

13. SÍNTESIS DE cDNA.

La síntesis de cDNA a partir de ARN total (RT-PCR) se realizó mediante el kit SuperScript™ III First-Strand Synthesis System for RT-PCR, partiendo de aproximadamente 2 µg de una muestra de ARN total. El procedimiento consistió en una primera etapa en la que se mezcla la muestra de ARN molde con oligo(dT)₁₂₋₁₈ y dNTPs y se procedió a la desnaturalización del ARN a 65° C durante 5 min. A continuación, la muestra se enfrió lentamente y se incubó en hielo durante al menos 1 min. Se añadió el volumen necesario de tampón de la retrotranscriptasa, DTT, MgCl₂ y RNaseOUT™ (a las concentraciones recomendadas por el fabricante). Las muestras se incubaron a 42° C, 2 min y, posteriormente, se añadió 2 µl de la enzima SuperScript III Retrotranscriptasa. La reacción de síntesis de cDNA se llevó a cabo a 50° C durante 50 min y se terminó incubando las muestras a 85° C, 5 min. Para eliminar el ARN restante, las muestras se incubaron con 1 µl de RNase H a 37° C durante 20 min.

14. CLONACIÓN DE GENES.

14.1. Obtención de mutantes de glicosilación del MC1R mediante mutagénesis dirigida.

Para determinar la importancia de la glicosilación en la función del MC1R, creamos mutantes del receptor que carecieran de uno o de ambos supuestos sitios de N-glicosilación presentes en la secuencia del gen. Para ello, se llevaron a cabo mutaciones puntuales mediante mutagénesis dirigida utilizando el kit QuickChange XL Site-Directed Mutagenesis.

Como molde para realizar la mutagénesis se utilizó el gen del MC1R, con el epitopo Flag en el extremo N-terminal, insertado en el vector de expresión pcDNA3. Se realizó una PCR usando la ADN polimerasa *PfuTurbo* suministrada por el kit y una pareja de cebadores complementarios formados por oligonucleótidos sintéticos

(uno directo y uno reverso), que contienen la mutación deseada (Tabla 2). Durante los sucesivos ciclos de la PCR, los cebadores se unen al vector y la ADN polimerasa va extendiendo la cadena nucleotídica hasta generar el plásmido mutado. Para digerir el ADN molde parental y seleccionar el ADN sintetizado con la mutación de interés, se incubó el producto de la PCR con la endonucleasa *Dpn* I, que digiere específicamente el ADN metilado y hemimetilado. Después, se realiza la transformación de células ultracompetentes XL10 Gold, y la selección de recombinantes.

Las PCR y la elección de cebadores, se realizaron siguiendo las instrucciones del fabricante (2.5 U de Pfu Turbo, 10 ng de ADN molde, y 125 ng de cada cebador, para cada reacción). En todos los casos, el programa de amplificación consistió en 18 ciclos y una vez completados, una extensión de 7 min a 68° C.

A continuación, el producto de la PCR se incubó con 10 U de la enzima de restricción *Dpn* I a 37° C durante 1 h y, después, se procedió a la transformación de las células ultracompetentes y a la selección de los recombinantes.

Para llevar a cabo la selección, se picaron varias colonias, se inocularon en 4 ml de medio SOB con 100 µg/ml de ampicilina y se dejaron crecer durante 18h a 37° C en agitación vigorosa. Transcurrido este tiempo, se aisló el ADN plasmídico utilizando el Plasmid Genelute HP Miniprep kit, que acopla el sistema de extracción por lisis alcalina con el uso de columnas de elevada afinidad por el ADN. Para la cuantificación de la concentración del ADN, se realizó la lectura de la absorbancia a 260 nm mediante un espectrofotómetro GeneQuant. La pureza del ADN se determinó mediante la relación entre las absorbancias a 260 y 280 nm. Posteriormente, se realizaron las digestiones con las endonucleasas apropiadas en cada caso y se verificó que estuviera presente la mutación deseada mediante secuenciación (Servicio de Secuenciación de la Universidad de Murcia).

Mutante	Secuencia de oligonucleótidos
N15Q	5'-GGGCTCCCT CAAT CCACCCCCACAGC-3'
N29Q	5'-GCTGGCTGCC CAAC AGACAGGAGCCCGG-3'
T31A	5'-TGCCAACCAG GCA GAGCCCGGTGC-3'

Tabla 2. Se muestran los cebadores directos (5') diseñados para la mutagénesis. Se utilizan junto con un cebador reverso complementario. El codón mutado se muestra en negrita. Para el doble mutante N15Q-N29Q, se realizaron 2 rondas consecutivas

de mutagénesis, usando como molde un mutante sencillo y el par de cebadores correspondiente al otro mutante.

14.2. Subclonación de c-KIT en pcDNA3.

Tras la adquisición comercial del cDNA codificante para c-KIT humano (Openbiosystems) clonado en el vector pBluescriptR, procedimos a la subclonación del inserto en el vector de expresión pcDNA3.1. Para llevar a cabo la subclonación, se realizó una PCR utilizando como molde el ADN plasmídico con el inserto de interés y los oligonucleótidos directo y reverso que se detallan en la Tabla 3. Estos cebadores contienen secuencias de restricción para las enzimas BamHI y XhoI, respectivamente, lo que permitirá su posterior clonación en pcDNA3.1.

Las condiciones empleadas en la PCR son: 10 ng del clon de c-KIT, 0.5 μ g de cada cebador, 0.2 mM de dNTPs y 1 unidad de Pfu polimerasa en un volumen final de 50 μ l. El programa de amplificación consistió en 30 ciclos, con una etapa de 65° C durante 1 min de la hibridación de los cebadores con el molde, seguido de una extensión de 10 min a 72° C. Mediante electroforesis en gel de agarosa del 1% se verificó la presencia del producto de amplificación del tamaño adecuado, que se purificó con el kit DNA Clean & Concentrator™ (Zymo Research), siguiendo las instrucciones del fabricante.

El producto de PCR purificado y el vector de expresión pcDNA3.1 se sometieron por separado a una doble digestión con las endonucleasas de restricción BamHI y XhoI, mediante incubación a 37° C durante 1h. Cada mezcla de digestión se separó por electroforesis en un gel de agarosa del 1% y las bandas correspondientes al vector y a c-KIT se extrajeron del gel con el Quiagen Extraction kit, según las instrucciones del fabricante. Posteriormente, los eluidos se cuantificaron mediante el aparato nanodrop del Servicio de Apoyo a la Investigación de la Universidad de Murcia.

La ligación se llevó a cabo usando 30 ng de vector, la cantidad de inserto necesaria para mantener una proporción molar de 1: 5 (vector: inserto) y 1 unidad de T4 DNA ligasa, mediante incubación a temperatura ambiente durante 4h. Finalmente, 10 ng de la mezcla de ligación se transformaron en las células competentes DH5 α F' resistentes a ampicilina. El procedimiento continúa como se ha descrito en el apartado 14.1.

Nombre	Secuencia (5' → 3')
c-Kit-Fw (d)	GCAGGATCCGCGATGAGAGGCGCTCGC (<i>Bam</i> HI)
c-Kit-Rv (r)	CTGACTCGAGTCAGACATCGTCGTGC (<i>Xho</i> I)

Tabla 3. Secuencia de los oligonucleótidos empleados en la subclonación de c-KIT en el vector de expresión pcDNA3.1. Se detalla el nombre del cebador, si es directo (d) o reverso (r) y su secuencia oligonucleotídica. La secuencia diana de la enzima de restricción elegida para clonar el producto aparece en negrita y el nombre de la enzima entre paréntesis.

15. SECUENCIACIÓN DE N-RAS, B-RAF Y MC1R EN LÍNEAS DE MELANOMA.

Para obtener la secuencia nucleotídica completa del cDNA codificante para N-RAS y MC1R de distintas líneas de melanoma, se realizó una RT-PCR. Para ello, se extrajo el ARN total de cada una de las líneas celulares y se sintetizó el cDNA, siguiendo el protocolo descrito en los apartados 10 y 11.

A partir del cDNA, se llevó a cabo la amplificación por PCR de los genes N-RAS y MC1R. Los cebadores empleados en la reacción de amplificación se detallan a continuación en la tabla 4, así como la temperatura de la etapa de hibridación de los cebadores.

En el caso de B-RAF, se analizó la secuencia nucleotídica completa del exón 15, en las distintas líneas de melanoma. En primer lugar, se realizó el aislamiento del ADN genómico de las líneas celulares siguiendo el protocolo descrito en el apartado 10 y, a continuación, se llevó a cabo la reacción de amplificación del exón 15, usando los cebadores directo y reverso especificados en la tabla 5 (Thomas et al., 2004). El programa de amplificación empleado consistió en 35 ciclos, con una etapa de 57° C durante 1 min correspondiente a la hibridación de los cebadores con el molde, seguidos de una extensión final de 10 min a 72° C.

Mediante electroforesis en gel de agarosa del 1% se verificó la presencia del producto de amplificación en el peso molecular esperado y el producto de la reacción de amplificación se purificó mediante el kit DNA Clean & Concentrator™ (Zymo Research), siguiendo las instrucciones del fabricante.

Nombre	Secuencia (5' → 3')	Temperatura hibridación (° C)
MC1R-Fw (d)	ATGGCTGTGCAGGGATCCCAG	60
MC1R-Rv (r)	TCACCAGGAGCATGTCAGCAC	60
NRAS-Fw (d)	CGGATCCGAAATGACTGAGTACAAAC	54
NRAS-Rv (r)	TGAATTCTTACATCACCACACATG	54
BRAF-Fw (d)	TCATAATGCTTGCTCTGATAGGA	57
BRAF-Rv (r)	GGCCAAAATTTAATCAGTGGA	57
<u>MC1Rint-Fw (d)</u>	AGCAACGTGCTGGAGAC	54
<u>MC1Rint-Rv (r)</u>	GCGTAGAAGATGGAGATGTAG	62
<u>NRASint-Fw (d)</u>	CCATGAGAGACCAATAC	50
<u>NRASint-Rv (r)</u>	GGTACATCATCCGAGTC	52

Tabla 4. Secuencia de los oligonucleótidos empleados en la secuenciación del cDNA codificante para MC1R y N-RAS, y del exón 15 de B-RAF. En negrita se indican los cebadores usados en la amplificación por PCR y en negrita y subrayado, los cebadores internos usados en la secuenciación. Se especifica el nombre del cebador, si es directo (d) o reverso (r), su secuencia oligonucleotídica y la temperatura empleada en la etapa de hibridación en el programa de amplificación por PCR.

Para la cuantificación de la concentración de ADN, se realizó la lectura de la absorbancia a 260 nm mediante un espectrofotómetro GeneQuant. La pureza del ADN se determinó mediante la relación entre las absorbancias a 260 y 280 nm. Posteriormente, se obtuvo la secuencia nucleotídica del gen de interés mediante secuenciación (Servicio de Secuenciación de la Universidad de Murcia) y comparación con la secuencia del gen silvestre, usando los mismos cebadores que para la amplificación por PCR. En el caso de la secuenciación del MC1R y N-RAS, se emplearon además cebadores internos, que se especifican en la Tabla 4. Todas las mutaciones se confirmaron mediante una segunda secuenciación a partir de un producto de PCR obtenido independientemente del anterior, para descartar mutaciones debidas a artefactos durante el procedimiento experimental.

16. MICROSCOPIA.

16.1. Microscopía óptica.

Para determinar el grado de dendritogénesis de las células PC12 y las células de melanoma humano HBL tras la incubación con distintos reactivos, se sembraron las células en placas de 12 pocillos. Cuando se encontraban aproximadamente al 80% de confluencia, se transfectaron transitoriamente con el plásmido de interés en los casos en los que fuera necesario y, posteriormente, se llevó a cabo el tratamiento requerido durante 48h. La adquisición de imágenes se realizó mediante el uso de una cámara fotográfica acoplada a un microscopio óptico, con el objetivo 20X y en contraste de fases. Para cada línea celular y para cada condición, se capturaron diferentes imágenes en zonas aleatorias del pocillo.

16.2. Microscopía confocal.

La microscopía confocal se utilizó para analizar el patrón intracelular de las distintas variantes de glicosilación del MC1R transfectadas transitoriamente en las células HEK293T.

16.2.1. Tinción inmunoquímica.

En todos los casos, se sembraron las células en placas de 24 pocillos en cuyo interior habíamos introducido cubreobjetos estériles necesarios para el tratamiento posterior. Cuando las células se encontraban aproximadamente a un 80% de confluencia, se realizó la transfección transitoria con un vector con el MC1R silvestre o con las distintas variantes de glicosilación del receptor marcadas con el epítipo Flag. Transcurridas 12h después de la transfección, las células se fijaron con p-formaldehído al 4% en PBS durante 10 min y, a continuación, se lavaron tres veces con PBS. La tinción inmunoquímica continúa mediante la incubación de las células con glicina 20 mM en PBS durante al menos 20 min, para bloquear los grupos amino libres. Posteriormente, para permeabilizar la membrana, las células se incubaron con Igepal CA630 0.5% en PBS durante 15 min, previamente a la incubación con el anticuerpo primario correspondiente (Tabla 5). Tras la incubación durante 30 min, las células se lavaron 3 veces durante 5 min con PBS-BSA 1% y se incubaron 30 min con el anticuerpo secundario correspondiente. Tras la incubación, las preparaciones se lavaron 3 veces con PBS-BSA 1%, y tras retirar el exceso de líquido, los cubreobjetos se montaron sobre portaobjetos, mediante el uso de 3 μ l de medio de montaje para fluorescencia. Tras el secado del medio sobrante, se sellaron los cubreobjetos con laca de uñas, quedando preparados para su observación.

16.2.2. Adquisición de imágenes.

Las imágenes fueron captadas mediante un microscopio de fluorescencia confocal True Confocal Scanner TCS-SP2 de Leica. Para cada tipo de línea celular y para cada condición, se capturaron diferentes imágenes en zonas aleatorias del cubreobjetos. Las imágenes representan múltiples series de cortes transversales en el eje Z, adquiridos a intervalos de entre 0.1 y 0.5 μm , desde el polo superior hasta el inferior de la célula, o bien imágenes de una sola sección en diferentes niveles del eje Z. Las secciones se obtuvieron a través de un objetivo de 63X en aceite de inmersión. La correcta adquisición de imágenes para una posterior colocación requirió que la selección de los filtros fuera la idónea y que la emisión espectral de los fluorocromos usados estuviera suficientemente separada para evitar el fenómeno de cruce de señales o "crosstalking". Otro de los requisitos importantes fue que la intensidad de fluorescencia del fondo fuera mínima y la intensidad de fluorescencia de la muestra no estuviera saturada. Para los estudios de colocación, la toma de las imágenes de una misma sección marcada con diferentes fluorocromos se realizó de manera secuencial para cada fluorocromo y ambas imágenes fueron adquiridas en condiciones espaciales idénticas.

Anticuerpo primario	Dilución	Anticuerpo secundario	Dilución
anti Flag (Sigma-Aldrich)	1:7000	α -ratón conjugado con Alexa 568 (Invitrogen)	1:400
α -calnexina (Santa Cruz)	1:1500	α -conejo conjugado con Alexa 488 (Invitrogen)	1:400

Tabla 5. Anticuerpos usados en la tinción inmunológica de preparaciones para microscopía confocal. Se indica para cada anticuerpo primario, su procedencia, la dilución empleada, el anticuerpo secundario empleado y su dilución. Todas las incubaciones con los anticuerpos tanto primario como secundario se realizaron en PBS-BSA 1%.

RESULTADOS Y DISCUSIÓN.

CAPÍTULO 1

N-glicosilación del receptor de melanocortinas 1. Ocupación de los sitios de glicosilación y papel funcional.

RESUMEN.

La estimulación del receptor de melanocortinas 1 (MC1R), receptor acoplado a la proteína Gs (GPCR) expresado en melanocitos, por la hormona estimulante del melanocito α (α MSH) conduce a la señalización a través de la vía del AMPc y la vía de las proteínas quinasas activadas por mitógenos ERK1 y ERK2. Por tanto, la señalización del MC1R es crítica para la proliferación y diferenciación del melanocito y el MC1R es uno de los principales determinantes de la pigmentación cutánea y del fototipo. Los GPCRs suelen estar N-glicosilados y el MC1R tiene dos sitios potenciales de N-glicosilación situados en su extremo N-terminal, Asn15 y Asn29. Se ha demostrado que el MC1R es una glicoproteína con una sensibilidad inusual a la digestión con endoglicosidasa H, pero la ocupación de cada sitio de glicosilación específico o la importancia funcional de esta modificación post-traduccional no se ha investigado. Demostramos que el MC1R está N-glicosilado en los residuos Asn15 y Asn29, con cadenas de glicanos estructuralmente diferentes. Además, mostramos que la N-glicosilación no es necesaria para la alta afinidad de unión del agonista o el acoplamiento funcional. Por el contrario, la glicosilación tiene un fuerte efecto sobre la disponibilidad de moléculas del MC1R en la membrana plasmática, muy probablemente por una combinación entre un mejor tráfico hacia la superficie celular y una menor internalización. Finalmente, encontramos que mutantes del MC1R tienen diferentes grados de glicosilación que no muestran una simple correlación con su grado de impedimento funcional ni con su tráfico intracelular.

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N-glycosylation of the human melanocortin 1 receptor. Occupancy of glycosylation sequons and functional role.

ABSTRACT.

Stimulation of the melanocortin 1 receptor (MC1R), a G_s-protein coupled receptor (GPCR) expressed in melanocytes, by α melanocyte-stimulating hormone (α MSH) triggers the cAMP pathway and activates the mitogen-activated protein kinases ER1 and ERK2. Thus MC1R signalling is critical for melanocyte proliferation and differentiation and MC1R is a major determinant of skin pigmentation and phototype. GPCRs are usually N-glycosylated and MC1R has two putative N-linked glycosylation sites located in its extracellular N-terminus, Asn15 and Asn29. It has been demonstrated that MC1R is a glycoprotein with an unusual sensitivity to endoglycosidase H digestion, but the occupancy of each specific glycosylation sequon or the functional importance of this post-translational modification has not been investigated. We demonstrate that MC1R is N-glycosylated at residues Asn15 y Asn29, with structurally different glycan chains. We also show that N-glycosylation is not necessary for high affinity agonist binding or functional coupling. Conversely, glycosylation has a strong effect on the availability of MC1R molecules on the plasma membrane, most likely by a combination of improved forward trafficking and decreased internalization. Finally, we found that MC1R mutants exhibit different degrees of glycosylation which do not show a simple correlation with their degree of functional impairment or their intracellular trafficking.

INTRODUCTION.

The human melanocortin 1 receptor (MC1R) is a G protein-coupled receptor (GPCR) preferentially expressed in epidermal melanocytes (1), where it acts to regulate the amount and type of melanin pigment production (2). Upon activation by α melanocyte stimulating hormone (α MSH), MC1R is positively coupled to stimulation of the cAMP pathway via the G_s protein, and to the extracellular signal-regulated mitogen activated protein kinases ERK1 and ERK2 (3). Accordingly, its signalling is critical for melanocyte differentiation and proliferation, and MC1R is a major determinant of skin phototype and skin cancer risk (4). Several variant alleles of the highly polymorphic *MC1R* gene are associated with red hair and fair skin (the RHC phenotype) (5-8), a high number of freckles (9) and increased risk for skin cancer (10-13).

GPCRs and most other plasma membrane proteins are synthesized in the endoplasmic reticulum (ER) and undergo subsequent processing during their trafficking through the biosynthetic-secretory pathway (14). This post-translational maturation usually includes N-glycosylation, the covalent attachment of glycan chains to Asn residues in the protein backbone, which involves a multistep pathway highly conserved in eukaryotic cells. N-glycosylation plays a critical role in protein folding (15-17), quality control (18) including ER-associated degradation (ERAD) as a consequence of mannose trimming (19), oligomerization (20), lectin-based sorting along the secretory pathway (21), protection from protease-mediated degradation (22) and lysosomal trafficking (23). In the case of GPCRs, N-linked carbohydrate chains may fulfil additional functions by contributing to high affinity ligand binding, cell surface expression and/or functional coupling (24-31). These effects are specific to each glycoprotein, thus requiring investigation in each receptor system.

N-glycosylation begins in the lumen of the ER by the one-step transfer of the mannose-rich glycan $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ - from the lipid carrier dolichol pyrophosphate onto an Asn residue of the nascent protein. This residue must be located within the rather strict consensus sequence Asn-X-Ser/Thr, where X stands for any amino acid except a Pro (32). After this transfer, subsequent processing reactions including cycles of glucose removal and readdition yield a high-mannose type chain (Fig. 1). The final structure is acquired in the Golgi apparatus by the trimming of several mannose residues and addition of galactosyl, fucosyl, sialyl and GlcNAc residues. The occurrence and extent of these reactions is variable, thereby accounting for the structural diversity of N-linked sugar chains in mature glycoproteins. These may include smaller high-mannose type chains, hybrid type oligosaccharides or larger complex carbohydrate chains (Fig. 1). The degree of processing and the contribution of the oligosaccharide moiety to the molecular weight of glycoproteins can be analyzed by means of two glycosidases, endo- β -acetylglucosaminidase H (EndoH) and peptide-N⁴-(acetyl- β -glucosaminyl)-asparagine amidase (PNGaseF). PNGaseF hydrolyses all common types of N-linked glycan chains and therefore comparison of the electrophoretic mobility of glycoproteins before and after PNGaseF treatment allows for an estimation of the molecular weight (Mr) of the protein backbone. Conversely, EndoH only cleaves core high-mannose N-glycan chains and hybrid-type chains. Accordingly, the immature, incompletely processed forms of most glycoproteins found in the ER are sensitive to EndoH but most often these proteins become resistant to EndoH following modification of the mannose-rich glycan chains to complex

oligosaccharides in the Golgi network. Therefore, acquisition of EndoH resistance can be considered an evidence of normal processing and trafficking beyond the ER.

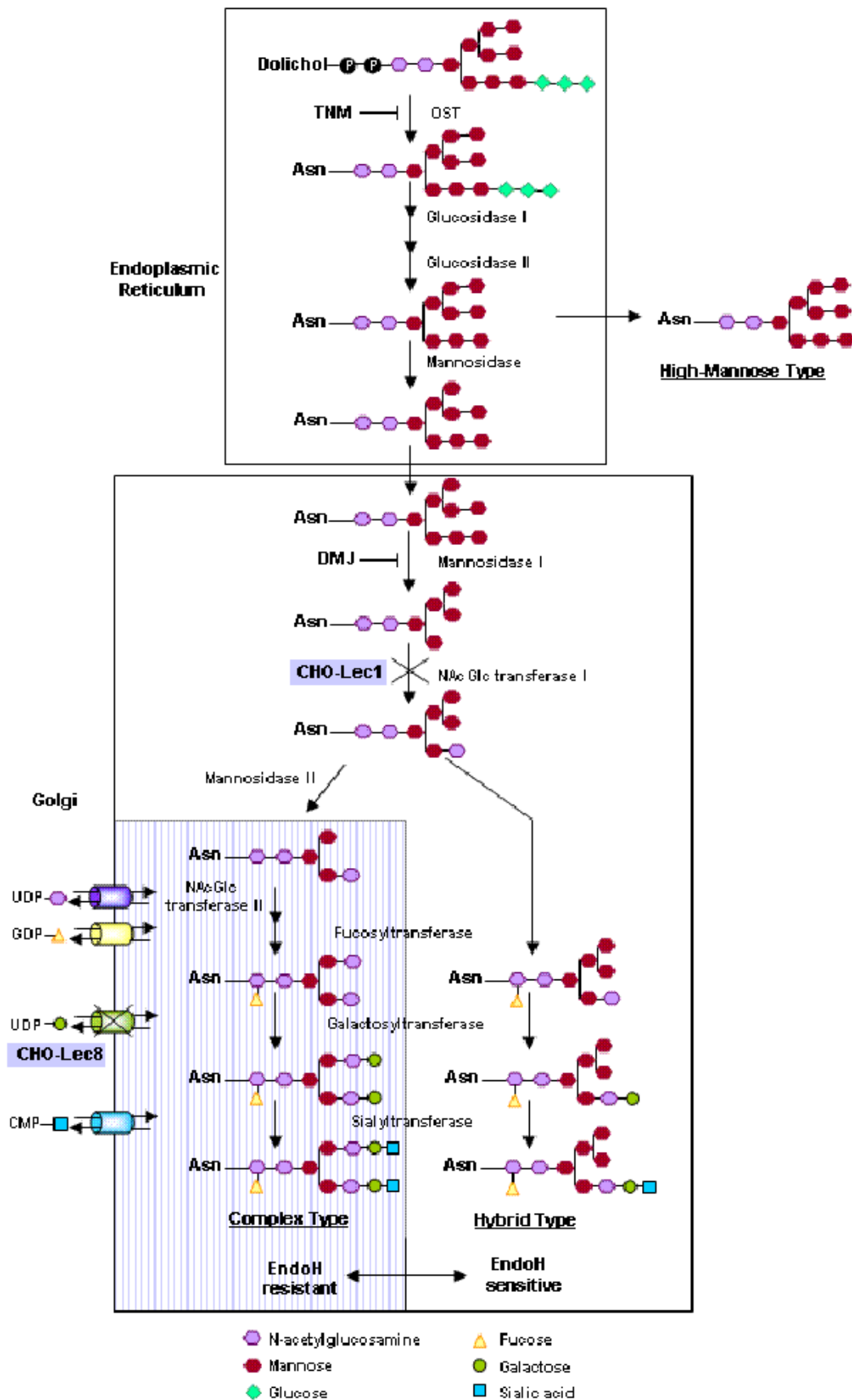


Fig. 1. Scheme of N-linked oligosaccharide, structural types and biosynthetic pathway in the

endoplasmic reticulum and the Golgi apparatus. OST stands for oligosaccharyl transferase, TNM for tunicamycin and DMJ for 1-deoxymannojirimycin. Forms included in the hatched box correspond to complex-type EndoH resistant oligosaccharides. All other forms are either high-mannose or hybrid type oligosaccharides and these forms correspond to EndoH sensitive glycoforms. Also shown are the steps of the pathway blocked by TNM and DMJ as well as CHO-Lec1 and CHO-Lec8 mutant cells.

The extracellular N-terminus of MC1R contains two potential N-glycosylation sequons ¹⁵NST¹⁷ and ²⁹NQT³¹. Previous EndoH and PNGaseF digestion studies and the comparison of the electrophoretic mobility of wildtype (WT) MC1R and a glycosylation-incompetent mutant where both Asn15 and Asn29 were mutated to Gln showed that MC1R is expressed as a glycoprotein both in heterologous systems (33) and in melanoma cells (34;35). However, these studies did not address the occupancy of each specific glycosylation sequon, or the functional consequences of glycosylation. Moreover, MC1R glycosylation pattern was highly unusual in that WT remained EndoH-sensitive. This feature would be expected for aberrantly processed mutant forms retained within intracellular compartments such as the R151C and R160W RHC variants (36) or other natural mutants (11;36), but it was surprising for a fully active WT protein.

In this study, we sought to compare the occupancy of each glycosylation sequon, to analyze the functional significance of this modification, and to establish the glycosylation pattern of different natural MC1R variants.

EXPERIMENTAL PROCEDURES.

Materials and pharmacological agents.

A radioimmunoassay kit for cAMP and [¹²⁵I]-NDP-MSH (2000 Ci/mmol) were from Amersham Pharmacia Biotech (Little Chalfont, UK). Lipofectamine 2000 and competent DH5 α cells were from Invitrogen, (Carlsbad, CA). Igepal CA-630, BSA, EDTA, PMSF, bicinchoninic acid, β -mercaptoethanol, SDS and anti-Flag M2 monoclonal antibody peroxidase conjugate were from Sigma Chemical Company (St. Louis, MO, USA). Rabbit antibodies against ERK2 and calnexin were from Santa Cruz Biotechnology (Santa Cruz, CA). SDS-PAGE and Western blot reagents were from Bio-Rad (Richmond, CA, USA), and other reagents from Merck (Darmstadt, Germany) or Prolabo (Barcelona, Spain). The α MSH analogue [Nle⁴, D-Phe⁷] α MSH (NDP-MSH) and forskolin were from Calbiochem (Darmstadt, Germany) and were routinely used at 10⁻⁷ and 10⁻⁵ M respectively. Deoxymannojirimycin (DMJ) and tunicamycin (TNM) were from Sigma (St. Louis,

MO, USA) and were used at 5 μ M and 5 μ g/ml, respectively. The endoglycosidase H and N-glycosidase F were from Roche Applied Sciences (Mannheim, Germany).

Expression construct and transfection.

All expression constructs were prepared in pcDNA3 (Invitrogen). The following expression constructs have been described: WT-MC1R, the Flag-tagged variants V38M, V51A and N281S (37), S41F, M128T and C289R (35), R162P (38), L93R and E94K (11), C35A, R151C, R160W and D294H (33), T308A-T316A, T308D-T316D (39). The V60L and V92M point mutants and the double mutants N15Q-N29Q, K226N-R227Q and R306Q-R307Q were obtained by mutagenesis with the QuickChange kit (Stratagene, La Jolla, CA) and WT Flag-MC1R as template. All constructs were verified by double strand automated sequencing. The Rab1-EGFP, Rab5-EGFP and Rab7-EGFP constructs were a gift from Prof. Miguel Seabra (Imperial College, London, UK).

Cell culture.

HEK293T cells, parental CHO and mutant CHO-Lec1 and CHO-Lec8 cells, and HBL human melanoma cells (a gift from Prof. G. Ghanem, Free University of Brussels, Belgium) were grown in DMEM, supplemented with 10% foetal bovine serum, 100 U/ml penicillin and 100 μ g/ml streptomycin sulfate. Stable transfectants derived from HBL cells were cultured in the presence of 800 μ g/ml G418 sulfate (40). Cells grown to 80% confluence were transfected using Opti-MEM to dilute DNA and Lipofectamine.

Functional assays.

Radioligand binding assays were done with 10^{-10} M [125 I]-NDP-MSH, and increasing concentrations of unlabelled NDP-MSH, up to 10^{-7} M, when required as previously described (41). For cAMP production assays, cells grown in 12-well plates were transfected, serum-deprived for 12-24 h and stimulated as required. The medium was aspirated and the cells quickly washed with 800 μ l ice-cold phosphate buffered saline (PBS). Cells were lysed with 200 μ l/well 0.1N HCl preheated at 70 $^{\circ}$ C, and scrapped. The mix was freeze-dried, washed with 100 μ l H₂O and freeze-dried again. cAMP was measured with a commercial radioimmunoassay, as per instructions. Parallel dishes were used for protein determination performed with the bicinchoninic acid method.

Western blot.

Cells were washed twice with PBS and solubilized at 4 $^{\circ}$ C in 200 μ l solubilization buffer (50 mM Tris-HCl pH 8, 1% Igepal, 1 mM EDTA, 0.1 mM PMSF, 10 mM iodoacetamide). Samples were centrifuged (105,000xg, 30 min) and a volume of

supernatant containing 10 µg protein was mixed (2:1 ratio) with electrophoresis sample buffer (180 mM Tris-HCl pH 6.8, 15% glycerol, 9% SDS, 0.075% bromophenol blue and 7.5% β-mercaptoethanol). Electrophoresis and Western blotting were performed as described (11;33;39). MC1R was detected with anti-Flag M2 monoclonal antibody peroxidase conjugate. Comparable loading was ascertained by stripping and reprobing the membranes with an anti-ERK2 antibody.

Endoglycosidase treatment.

Cell lysates were incubated (37° C, 4h) with 15 mU EndoH or 2 U PNGaseF in 50 mM phosphate buffer, pH 7.0, 10 mM EDTA 0.1% SDS. For PNGaseF digestion, samples were previously denatured by heating at 95° C for 5 min. Reactions were stopped with electrophoresis sample buffer, and the samples were analyzed by Western blot.

Immunofluorescence microscopy.

Cells grown on coverslips were transfected as required, fixed with paraformaldehyde (4% in PBS) and permeabilized (0.1% Igepal). To detect MC1R, cells were labelled with anti-Flag M2 monoclonal antibody (1:7000), followed by an Alexa 568-conjugated secondary antibody. For co-localization of Flag-MC1R and calnexin, cells were incubated simultaneously with anti-Flag monoclonal and anti-calnexin rabbit polyclonal, followed by Alexa 568-conjugated anti-mouse and Alexa 488-conjugated anti-rabbit secondaries. Rab1-EGFP, Rab5-EGFP and Rab7-EGFP were detected by their intrinsic fluorescence. Samples were mounted with a mounting medium from DakoCytomation (Carpinteria, USA) and examined with a Leica laser scanning confocal microscope.

RESULTS.

Two potential glycosylation sites in MC1R are occupied by structurally different, EndoH-sensitive glycan chains.

We confirmed our previous data on the occurrence of WT MC1R as an EndoH-sensitive glycoprotein and extended these observations by analyzing the occupancy of each one of the two potential glycosylation sites in the protein. WT MC1R and the glycosylation-incompetent double mutant N15Q-N29Q (35) were expressed in HEK293 cells, and their electrophoretic pattern before and after EndoH and PNGaseF treatment was compared (Fig. 2A). As previously reported (33), MC1R expressed in HEK cells migrated as a doublet with a majority band of apparent Mr ~ 29 kDa and a minority band of Mr ~ 34 kDa. Both EndoH and PNGaseF fully digested the upper 34 kDa band but the mobility of the 29 kDa form

remained unchanged, and identical to the one of the glycosylation-null mutant. The same pattern was observed for HBL melanoma cells stably expressing physiological levels of WT receptor (36) (Fig. 2B). This confirmed that the ~ 29 kDa form corresponded to "de novo" WT MC1R, whereas the 34 kDa isoform was a fully glycosylated but EndoH-sensitive protein (33;35;36).

We prepared point mutants where the putative glycosylation sequons were abolished independently by mutating either Asn15 or Asn29 to Gln. These N15Q and N29Q point mutants were expressed in HEK293T cells and their electrophoretic pattern was compared with WT MC1R and the double mutant N15Q-N29Q (Fig. 2C). Both single mutants migrated as doublets with one band corresponding to the ~ 29 kDa protein backbone and the other to a partially glycosylated form. The Mr of these glycoforms was significantly lower than the one of glycosylated WT MC1R. Moreover, the electrophoretic mobility of the glycosylated N29Q mutant was higher compared with glycosylated N15Q. These data proved that the Asn15 and the Asn29 sites were both occupied by inequivalent glycan chains.

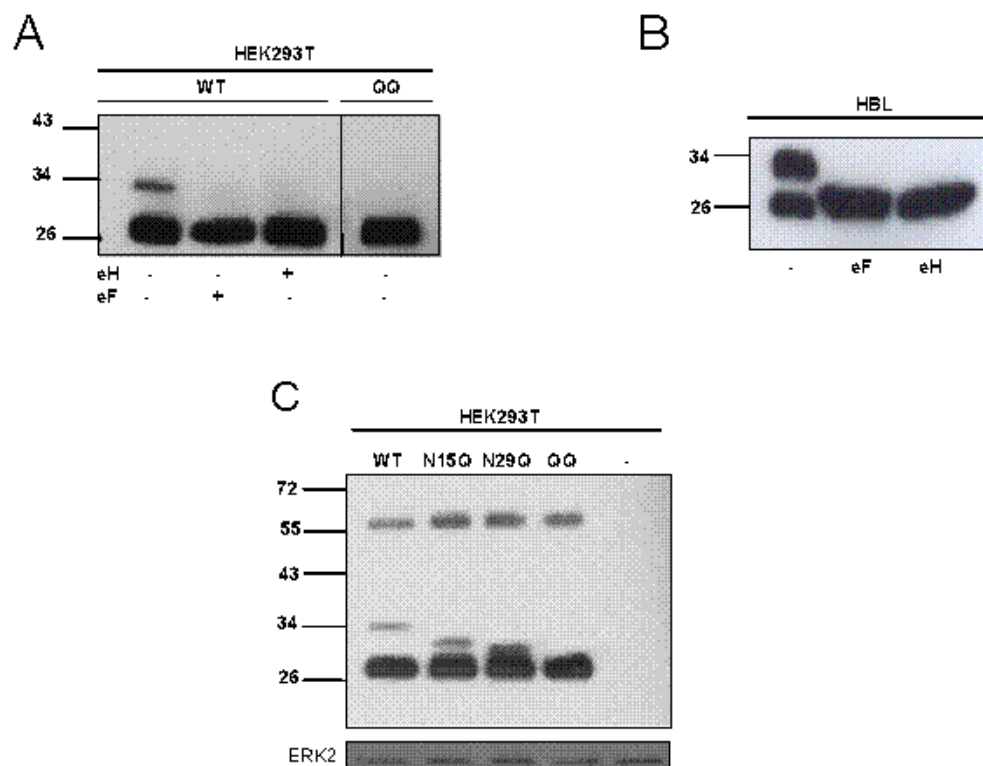


Fig. 2. Usage of glycosylation sequons in the MC1R. A, EndoH and PNGaseF digestion of WT MC1R expressed in HEK293T cells and electrophoretic pattern of a glycosylation-null MC1R mutant. Cell extracts were digested at 37 °C for 3 h with 15 mU EndoH (eH) or 2 U PNGaseF (eF). The electrophoretic pattern of the digested extracts was analyzed by

Western blot and compared with the glycosylation-incompetent N15Q/N29Q mutant (lane labelled QQ). B, Endoglycosidase digestion of Flag-labelled WT MC1R stably expressed in HBL human melanoma cells. Extracts of HBL cells were digested with EndoH or PNGaseF and analyzed by Western blot. C, Electrophoretic pattern of glycosylation-deficient MC1R mutants. Flag-labelled WT MC1R, N15Q, N29Q and N15Q/N29Q (QQ) were expressed in HEK cells and their electrophoretic pattern was compared by Western blot. ERK2 blot was used as loading control.

Effect of glycosylation on MC1R cell surface expression and functional coupling.

To assess the effect of glycosylation on MC1R cell surface expression we performed radioligand binding studies using ^{125}I -labelled NDP-MSH and HEK cells transfected with WT MC1R or the glycosylation mutants. To exclude possible artefacts due to misfolding of the mutant proteins, we used two complementary strategies: i) analysis of new mutants where the NXS/T glycosylation sites were abolished by replacement of the Ser/Thr hydroxylated residue, rather than the Asn acceptor and, ii) parallel studies performed with HBL cells expressing WT receptor and treated with nucleoside antibiotic tunicamycin (TNM) or with 1-deoxymannojirimycin (DMJ). TNM blocks the initial transfer of the dolichol phosphate-bound oligosaccharide chain to Asn acceptors in proteins by inhibition of ER phosphosugar transferases. DMJ inhibits Golgi-resident mannosidases, which allows loading of the Asn acceptor with the initial carbohydrate chain, but prevents trimming and processing to hybrid or complex type oligosaccharides (Fig. 1).

HEK293 cells transfected with WT or mutant MC1R were incubated with a fixed concentration of ^{125}I -labelled NDP-MSH (10^{-9} M corresponding to $\sim 10^5$ cpm), washed and counted for bound ligand. Cells expressing WT or the N15Q mutant bound similar levels of agonist, whereas ligand binding was much lower for the N29Q and N15Q-N29Q mutants (Fig. 3A). We also abolished glycosylation of Asn29 by mutating Thr31 to Ala. The T31A mutant behaved as N29Q or N15Q-N29Q. Thus, occupancy of the $^{29}\text{NQT}^{31}$ sequon was crucial for efficient ligand binding, whereas glycosylation of the $^{15}\text{NST}^{18}$ site was dispensable. Accordingly, the two glycan chains in MC1R were not only different in structure, but also functionally inequivalent.

Consistent with the importance of glycosylation for normal agonist binding, HBL cells treated with either TNM or DMJ bound significantly lower amounts of the MC1R radioligand (Fig. 3B). We also tested the effect of the drugs on the electrophoretic pattern of the MC1R (Fig. 3C). Under our experimental conditions,

TNM completely abolished receptor glycosylation as assessed by the presence of the 29 kDa protein band exclusively. Significantly, DMJ had no noticeable effect on the electrophoretic pattern, in spite of the strong decrease in agonist binding observed for cells treated with the mannosidase inhibitor.

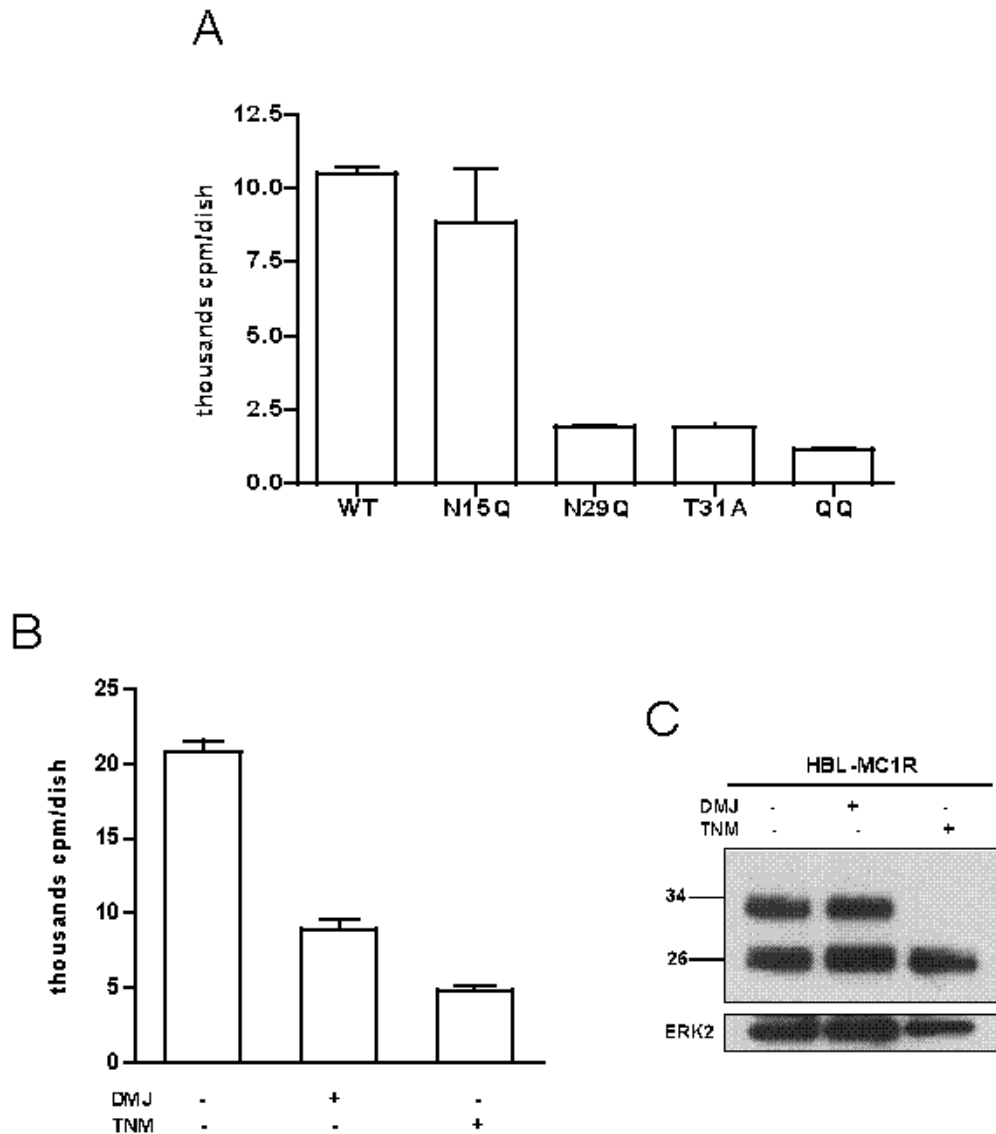


Fig. 3. Effect of glycosylation on MC1R cell surface expression. A, Specific binding of ¹²⁵I-NDP-MSH (10^{-9} M, 10^5 cpm) to HEK293 cells expressing the indicated MC1R forms. Results are given as mean \pm sem, $n \geq 3$. B, Effect of TNM and DMJ on ¹²⁵I-NDP-MSH binding to HBL melanoma cells. HBL cells expressing WT MC1R were treated with DMJ (5 μ M, 48h) or TNM (5 μ g/ml, 18h), before determination of radioligand binding. Results are given as mean \pm sem, $n \geq 3$. C, Effect of TNM and DMJ on the electrophoretic pattern of MC1R in HBL cells. Cells were treated as above, and detergent-solubilized extracts were analyzed by Western blot using an anti-Flag monoclonal antibody for MC1R detection.

Changes in the amount of agonist bound at a single ligand concentration can be determined by differences in receptor cell surface expression and/or binding affinity. To distinguish these possibilities, we performed saturation binding experiments at increasing agonist concentrations, from 10^{-12} to 10^{-7} M. Data were analyzed by non-linear regression to obtain the total number of binding sites (B_{max}), and the dissociation constant (K_d). The results are summarized in Table 1. The glycosylation mutants and WT MC1R showed comparable binding affinity. WT and mutant N15Q were expressed at similar plasma membrane levels. In contrast, N29Q and N15Q-N29Q showed significant reductions in B_{max} relative to WT. Accordingly, decreased agonist binding in cells expressing N29Q or the glycosylation-null mutant was due to lower cell surface expression, rather than impaired affinity. Nonetheless, a significant fraction of unglycosylated receptor molecules was trafficked to the cell surface, thus showing that N-glycosylation at Asn29 is an important but not an absolute requirement for expression on the plasma membrane.

TABLE 1. Binding parameters of WT and glycosylation MC1R mutants in HEK293T cells.

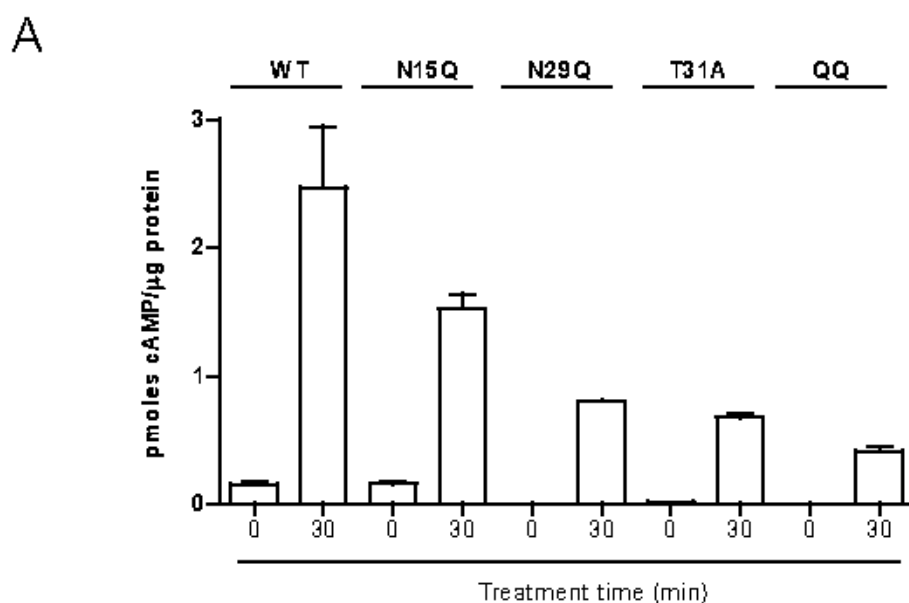
MC1R Variant	B_{max} (fmoles/ μ g protein)	K_d (nM)
WT	3.6 ± 0.4	1.541 ± 0.747
N15Q	3.5 ± 0.3	1.605 ± 0.668
N29Q	0.64 ± 0.05	2.368 ± 0.875
QQ	0.44 ± 0.03	1.135 ± 0.385

Data are given as mean \pm SEM, for at least three independent experiments.

The effect of glycosylation on functional coupling to the cAMP synthesizing system was next considered. Cells expressing WT MC1R or the glycosylation mutants were challenged with 10^{-7} M NDP-MSH and intracellular cAMP levels were measured (Fig. 4A). The cAMP response was strongly decreased for N29Q, T31A and N15Q-N29Q. Additionally, we examined cAMP production after NDP-MSH stimulation of HBL cells in the presence or absence of DMJ or TNM (Fig. 4B). cAMP generation was also significantly inhibited by the drugs. Moreover, residual levels of agonist-dependent cAMP production were consistent with residual cell surface expression, suggesting that the fraction of mutant receptor molecules reaching the plasma membrane could activate the cAMP pathway in response to the agonist.

The strong effect of DMJ on MC1R availability on the cell surface suggested that some degree of carbohydrate trimming and processing was necessary for normal trafficking to the plasma membrane. We tested this possibility by comparing

the functional behaviour of MC1R expressed in CHO cells and in two derived mutant cell lines defective in carbohydrate processing, CHO-Lec1 and CHO-Lec8 (Fig. 1). CHO-Lec1 cells have no detectable N-acetylglucosaminyl-transferase activity, and therefore glycoproteins expressed in these cells bear exclusively high mannose type oligosaccharide chains since further modification is blocked (42;43). CHO-Lec8 cells are unable to transport UDP-galactose from the cytosol to the Golgi, because they lack the Golgi UDP-galactose translocase (44;45). Therefore, in CHO-Lec8 cells, addition of galactose residues to previously trimmed glycan chains is negated, thereby abrogating subsequent addition of sialic acids. In preliminary experiments parental CHO, CHO-Lec1 and CHO-Lec8 cells showed significant differences in forskolin-stimulated adenylyl cyclase activity. Accordingly, functional coupling data were expressed as the percentage of cAMP levels in agonist-stimulated cells relative to the maximal levels of cAMP achieved upon stimulation with forskolin (46). As shown in Fig. 4C, MC1R expressed in CHO-Lec1 cells was approximately 50% less active than in parental CHO cells. Comparable expression of MC1R was ascertained by Western blot (not shown). This was consistent with the functional loss observed in HBL cells treated with DMJ, suggesting that trimming (inhibited by DMJ) and subsequent addition of N-acetylglucosamine (blocked in CHO-Lec1 cells) were both required for function. Conversely, the functional response was similar in CHO-Lec8 and parental CHO cells. This suggested that further addition of galactose was not required for normal function, consistent with EndoH sensitivity of glycosylated MC1R and with the minor effect of DMJ on the electrophoretic pattern of MC1R.



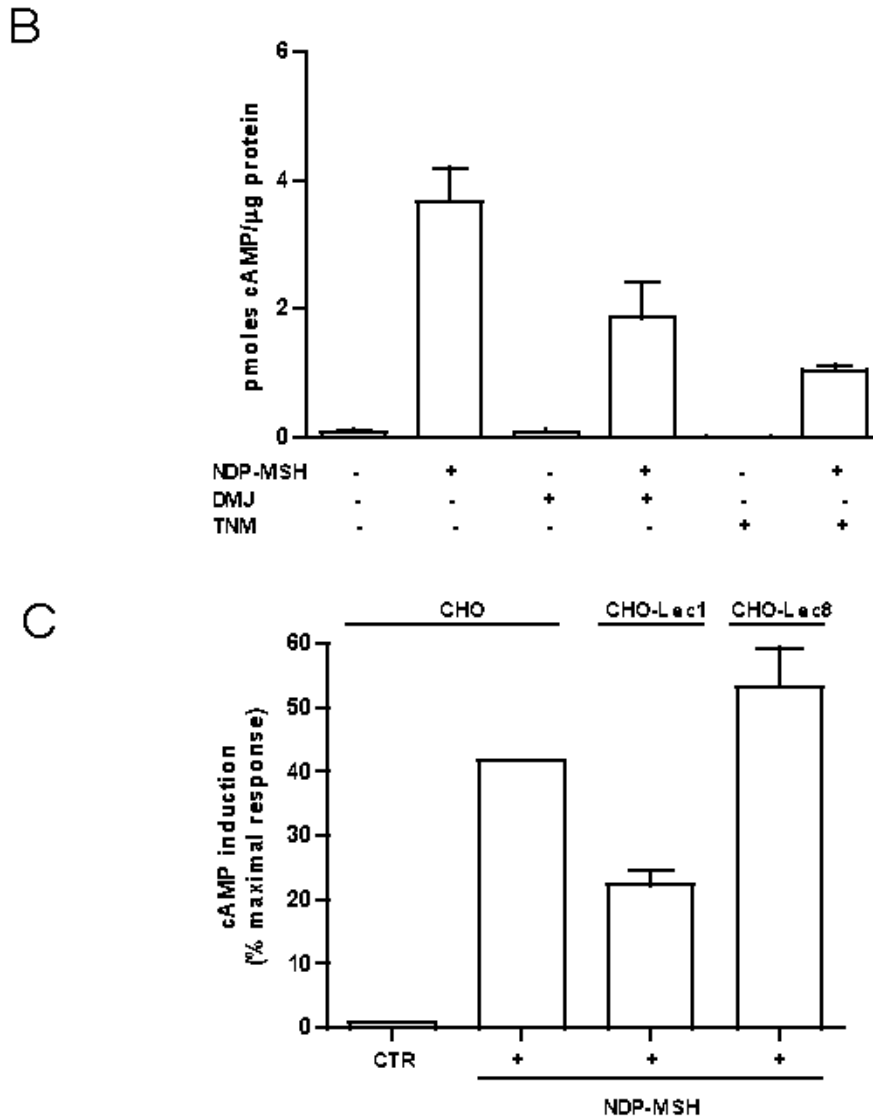
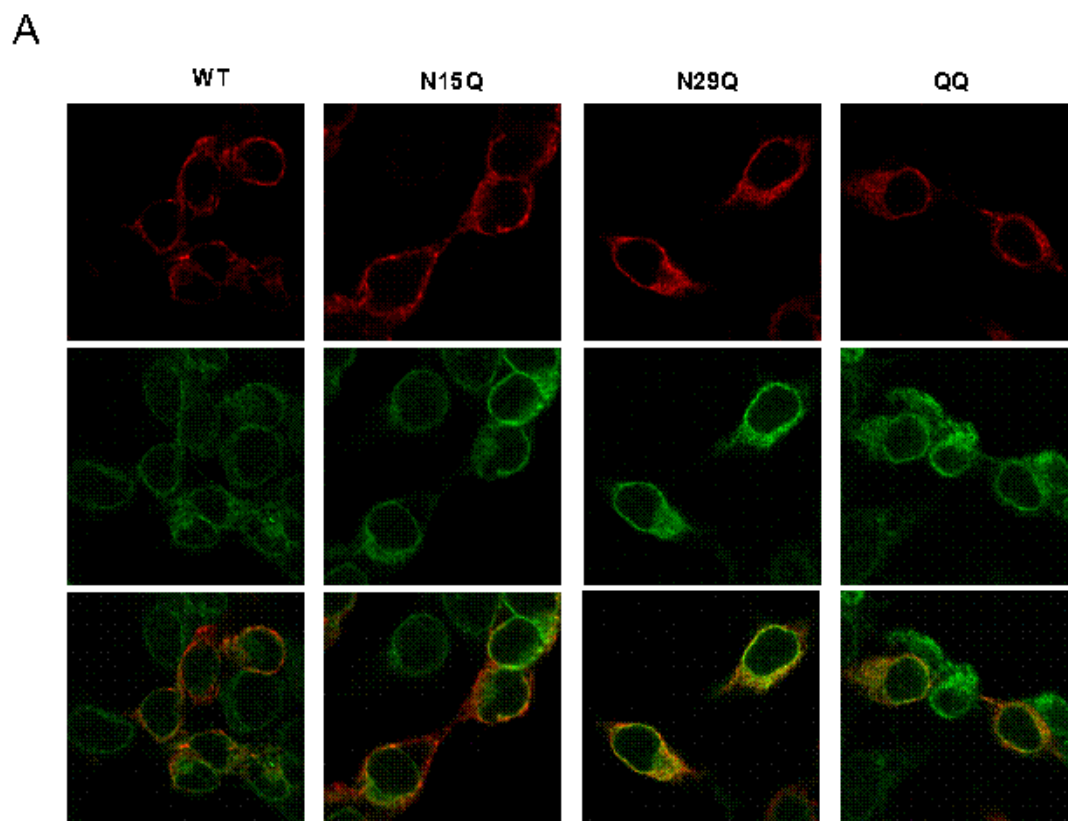


Fig. 4. Effects of N-glycosylation on the functional coupling of MC1R to the cAMP pathway. A, Agonist-induced cAMP production in HEK293 cells expressing WT or glycosylation-deficient mutants. Cells were stimulated with 10^{-7} M NDP-MSH for 30 min. cAMP contents were determined by radioimmunoassay. B, Effect of DMJ or TNM on agonist-stimulated cAMP production in HBL cells. Cells were treated with DMJ ($5 \mu\text{M}$, 48h) or TNM ($5 \mu\text{g/ml}$, 18h), as indicated, before stimulation with NDP-MSH (10^{-7} M, 30 min) C, cAMP generation in CHO cells and mutant CHO-Lec1 and CHO-Lec8 cells expressing MC1R. Cells were transfected with MC1R and treated with 10^{-7} M NDP-MSH or 10^{-5} M forskolin for 30 min. cAMP levels were measured and values were referred to the maximal levels obtained after forskolin treatment. Results are the mean \pm sem ($n \geq 3$).

Intracellular trafficking pattern of MC1R glycosylation mutants.

Cell surface expression of GPCRs depends upon the balance between forward trafficking from the ER to the plasma membrane on one hand, and

retrograde transport by endocytosis on the other (47). We looked for alterations in forward or retrograde transport of MC1R glycosylation mutants by comparing their subcellular distribution by confocal microscopy. HEK293T cells transfected with WT MC1R or glycosylation mutants were fixed, permeabilized and stained for receptor and calnexin, an ER-resident chaperone that assists the folding of nascent membrane proteins of the secretory pathway (Fig. 5A). As previously reported (36), WT MC1R showed low co-localization with the ER marker. A similar pattern was obtained for single mutant N15Q. In contrast, N29Q and N15Q-N29Q displayed a significantly higher co-localization with calnexin, indicating their accumulation in the ER. MC1R mutants were also co-expressed with Rab1-EGFP. Rab1 is a small GTPase that regulates forward trafficking from the ER to the Golgi (48) and can be used as a marker of the ERGIC and the *cis* side of the Golgi. All mutants showed low co-localization with Rab1, similar to WT (Fig. 4B). Therefore, the labelling pattern of N29Q and N15Q-N29Q was indicative of association with the ER, whereas staining for N15Q was consistent with normal forward trafficking and cell surface expression.



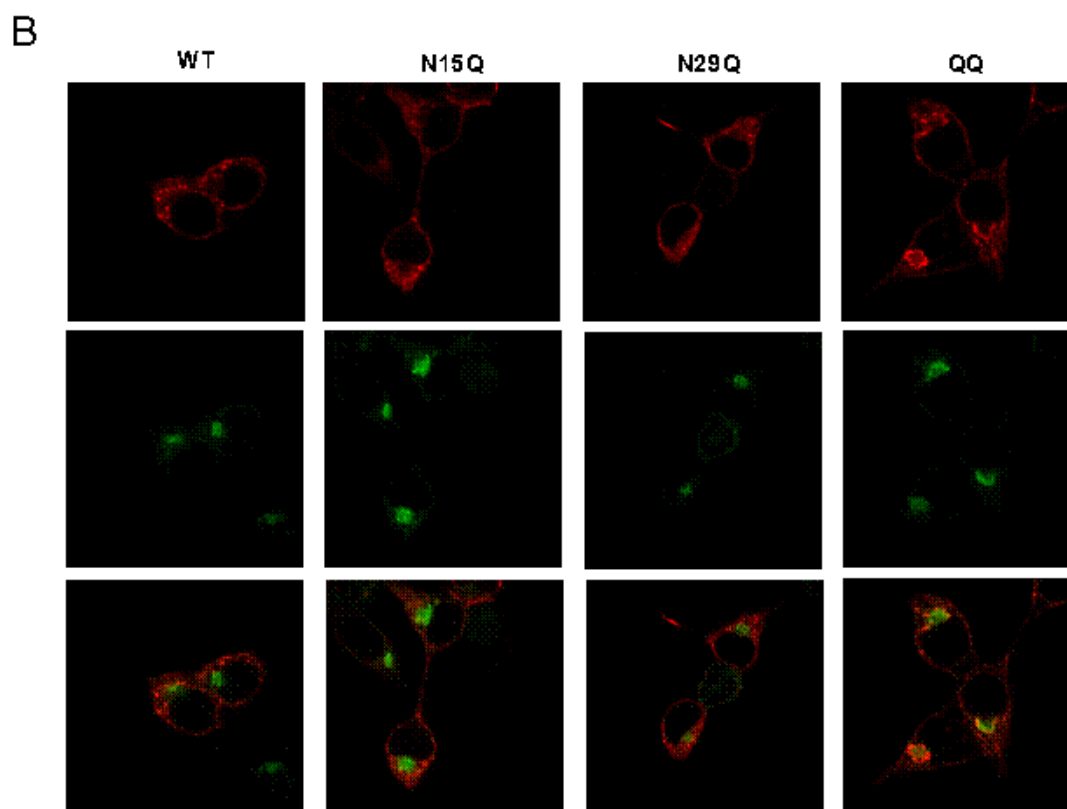
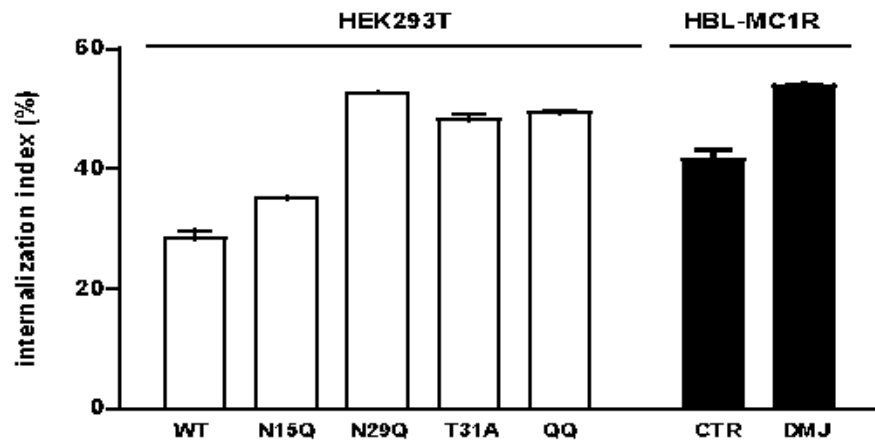
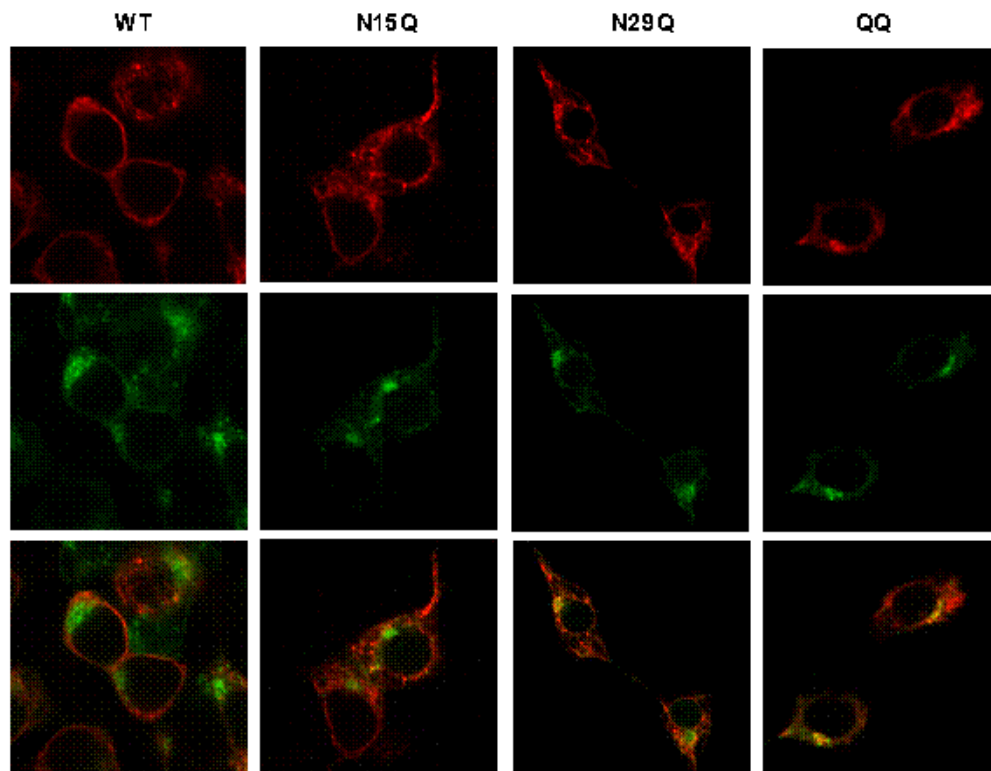


Fig. 5. Intracellular localization of glycosylation MC1R mutants. A, HEK293T cells were transfected with WT MC1R or glycosylation mutants N15Q, N29Q or QQ. Cells were fixed, permeabilized and stained for MC1R and calnexin. MC1R is shown in red (upper) and calnexin (middle) in green. In merged images (lower) co-localization is shown in yellow. B, HEK293T cells were co-transfected with the indicated MC1R forms and Rab1-EGFP. Cells were stained for MC1R (shown in red) and Rab1-EGFP was detected directly (green). Co-localization in merged images is shown in yellow.

We also checked whether MC1R glycosylation had any effect on receptor internalization. We performed radioligand binding studies using an acid wash procedure that distinguishes agonist bound to MC1R at the cell surface and internalized ligand-receptor complexes (49). We found that the rate of internalization was similar for WT and N15Q (Fig. 6A). However, N29Q and N15Q-N29Q showed a significant increase in MC1R internalization, which suggested that the stability of these forms on the cell surface was impaired. The internalization index for the T31A mutant was the same as for N29Q or the double mutant, confirming the relevance of occupancy of the second sequon. A similar increase in the rate of internalization was observed for HBL cells treated with DMJ (Fig. 6A).

We confirmed changes in the rate of internalization by comparing co-localization of MC1R forms with markers of endocytic vesicles (50) by confocal microscopy. HEK293T cells were co-transfected with WT or mutant MC1R, and the

markers of endosomal compartments Rab5-EGFP or Rab7-EGFP. Rab5 shows a preferential association with early endosomes, whereas Rab7 is a late endosome/lysosome marker (51). Consistent with their higher internalization rate, mutants N29Q and N15Q-N29Q displayed a significant colocalization with both endocytic markers, as opposed to WT and the N15Q mutant (Fig. 6B and C).

A**B**

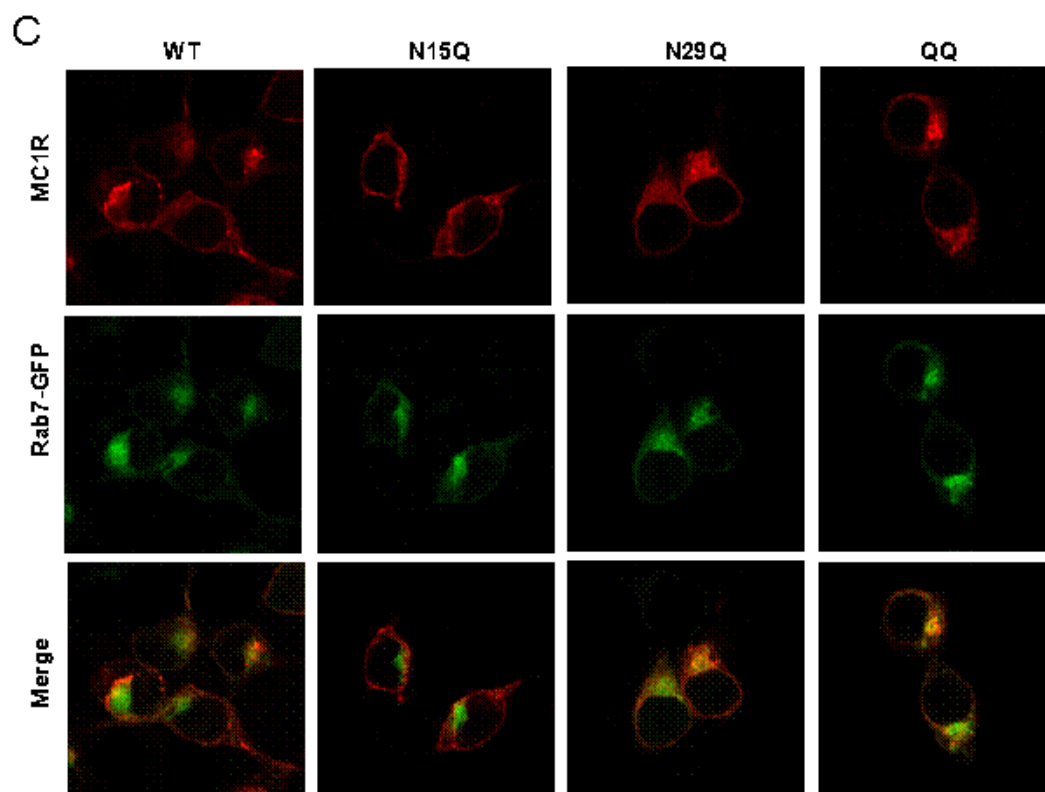


Fig. 6. Effect of glycosylation on MC1R internalization. A, HEK cells were transfected to express the indicated MC1R forms and incubated with radiolabelled NDP-MSH. Internalized ligand was separated from the ligand bound on the cell surface by an acid wash procedure, and an internalization index was calculated as the percentage of ligand inside the cells relative to total bound ligand. Also, HBL treated with vehicle or with DMJ (5 μ M, 48h) were incubated with 10^{-10} M 125 I-NDP-MSH and treated in parallel. CTR stands for untreated control. Results are the mean \pm sem ($n \geq 3$). B, HEK293T cells were transfected with WT MC1R or glycosylation mutants and the early endosome marker Rab5 fused to the fluorescent protein EGFP. Cells were stained for MC1R (red) and Rab5-EGFP was detected directly (green). Co-localization in merged images is shown in yellow. C, Same as in B, except that cells were transfected with the late endosome marker Rab7-EGFP.

GPCR endocytosis can be followed by dephosphorylation and recycling to the plasma membrane (resensitization) or targeting to lysosomes or proteasomes for destruction (down-regulation) (52;53). We tested whether defective glycosylation and the resulting increase in internalization had any effect on the intracellular stability of the protein. This was done by comparing the half-life of MC1R in HBL cells grown in the presence or absence of DMJ. Control cells or cells grown with the mannosidase inhibitor were treated with the protein synthesis inhibitor cycloheximide for various times up to 8h, and cell lysates were blotted to compare residual levels of MC1R (Fig. 7). With this approach, we could not only assess the

effect of DMJ on the stability of the protein, but also compare the relative stability of the "de novo" receptor and the glycosylated protein, at physiological levels of expression and in a homologous environment. The relative rate of disappearance of "de novo" and glycosylated proteins in control cells suggested that glycosylation increased only very slightly the half life of the protein. On the other hand, DMJ treatment had no effect on the intracellular stability of the MC1R. Accordingly, increased internalization of defectively glycosylated MC1R did not accelerate its degradation, thus suggesting its recycling.

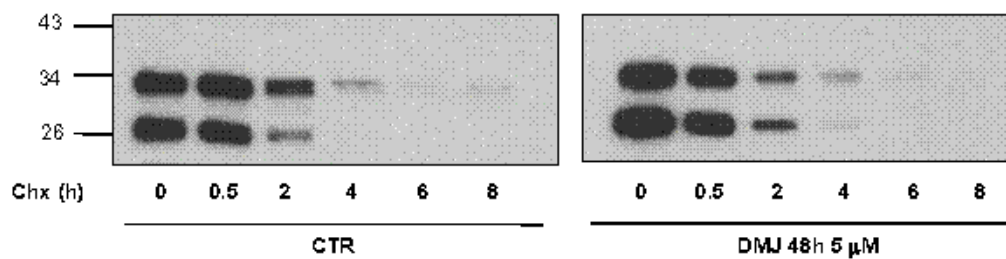


Fig. 7. Effect of DMJ on the intracellular stability of MC1R in HBL melanoma cells. HBL cells treated with vehicle (left blot, CTR) or DMJ (5 μ M, 48h, right blot) were incubated with the protein synthesis inhibitor cycloheximide (Chx) for the times shown on top of each lane, and residual levels of MC1R in detergent-solubilized extracts were detected by Western blot.

Glycosylation of MC1R mutants

We compared the glycosylation pattern of a panel of natural and artificial MC1R mutants expressed in HEK cells (Fig. 8A). In all cases, the usual doublet of ~29 and 34 kDa bands was detected, but significant differences in the relative proportion of *de novo* and glycosylated proteins were found. A series of mutants displayed a noticeable impairment of glycosylation as assessed by the lower proportion of the 34 kDa band. This was the case of L93R variant, which shows extensive intracellular retention with dramatically reduced cell surface expression (11). Interestingly, this was also the case of the D294H and T308A-T316A mutants, which are expressed at plasma membrane densities even higher than WT (39). On the other hand, several other mutants showed a gain-of-function glycosylation phenotype manifested by a higher percentage of the fully glycosylated isoform. Again, this phenotype did not show a simple relationship with the known intracellular trafficking properties of the mutants. It was observed for variants displaying extensive intracellular retention, such as R160W, but also for M128T who is correctly expressed on the cell surface, or the T308D-T316D double mutant with a high rate of internalization and extensive association with endocytic vesicles (39). In order to rule out that these unusual observations could be influenced by expression

in an heterologous cell system, we analyzed the pattern of glycosylation and the sensitivity to EndoH of the major RHC variants stably expressed at near-physiological levels in clones of HBL melanoma cells (Fig. 8B) (36). The results were comparable, with a higher percentage of glycosylated protein for the R160W form, intermediate for R151C and dramatically lower for the D294H variant. Moreover, all glycosylated forms were equally sensitive to EndoH.

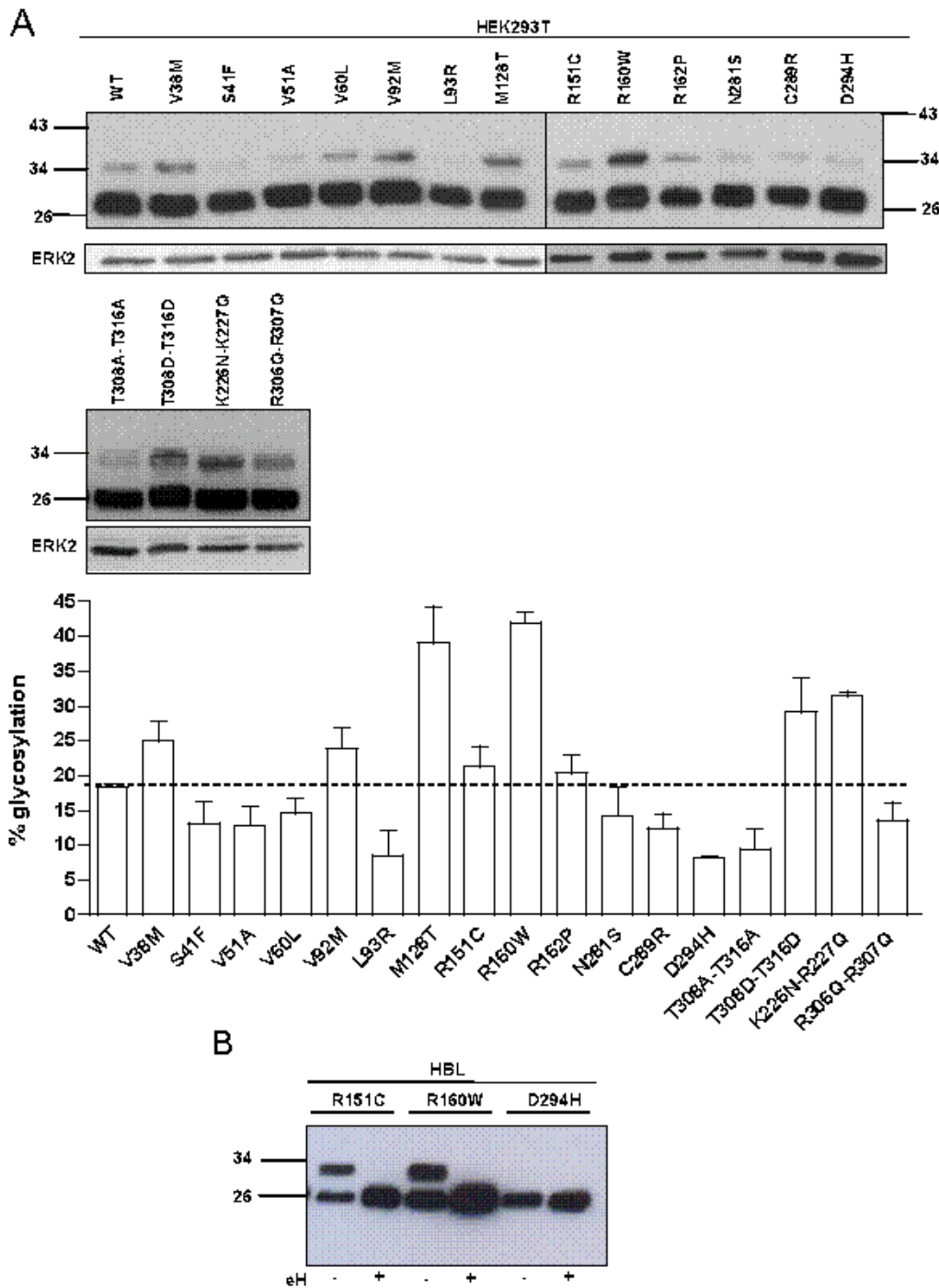


Fig. 8. Glycosylation of MC1R mutants. A, Electrophoretic pattern of MC1R point mutants expressed in HEK293T cells. Comparable loading control was ascertained by reprobating the membranes with an anti-ERK2 antibody. MC1R was detected by Western blot and the

intensity of the glycosylated and *de novo* bands was quantified in order to calculate the ratio of glycosylated MC1R (results given as mean \pm sem for three independent experiments). B, Glycosylation pattern of MC1R stably expressed in HBL cells. HBL cells expressing physiological levels of Flag epitope-labelled WT MC1R or the RHC variants R151C, R160W and D294H were solubilized and extracts were digested with EndoH.

DISCUSSION.

N-glycosylation is a common post-translational modification found in most membrane proteins, including GPCRs (14). The functional consequences of the presence of a carbohydrate moiety in GPCRs are specific for each receptor and span a variety of roles related with the folding, intracellular trafficking and stability and acquisition or modulation of functional properties (24-31). MC1R contains two asparagine residues, Asn15 and Asn29, which are potential *N*-linked glycosylation sites. Previous studies using glycosidase treatment demonstrated that MC1R expressed in HEK293T cells and in HBL human melanoma cells migrates as a doublet comprising a glycosylated band and a non-glycosylated protein of lower Mr (33-35). However, it has been shown that a relatively large deletion of the 27 N-terminal amino acids removing the N-glycosylation sequons in the MC1R did not affect ligand binding or expression levels when the deleted mutant was expressed in COS-1 cells, thus suggesting a minor role for glycosylation (54). Moreover, we found that the WT and fully active glycosylated receptor could be completely digested by EndoH (33;35), although other researchers reported resistance of the WT but not mutant MC1R to the endoglycosidase (34). This prompted us to analyze the MC1R for sequon occupancy, functional effects of glycosylation and changes in the glycosylation pattern of mutant forms.

We used site-directed mutagenesis to ablate the putative glycosylation sites (Asn15 and Asn29), alone or in combination, and found that both sequons are occupied by structurally inequivalent sugar chains. Moreover, comparison of the electrophoretic mobility and apparent Mr of the mutants showed that the oligosaccharide bound to the Asn29 was bulkier than the one attached to Asn15. This suggested a different processing for the *N*-oligosaccharide bound to each residue. For the bulkier N-glycan in Asn29, EndoH sensitivity excluded processing to a complex type oligosaccharide, but its contribution to the overall Mr of the protein was consistent with a hybrid type structure. On the other hand, the small size and EndoH sensitivity of the glycan in Asn15 was compatible with a core high mannose-type structure (14).

Ligand binding and functional coupling experiments showed that glycosylation was important for cell surface expression but had no effect on the

ligand binding and signalling properties of MC1R molecules available on the plasma membrane. The effect on cell surface expression most likely resulted from a combination of defective forward trafficking, as shown by accumulation of the glycosylation-null N15Q-N29Q and N29Q mutants in the ER, and increased retrograde uptake, as shown by a higher rate of internalization and a stronger co-localization with endosomal markers. Concerning the role of each one of the two sugar chains, the oligosaccharide bound to Asn15 was dispensable for cell surface expression and agonist binding affinity as shown by the very similar behaviour of the WT receptor and the N15Q mutant. On the other hand, the glycan chain N-linked to Asn29 had an important role on cell surface expression, as its removal accounted for virtually all the loss of binding sites in the glycosylation-null mutant. The finding of a functional effect of DMJ, as well as the different behaviour of MC1R expressed in parental CHO and mutant CHO-Lec cells showed glycan chain(s) are partially processed by Golgi mannosidases and that this processing is important for full activity. However, the undetectable effect of DMJ treatment on the electrophoretic mobility of the protein showed that the oligosaccharide was not extensively processed by addition of complex residues after the initial removal of terminal mannose, consistent with its sensitivity to EndoH.

The lower but significant number of receptor molecules available on the surface of cells transfected with the glycosylation-null mutant proved that even a completely deglycosylated MC1R could be trafficked to the cell surface, although less efficiently than the normal protein. This observation was consistent with the finding of an aberrant glycosylation for certain mutants such as D294H which is well expressed on the cell surface (39) but was severely underglycosylated. A similar situation was found for the double mutant T308A-S316A. Interestingly, comparable and low rates of internalization have been reported for D294H and T308A-S316A (39). The marked hypoglycosylation of these mutants suggests the occurrence of a cycle whereby part of the *de novo* MC1R could reach the cell surface in a fully unglycosylated form. Absence of an oligosaccharide moiety would lead to a less stable association with the cell surface and would promote internalization, unless a specific mutation prevents sequestration, as in D294H or T308A-S316A. For the WT receptor, retrograde transport to the ER would allow for a new round of exposure to the N-glycosylation machinery and forward trafficking to the cell surface. This putative cycle accounts for the hypoglycosylation of mutant forms with impaired forward trafficking due to retention in the ER (such as S41F or L93R) as well as variants with normal forward trafficking but deficient internalization, such as D294H. It is also consistent with the observation that the half life of MC1R was not affected

by DMJ treatment, which showed that increased internalization did not lead to decreased stability. It also accounts for the different glycoform distribution of the hypoglycosylated T308A-S316A mutant with decreased internalization compared with the hyperglycosylated T308D-S316D with a high constitutive internalization rate (39). However, the evidence suggesting this cycle is indirect for the moment, and further work is required to demonstrate or rule out its occurrence.

In any case, we have demonstrated that the extracellular N-terminus of the MC1R bears two structurally and functionally inequivalent oligosaccharide chains, most likely of the high mannose and hybrid type. We have also shown that the efficiency of N-glycosylation does not display a simple correlation with cell surface expression or forward trafficking of MC1R variants, in that hypoglycosylation can be found for mutants with a strong intracellular retention as well as mutants with a normal cell surface expression. Moreover, the sensitivity to EndoH digestion of the WT mature protein excludes the use of the endoglycosidase as a tool to assess the degree of post-translational maturation of the MC1R. It will be important to determine if similarly unusual glycosylation patterns are found in other GPCRs.

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CAPÍTULO 2

Mutaciones en el receptor de melanocortinas 1 tienen un impacto diferencial a través de la vía del AMPc y a través de la vía de las proteínas quinasas activadas por mitógenos ERK.

RESUMEN.

El receptor de melanocortinas 1 (MC1R), receptor acoplado a la proteína Gs expresado en melanocitos, es uno de los principales determinantes de la pigmentación cutánea, del fototipo y del riesgo de padecer cáncer. El MC1R activa la vía de señalización del AMPc y la vía de las proteínas quinasas activadas por mitógenos ERK1/ERK2. Cuando se expresaron en la línea celular de feocromocitoma de rata PC12, las variantes alélicas del MC1R R151C, R160W y D294H, asociadas con melanoma y con una señalización reducida vía AMPc, mediaron la activación de ERK y el crecimiento de neuritas inducido por el agonista, dependiente de ERK comparable con la mediada por el MC1R silvestre. Las curvas dosis-respuesta para la activación de ERK y para la producción de AMPc indicaron una mayor sensibilidad en la respuesta de las ERK. Por tanto, las mutaciones del MC1R asociadas a melanoma inciden de forma diferente en la señalización vía AMPc y vía ERK, lo que sugiere que el AMPc no es el responsable del acoplamiento funcional del MC1R a la cascada de las ERK.

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Melanocortin 1 receptor mutations impact differentially on signalling to the cAMP and the ERK mitogen-activated protein kinase pathways.

ABSTRACT.

MC1R, a Gs protein-coupled receptor expressed in melanocytes, is a major determinant of skin pigmentation, phototype and cancer risk. MC1R activates cAMP and mitogen-activated protein kinase ERK1/ERK2 signalling. When expressed in PC12 cells, the R151C, R160W and D294H MC1R variants associated with melanoma and impaired cAMP signalling mediated ERK activation and ERK-dependent, agonist-induced neurite outgrowth comparable with wild-type. Dose-response curves for ERK activation and cAMP production indicated higher sensitivity of the ERK response. Thus, the melanoma-associated MC1R mutations impact differently on cAMP and ERK signalling, suggesting that cAMP is not responsible for functional coupling of MC1R to the ERK cascade.

INTRODUCTION.

The human melanocortin 1 receptor (MC1R) is a G protein-coupled receptor (GPCR) expressed in epidermal melanocytes (1), where it regulates the amount and type of melanin pigments produced. MC1R is a major determinant of skin phototype, sensitivity to ultraviolet radiation and melanoma and nonmelanoma skin cancer risk (2). Upon stimulation by α melanocyte stimulating hormone (α MSH) or other related proopiomelanocortin-derived peptides expressed in skin cells (3), MC1R triggers cAMP synthesis leading to activation of the rate-limiting melanogenic enzyme tyrosinase and increased production of dark, photoprotective eumelanins (4;5). MC1R also activates the mitogen-activated protein kinase (MAPK) module leading to the Ser/Thr kinases ERK1 and ERK2 that control key cellular decisions such as proliferation or differentiation (6). The ERK pathway is most often initiated by binding of growth factors to cell surface tyrosine kinase receptors, followed by sequential activation of RAS, then members of the RAF family of kinases, the MAPK kinase MEK and finally ERK1 and ERK2 (7). In addition to this classical tyrosine kinase receptor pathway, the RAS/RAF/MEK/ERK module is also regulated by GPCRs (8;9). Depending on the cellular context, GPCRs that trigger the cAMP pathway can either inhibit or activate ERK signalling by partially understood mechanisms (10). In most cell types, PKA-dependent events such as activation of the small GTPase Rap1 or phosphorylation of the C-RAF isoform of RAF result in C-RAF inhibition and lower ERK activity (10). However, ERK activation by cAMP has been reported in a few cell types including PC12 cells and mouse melanoma cells (6).

Human *MC1R* is extremely polymorphic (1;11) and several relatively frequent alleles are associated with red hair and fair skin (the RHC phenotype) and increased risk for melanoma and other skin cancers (12-14). Three frequent and penetrant melanoma-associated alleles, R151C, R160W, and D294H, are hypomorphic variants with reduced functional coupling to the cAMP cascade (1;15-17). Since cAMP was reported to trigger ERK activation in melanocytes (6), signalling to the ERKs might also be impaired for these variants. However, this possibility has never been investigated.

We have analyzed ERK activation by the melanoma-associated *MC1R* variants. We show that the R151C, R160W and D294H variants expressed in PC12 cells activate the ERKs as effectively as wild-type *MC1R* (wt*MC1R*) in spite of a strong impairment of cAMP production. We also show that the adenylyl cyclase activator forskolin increases cAMP levels in human melanoma cells but does not trigger ERK activation. These results open unexpected perspectives on the functional coupling of *MC1R* to ERK signalling in melanocytes.

EXPERIMENTAL PROCEDURES.

Cell culture and transfection.

Cell culture reagents were from Gibco BRL-Life Technologies (Gaithersburg, MD). HBL human melanoma cells were cultured as described previously (18). PC12 cells grown in DMEM supplemented with 15% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin sulphate, were transfected with 0.3 µg plasmid DNA/well, using Lipofectamine (Invitrogen, Carlsbad, CA). Expression constructs were prepared in pcDNA3 (Invitrogen) (17;18).

Functional assays.

Cells grown in 12-well plates were transfected, serum-deprived for 12-24h and stimulated as required with the α MSH analogue [Nle⁴, D-Phe⁷] α MSH (NDP-MSH) or nerve growth factor (NGF) (Calbiochem, Darmstadt, Germany) at 10⁻⁷ M and 100 ng/ml final concentrations, respectively, unless stated otherwise. Media were aspirated and the cells washed with 800 µl ice-cold phosphate buffered saline (PBS), lysed with 200 µl/well 0.1N HCl preheated at 70° C, and scrapped. The mix was freeze-dried, washed with 100 µl H₂O and freeze-dried again. cAMP was measured with a commercial radioimmunoassay from Amersham Pharmacia Biotech (Little Chalfont, UK). Parallel dishes were used for protein determination

with bicinchoninic acid. To estimate ERK activation, the levels of phosphorylated ERK (pERK) were analyzed by Western blot. Cells were solubilized in 75 μ l PBS supplemented with PMSF 100 ng/ml, 1% Igepal and 1% phosphatase inhibitor mix from Calbiochem. Samples were centrifuged (105,000xg, 30 min) and a volume of supernatant containing 30 μ g protein was electrophoresed and blotted as described (17). Blots were probed with an anti-pERK1/2 rabbit polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and stained with a chemiluminescent substrate. Comparable loading was ascertained by stripping and reprobing the membranes with an anti-ERK2 antibody. Quantification of band intensity was performed with ImageJ (available at rsb.info.nih.gov/ij).

RESULTS AND DISCUSSION.

MC1R signalling to ERK in heterologous systems.

In order to find an appropriate cellular model to study MC1R-mediated ERK activation, we measured pERK levels and cAMP concentrations in HBL human melanoma cells and PC12 cells transiently expressing wtMC1R, following stimulation with NDP-MSH. HBL cells were selected because they are wild-type for MC1R (18), N-RAS and B-RAF (our unpublished results) and PC12 cells because they are one of the few cell types where cAMP triggers ERK activation, as reported for melanocytes (6;10;19;20). ERK1/2 activation was detected by Western blot using an antibody specific for the active enzyme phosphorylated at Thr202 and Tyr204, and cAMP was determined by radioimmunoassay (17). In HBL cells, pERK signals increased rapidly and transiently upon treatment with NDP-MSH, with maximal activation 5 min after agonist challenge (Fig. 1A). PC12 cells expressing MC1R also showed an increase in ERK phosphorylation, with maximal values 15 min after stimulation followed by a slower decrease towards baseline (Fig. 1B). NDP-MSH strongly increased intracellular cAMP in both cell types (Fig. 1C). Accordingly, transient transfection of PC12 cells was used to compare signalling from wild-type or variant MC1R to both the cAMP and the ERK pathways, under identical conditions of cellular setting and receptor expression levels.

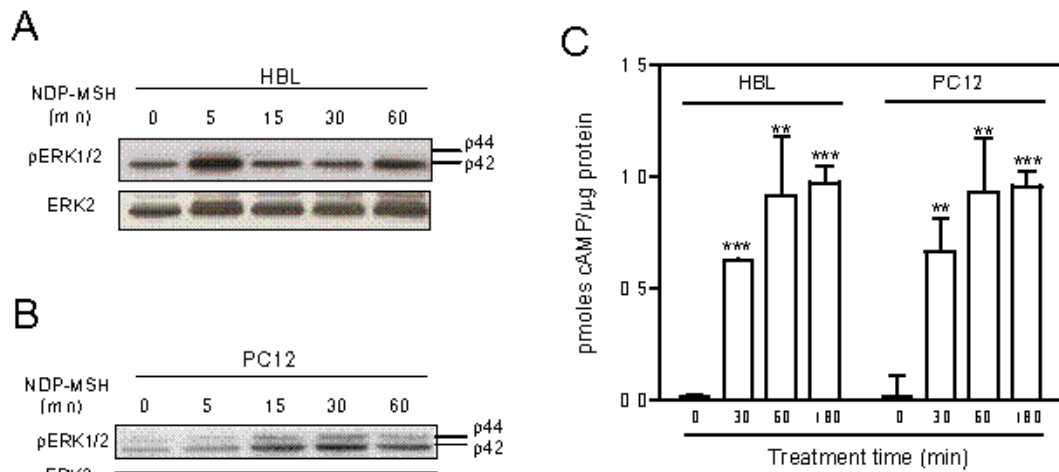


Fig. 1. MC1R-mediated ERK activation in melanocytic and heterologous cells. A, HBL human melanoma cells were serum-deprived and stimulated with 10^{-7} M NDP-MSH for the times shown. Cell lysates were Western blotted for pERK1/2 and total ERK2 as loading control. B, PC12 cells transfected to express wtMC1R were serum-deprived, stimulated with NDP-MSH and probed for ERK activation. C, Agonist-induced cAMP production in HBL and PC12 cells expressing wtMC1R. ** $p < 0.005$, *** $p < 0.0001$.

Similar activation of ERKs by wild-type and variant MC1R.

The melanoma-associated R151C, R160W and D294H mutants show diminished ability to stimulate cAMP synthesis in melanocytic cells (1;15-17). Functional assays in PC12 cells yielded similar results, confirming that these alleles are hypomorphic variants (Fig. 2A). D294H was the most severely impaired mutant, consistent with results obtained in other cell types (17;21). Surprisingly, ERK activation was equally intense in cells expressing variant or wtMC1R (Fig. 2B). Moreover, the kinetics of ERK phosphorylation was also similar for wtMC1R and the RHC variants, with maximal activation occurring roughly 15 min after agonist challenge. Stripping of membranes and reprobing with an anti-Flag monoclonal antibody confirmed comparable levels of expression of all MC1R forms (not shown). The ERK1/2 cascade was not activated by NDP-MSH in cells transfected with two complete loss-of-function mutants unable to reach the plasma membrane, L93R and R162P (1) or with empty vector (not shown), thus confirming the specificity of the response.

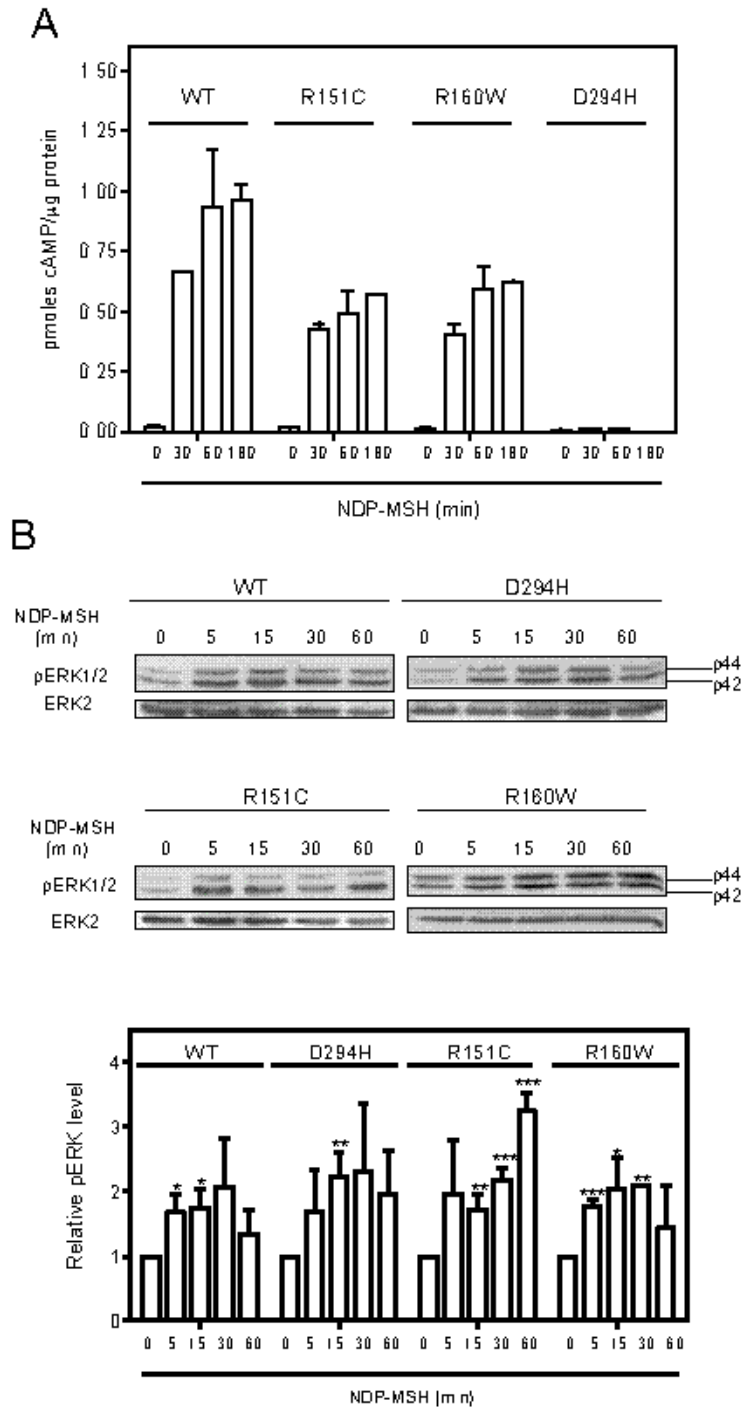


Fig. 2. Efficient signalling of RHC variants to the ERKs. PC12 cells transfected with wtMC1R or the RHC variants R151C, R160W or D294H were challenged with 10^{-7} M NDP-MSH. A, Kinetics of cAMP generation upon stimulation with 10^{-7} M NDP-MSH. For all time points, cAMP increases over untreated controls were statistically significant ($p < 0.005$) except for the D294H variant, that did not display significant responses. B, Kinetics of ERK activation by wild-type or variant MC1R. Representative blots and quantification of 3 experiments (mean \pm standard deviation, right) are shown. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$.

Sustained ERK activation in PC12 cells is associated with their differentiation into sympathetic cells, a process characterized by neurite outgrowth (19;22). To further demonstrate wtMC1R and RHC variant signalling to the ERKs, we analyzed NDP-MSH-mediated neurite outgrowth. PC12 cells expressing wtMC1R and stimulated with NDP-MSH or NGF as a positive control developed neurites (Fig. 3A). Neurite outgrowth was dependent on ERK activation since it was abolished by the MEK inhibitor PD98059. The percentage of cells developing neurites increased significantly upon NDP-MSH treatment ($28 \pm 4 \%$, $p < 0.005$) compared with untreated controls and was roughly consistent with the transfection efficiency, suggesting that most MC1R-expressing cells were responsive. NDP-MSH failed to induce neurite outgrowth in cells transfected with empty vector (not shown). Statistically significant ($p < 0.005$) NDP-MSH-induced neurite outgrowth was also observed in cells expressing the RHC variants. Although the length of the dendritic processes was similar, the percentage of cells developing neurites was lower compared with wild-type (Fig. 3B). This smaller efficiency might be related with their impaired cAMP signalling, since cAMP has been shown to cooperate with ERK signalling in promoting neuritogenesis in PC12 cells (19).

Thus, although the RHC variants showed reduced functional coupling to the cAMP pathway, they efficiently stimulated ERK activity.

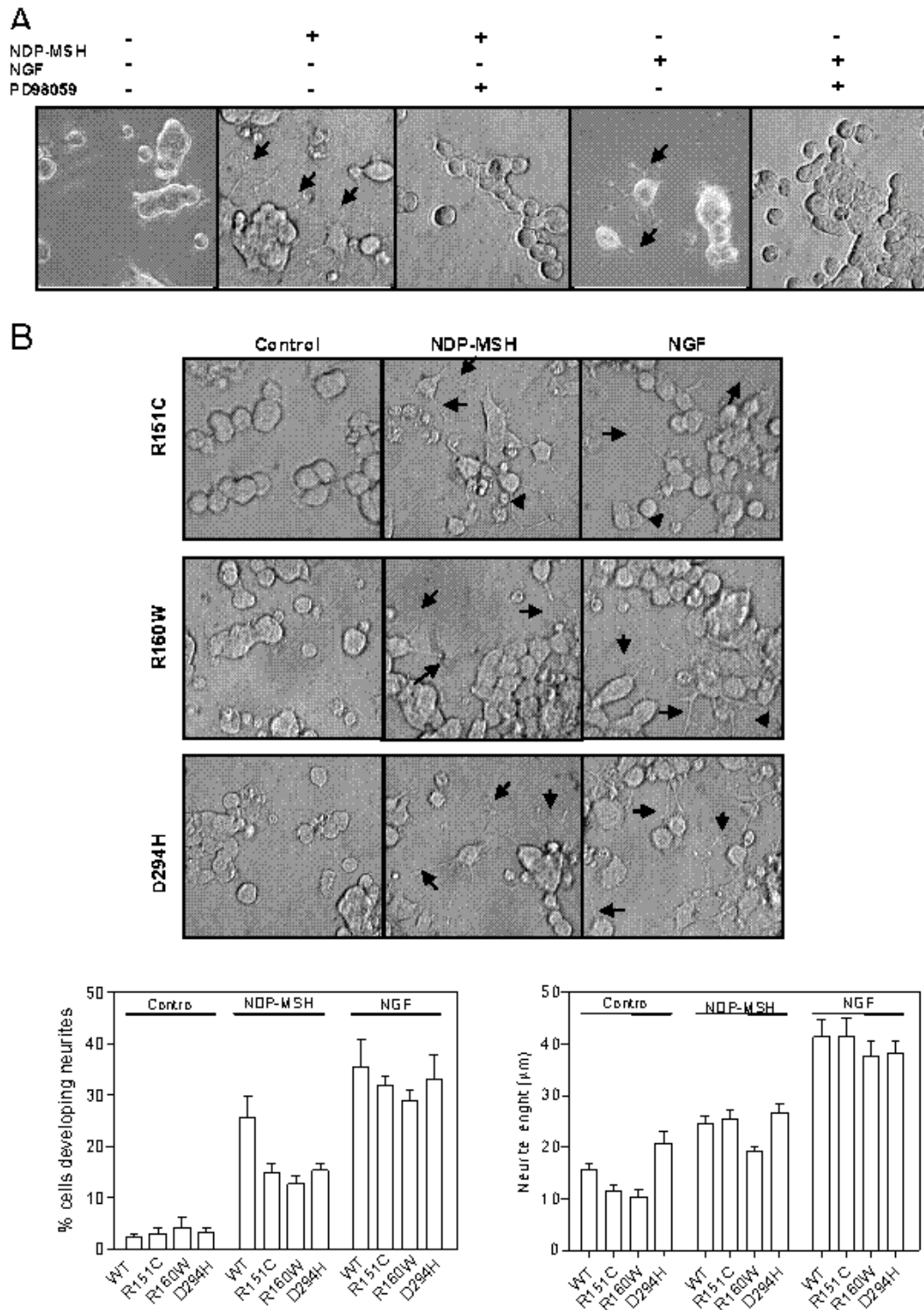


Fig. 3. MC1R-dependent neurite outgrowth in PC12 cells. A, Cells expressing wtMC1R were treated with NDP-MSH (10^{-7} M) or NGF (100 ng/ml) for 48h, with or without PD98059 (50 μ M). B, Neurite outgrowth in cells expressing variant MC1R. Cells expressing the variants indicated on the left of each row were treated with NDP-MSH or NGF as indicated. The lower graphs show the percentage of cells developing neurites (left), and the mean length of dendritic processes (right, results shown as mean \pm sem).

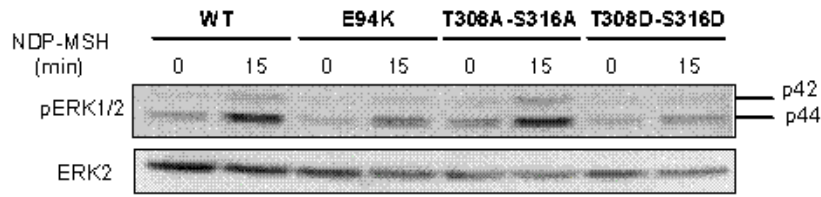
ERK activation is induced at lower agonist concentration than cAMP synthesis.

Treatment of B16 mouse melanoma cells with α MSH or cAMP elevating agents has been reported to activate ERK signalling in a RAS and B-RAF-dependent fashion (6), suggesting that MC1R-dependent ERK activation in these cells is mediated by cAMP. However, the differential effect of the RHC mutations on signalling to cAMP or the ERKs indicated that ERK activation by MC1R might, in fact, be independent on cAMP production. To further explore the relationship between cAMP and ERK signalling, we analyzed the functional coupling of 3 artificial variants with altered signalling to cAMP: E94K, a mutant that signals constitutively to cAMP in the absence of agonist (18), T308A-S316A and T308D-S316D, two double mutants where the Thr308 and Ser316 targets of G protein coupled receptor kinase 6 are mutated to Ala and Asp respectively. These double mutants display a dramatically reduced functional coupling to cAMP (17). Moreover, whereas T308A-S316A is resistant to internalization due to inability to undergo GRK-dependent phosphorylation, T308D-S316D is constitutively internalized and associated with endocytic vesicles (17). As shown in Fig. 4A, the internalization-resistant T308A-S316A mutant with impaired signalling to cAMP was as efficient as wtMC1R in triggering ERK phosphorylation following stimulation with NDP-MSH. Moreover, neither the constitutively active E94K variant nor the constitutively internalized T308D-S316D mutant was more potent than wtMC1R in activating the ERKs. These data suggest that MC1R-mediated ERK activation is independent on receptor internalization, and does not involve cAMP synthesis. Consistent with independence on cAMP, treatment of HBL human melanoma cells with the potent adenylyl cyclase activator forskolin (10 μ M, 15 min) strongly increased intracellular cAMP levels, but failed to activate the ERKs (Fig. 4B). Conversely NDP-MSH promoted ERK phosphorylation while yielding a smaller stimulation of cAMP synthesis. The inability of forskolin to trigger ERK phosphorylation was confirmed in kinetic experiments where HBL cells were treated with the drug from 5 to 60 min (not shown).

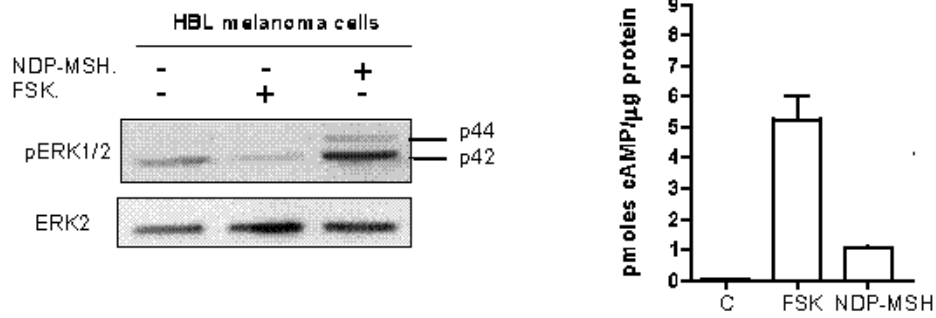
Since activation of the cAMP and ERK pathways are most likely independent events, they might display different NDP-MSH dose-response curves. To check this possibility, PC12 cells expressing wtMC1R were stimulated with NDP-MSH concentrations from 10^{-12} to 10^{-7} M, and pERK and cAMP intracellular levels were measured. ERK phosphorylation was detectable at concentrations as low as 10^{-12} M (Fig. 4C), whereas cAMP increases were only detected at 10^{-10} M or higher (Fig. 4D). Accordingly, the dose-response curve for ERK phosphorylation was

dramatically left-shifted by up to 3 logs relative to the cAMP curve. These differences in dose-response curves suggest that ERK activation might involve effector(s) with higher affinity for MC1R than the G_s protein responsible for coupling to cAMP. Experiments aiming at the identification of this effector have excluded the involvement of PKC or calcium fluxes (not shown). Moreover, the different dose-response curves for ERK activation and G_{αs}-dependent activation of cAMP synthesis (Fig. 4D) argue against involvement of free βγ dimers in triggering ERK phosphorylation, although a role for βγ dimers cannot be formally excluded based on the results reported here. Finally, data obtained with the T308A-S316A and T308D-S316D mutants also suggest that ERK activation is not related with the arrestin-dependent formation of endocytic vesicles (8). Therefore, the precise mechanisms coupling MC1R activation to ERK phosphorylation remain unknown.

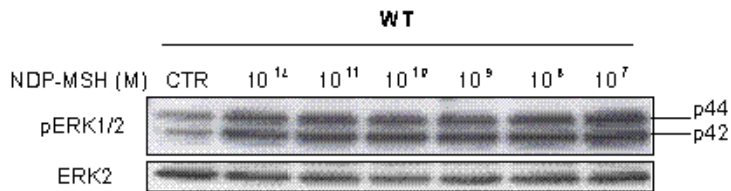
A



B



C



D

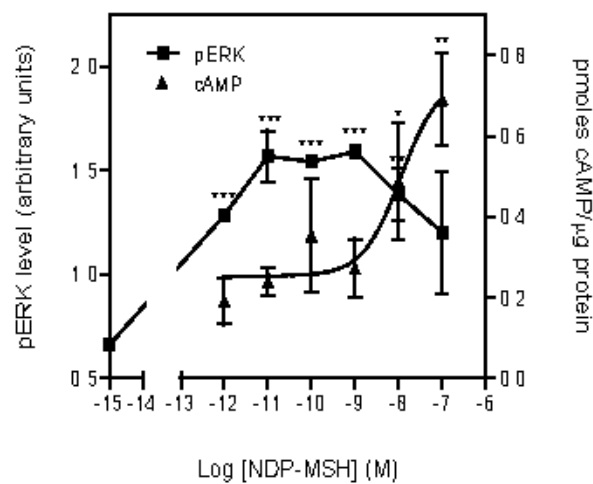


Fig. 4. A, Agonist-induced ERK activation by MC1R mutants with altered signalling to cAMP and/or internalization. PC12 cells were transfected with a constitutively active MC1R variant (E94K), or variants with decreased signalling to cAMP and impaired (T308A-S316A) or

enhanced (T308D-S316D) internalization. Cells were stimulated (10^{-7} M NDP-MSH, 15 min) and Western blotted for pERK. A blot representative of 3 experiments is shown. B, Activation of cAMP synthesis but not the ERKs in HBL human melanoma cells stimulated with Fsk. HBL cells were stimulated with forskolin (Fsk, $10 \mu\text{M}$, 15 min) or NDP-MSH (10^{-7} M, 5 min), lysed, and probed for pERK levels (left) or cAMP (right). C stands for control. Similar results have been obtained in 3 independent experiments. C, ERK phosphorylation in wtMC1R-expressing PC12 cells challenged with increasing concentrations of NDP-MSH (15 min). CTR, control. D, Comparison of pERK (squares, left axis) and cAMP levels (triangles, right axis) in cells treated with increasing concentrations of NDP-MSH. Results are the mean \pm standard deviation ($n = 4$). * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$.

In summary, we showed that the frequent melanoma-associated MC1R variants R151C, R160W and D294H are loss-of-function forms in signalling to cAMP, but activate the ERKs as efficiently as wild-type. This suggests that coupling of MC1R activation to cAMP or to ERKs are independent events, and provides a remarkable example of differential impact of point mutations on two signalling pathways. Moreover, ERK phosphorylation is triggered at much lower agonist concentrations than cAMP synthesis, suggesting that melanocytes might display a graded spectrum of responses to melanocortin ligands of MC1R, with ERK-dependent processes occurring at lower agonist levels than cAMP-dependent events. Finally, it also suggests that melanocytes harbouring variant MC1R might display an imbalanced functional response to melanocortins, with normal signalling to the ERKs but reduced coupling to cAMP.

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CAPÍTULO 3

La señalización del receptor de melanocortinas 1 a las proteínas quinasas activadas por mitógenos ERK1 y ERK2 implica la transactivación de c-KIT.

RESUMEN.

El receptor de melanocortinas 1 (MC1R), receptor acoplado a la proteína G_s expresado en melanocitos, es uno de los principales determinantes de la pigmentación cutánea, del fototipo y del riesgo de padecer cáncer. Tras la estimulación con la hormona estimulante del melanocito (α MSH), el MC1R señala a través de la vía del AMPc y la vía de las proteínas quinasas activadas por mitógenos ERK1 y ERK2. En melanocitos de ratón, la activación de las ERK por la unión de α MSH al Mc1r depende del AMPc y, los melanocitos se consideran un paradigma para la activación de ERK dependiente de AMPc. Sin embargo, variantes alélicas del MC1R humano asociadas con pelo rojo, piel clara (fenotipo RHC) y un riesgo aumentado de cáncer de piel muestran una señalización reducida vía AMPc, pero activan las ERK tan eficientemente como el receptor silvestre en células heterólogas, lo que sugiere que en melanocitos humanos la señalización a través del AMPc y la señalización a través de las ERK son dos vías independientes. En el presente trabajo, mostramos que la señalización del MC1R activó la vía de las ERKs en melanocitos normales humanos y células de melanoma que expresan niveles fisiológicos de las variantes RHC endógenas. La activación de ERK fue comparable tanto para el MC1R silvestre como para las variantes alélicas del receptor y, además, independiente del AMPc; ya que no se activó ni por estimulación de la síntesis de AMPc con forskolina, ni se bloqueó por el inhibidor de la adenilato ciclasa 2', 5'-dideoxiadenosina. La estimulación del MC1R con α MSH no condujo a la activación de PKC y la activación de ERK no se vio afectada por inhibidores de PKC. Por el contrario, la interferencia farmacológica, estudios de ARN pequeños de interferencia (siRNA), los perfiles de expresión y los experimentos de reconstitución funcional mostraron que la activación de ERK inducida por α MSH resultó de la transactivación mediada por la tirosina quinasa Src de c-KIT, un receptor tirosina quinasa esencial para la proliferación, diferenciación y supervivencia de precursores del melanocito, lo que demuestra la unión funcional entre c-KIT y el MC1R. Además, este fenómeno de transactivación es único, ya que no

se ve afectado por mutaciones naturales que reducen la señalización canónica del MC1R a través de la vía del AMPc.

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Signalling from the Human Melanocortin 1 Receptor to ERK1 and ERK2 Mitogen-Activated Protein Kinases Involves Transactivation of c-KIT.

ABSTRACT.

MC1R, a Gs protein-coupled receptor expressed in melanocytes, is a major determinant of skin pigmentation, phototype and cancer risk. Upon stimulation by α melanocyte-stimulating hormone (α MSH), MC1R triggers the cAMP and ERK1/ERK2 mitogen-activated protein kinase pathways. In mouse melanocytes, ERK activation by α MSH binding to Mc1r depends on cAMP, and melanocytes are considered a paradigm for cAMP-dependent ERK activation. However, human MC1R variants associated with red hair, fair skin (RHC phenotype) and increased skin cancer risk display reduced cAMP signalling, but activate ERKs as efficiently as wild-type (WT) in heterologous cells, suggesting independent signalling to ERKs and cAMP in human melanocytes. We show that MC1R signalling activated the ERK pathway in normal human melanocytes and melanoma cells expressing physiological levels of endogenous RHC variants. ERK activation was comparable for WT and mutant MC1R and was independent on cAMP, as it was neither triggered by stimulation of cAMP synthesis with forskolin, nor blocked by the adenylyl cyclase inhibitor 2',5'-dideoxyadenosine. Stimulation of MC1R with α MSH did not lead to PKC activation and ERK activation was unaffected by PKC inhibitors. Conversely, pharmacological interference, siRNA studies, expression profiles and functional reconstitution experiments showed that α MSH-induced ERK activation resulted from Src tyrosine kinase-mediated transactivation of c-KIT, a receptor tyrosine kinase essential for proliferation, differentiation and survival of melanocyte precursors, thus demonstrating a functional link between c-KIT and MC1R. Moreover, this transactivation phenomenon is unique as it is unaffected by natural mutations impairing canonical MC1R signalling through the cAMP pathway.

INTRODUCTION.

Melanocytes are skin cells specialized in the biosynthesis of photoprotective melanin pigments. Their proliferation and the synthesis of melanins are tightly controlled by interacting chemical and physical cues (1,2). Among the signalling cascades triggered by these signals, the cAMP and the mitogen-activated protein kinase (MAPK) extracellular signal-regulated (ERK) ERK1 and ERK2 pathways have been intensively analyzed. cAMP induces melanocyte differentiation *in vitro* (3) and *in vivo* (4), and ERK signalling is crucial for the control of both proliferation (5) and

melanogenesis, through the activation of the cyclic AMP response element binding protein (CREB) (6) and the microphthalmia-associated transcription factor (MITF), a master regulator of melanocyte development, differentiation and proliferation (7).

cAMP production in melanocytes is strongly stimulated by α melanocyte stimulating hormone (α MSH) and related peptide hormones collectively named melanocortins (MCs). MCs activate the melanocortin 1 receptor (MC1R), a G protein-coupled receptor (GPCR) that regulates the amount and type of melanin pigments and is a major determinant of skin phototype, sensitivity to ultraviolet radiation and skin cancer risk (8). cAMP is responsible for key melanogenic effects of α MSH (3), namely activation of the rate-limiting enzyme tyrosinase, and a switch from production of light-coloured and poorly photoprotective pheomelanins to darker and more photoprotective eumelanins (9). Many of these cAMP-dependent differentiation effects are due to transcriptional induction of MITF.

On the other hand, the MAPK module involving ERK1 and ERK2 is a major intracellular signalling pathway that controls key cellular decisions such as proliferation, differentiation or migration (10). This pathway is normally initiated by binding of growth factors to cell surface tyrosine kinase receptors (RTKs) and recruitment of Grb2 and the guanine nucleotide exchange factor Sos leading to activation of RAS. The GTP-bound active form of RAS then activates members of the RAF family of protein kinases, which in turn phosphorylate and thereby activate the MAPK kinase MEK (11;12). MEK phosphorylates and activates ERK1 and ERK2. Active ERKs phosphorylate cytoplasmic and cytoskeletal proteins, and translocate to the nucleus to regulate the activity of several transcription factors (13). The crucial role of this pathway in melanocytes is demonstrated by the occurrence of mutations in *BRAF* or *NRAS* in roughly 50% or 20-30% of human melanomas, respectively (14-17). Since *NRAS* and *BRAF* mutations are mutually exclusive in melanoma (18), they collectively account for ERK hyperactivation in over 75% of these tumours.

Within melanocytes, the ERK module is activated by c-KIT, a RTK crucial for melanogenesis, proliferation, migration, and survival of the pigment-producing cells (19). In mice, c-KIT maps to the dominant *white spotting* (*w*) locus, and its endogenous ligand SCF (stem cell factor, KIT-ligand, mast cell growth factor) to the *s/l* locus. Mutations in either one of these loci cause a pleiotropic phenotype with white spotting of the fur due to absence of melanocytes, lack of mast cells and defects in haematopoiesis and germ cell development (20). c-KIT mutations have also been identified in human piebaldism (21;22) and at low frequency in human melanomas (23), and c-KIT expression is often lost in these tumors (24;25).

Several GPCRs can activate the ERK module by a variety of mechanisms that rely on the activity of second messenger regulated protein kinases such as PKC, non-receptor tyrosine kinases (non-RTKs) such as Src, or on the generation of intracellular signalling complexes formed by binding of β -arrestins or other scaffolds to the activated GPCRs (26-28). Moreover, GPCRs can stimulate cytosolic or membrane-bound metalloproteases which cleave the membrane anchoring domain of RTK ligands and release the active agonist, thus promoting receptor activation (28-30).

In mouse melanoma cells, MC1R activation by MCs is positively coupled to cAMP and ERK signalling. Based on data from the B16 mouse melanoma model it has been proposed that ERK activation in melanocytic cells is due to cAMP-dependent but PKA-independent activation of NRAS and BRAF (31-33). However, recent findings suggest that the situation in human melanocytes might be different. The human *MC1R* gene is extremely polymorphic (34) and several variant alleles are associated with red hair and fair skin (the RHC phenotype) (8;35-37) and increased risk for melanoma and nonmelanoma skin cancer (38;39). Three frequent and highly penetrant RHC alleles, R151C, R160W, and D294H (36;40) are diminished function forms with reduced functional coupling to the cAMP cascade (41-47). At least for R151C and R160W this functional impairment results mainly from aberrant trafficking with intracellular retention and reduced cell surface expression, rather than from binding or signalling defects (41;44;46). However, these hypomorphic variants retain normal signalling to the ERKs when expressed in heterologous PC12 cells (48). Moreover, attempts to detect ERK activation in normal human melanocytes (NHM) treated with the strong cAMP inducer forskolin (FSK) have been unsuccessful (49). In addition, activation of CREB by ERK-dependent phosphorylation has been reported in NHMs treated with mitogenic RTK ligands, but not with cAMP analogs (6). These observations suggest that activation of the ERK module by human MC1R might be due to unknown cAMP-independent events.

Here we show that the RHC variants of the MC1R with reduced or absent signalling to cAMP activate the ERKs as efficiently as wild type (WT) MC1R in a physiological setting, both in human melanoma cells and NHMs. We also show that ERK activation is independent on cAMP, PKA, PKC or Ca^{2+} . Instead, positive functional coupling of MC1R to the ERKs relies on the transactivation of c-KIT. Moreover, our data show that α MSH activates the Src non-RTK in melanoma cells independently on cAMP, and that Src is involved in activation of the ERKs downstream of MC1R, most likely by mediating α MSH-induced c-KIT transactivation. These findings might have important and unexpected implications for our understanding of the functional connections of the main signalling pathways controlling human melanocyte proliferation

and differentiation. Moreover they provide new insights for the rational design of photoprotective strategies (4).

EXPERIMENTAL PROCEDURES.

Materials.

A radioimmunoassay kit for cAMP was from Amersham Pharmacia Biotech (Little Chalfont, UK). The transfection reagent Lipofectamine 2000 and competent DH5 α cells were from Invitrogen, (Carlsbad, CA). α MSH, ET-1, 2',5'-DDA, PMA, nifedipine, PD98059, (\pm)-Bay K8644, Igepal CA-630, BSA, EDTA, PMSF, bicinchoninic acid, ampicilin, β -mercaptoethanol and SDS were from Sigma Chemical Company (St. Louis, MO, USA). The synthetic α MSH analogue [Nle⁴, D-Phe⁷] α MSH (NDP-MSH), FSK, Ro31-8425, AG 1478, ISCK03, GTP-14564, PD153035, NA-PP-1 and PP-2 were from Calbiochem (Damstadt, Germany). The c-KIT inhibitor Sunitinib malate was from Tocris Bioscience (Bristol, UK), and the pan-Src inhibitor Dasatinib from Biotang Inc (Waltham, MA, USA). The cAMP analogue Rp-cAMP was from Biolog (Bremen, Germany). Recombinant human SCF was from Genscript (Piscataway, NJ). The anti-pERK1/2 (pERK1/2) rabbit polyclonal IgG, the anti-ERK2 rabbit polyclonal IgG, the anti-pTyr (PY99) mouse monoclonal antibody, the anti-phospho-c-KIT (Tyr 721) rabbit polyclonal antibody, the anti-p-c-Src (Tyr 530) rabbit polyclonal antibody and the anti-c-Src mouse monoclonal antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-c-KIT (D13A2) rabbit monoclonal antibody and the anti-phospho-c-KIT (Tyr703) rabbit monoclonal antibody were from Cell Signaling. Reagents for SDS-PAGE and Western blot were from Bio-Rad (Richmond, CA, USA). Other reagents were from Merck (Darmstadt, Germany) or Prolabo (Barcelona, Spain).

Cell culture.

Cell culture reagents were from Gibco BRL-Life Technologies (Gaithersburg, MD). PC12 cells were grown in 12-well dishes using DMEM, supplemented with 15% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin sulfate. HEK293 cells were grown in RPMI 1640, with 10% FBS, and the same antibiotics. HBL (LOCE-MM1) human melanoma cells were grown in MEM with antibiotics and 10% FCS. LOCE-MM61, 79, 94, 98, 104 and 117 human melanoma cells (established in the LOCE, Université Libre de Bruxelles, Belgium) were cultured in HAM F-10 medium supplemented with 10% FBS and antibiotics (100 U/ml penicillin, 100 μ g/ml kanamycin sulphate, 100 μ g/ml streptomycin). Primary NHM cultures were obtained from neonatal foreskins as described (86;87), with the approval of the University of Cincinnati Medical Centre Institutional Review Board. Cultures were grown in melanocyte growth medium

containing bovine pituitary extract. This was removed 2 to 3 days prior to, and for the duration of, each experiment.

Expression constructs and transfection.

All expression constructs were prepared in pcDNA3 (Invitrogen). The following expression constructs have been previously described: WT-MC1R, the Flag-tagged RHC variants R151C, R160W and D294H (44;45;88). NRAS Q61R was amplified by PCR and cloned into pcDNA3. cDNA encoding for human c-KIT was obtained from Openbiosystems and was subcloned into pcDNA3. All constructs were verified by double strand automated sequencing as described (50).

Cells grown to approximately 80% confluence were transfected with 0.3 µg plasmid DNA/well, using Opti-MEM to dilute DNA and Lipofectamine. Stable transfectants were obtained as described (45;52), and were cultured in the presence of 800 µg/ml G418 sulfate. Transfections with siRNA were performed as previously described using a pool of 4 target-specific 20-25 mer siRNAs (Santa Cruz Biotechnology) and two individual c-KIT specific siRNA (Applied Biosystems/ Ambion, CA, USA) to knock down c-KIT expression. As a negative control we used a scrambled sequence that will not lead to the specific degradation of any mRNA. Cells were incubated with the transfection mixture for 6h and then normal growth medium was replaced, and cells were further grown for 48h.

Functional assays.

Cell surface expression of WT and variant MC1R was determined by radioligand binding analysis as described previously (46;47). For cAMP measurements, cells grown in 12-well plates were transfected, serum-deprived for 12-24h and stimulated as required. The medium was aspirated and the cells quickly washed with 800 µl ice-cold PBS. Cells were lysed with 200 µl/well 0.1N HCl preheated at 70° C, and scrapped. The mix was freeze-dried, washed with 100 µl H₂O and freeze-dried again. cAMP was measured with a commercial radioimmunoassay, as per instructions. All cAMP assays were repeated at least twice and for each independent experiment, duplicate or triplicate dishes were analyzed. Parallel dishes were used for protein determination with the bicinchoninic acid method.

Western blot.

Cells were washed twice with PBS and solubilized in 75 µl solubilization buffer (PMSF 100 ng/ml, 1% Igepal and 1% phosphatase inhibitor mix containing 200 mM imidazole, 100 mM NaF, 115 mM sodium molybdate, 100 mM sodium o-vanadate and 400 mM sodium tartrate). Samples were centrifuged (105,000xg, 30 min) and a volume of supernatant containing 30 µg protein was mixed (2:1 ratio) with electrophoresis

sample buffer (180 mM Tris-HCl pH 6.8, 15% glycerol, 9% SDS, 0.075% bromophenol blue and 7.5% β -mercaptoethanol). Electrophoresis and Western blotting were performed as described (44;45;47). Blots were probed with the required antibodies and stained with a chemiluminescent substrate (Amersham). Comparable loading was ascertained by stripping and reprobing the membranes with an anti-ERK2 antibody. Stripping was performed by washing the membranes with PBS, followed by treatment with 0.5 N NaOH, 10 min at room temperature, and a final 10 min wash with PBS.

RESULTS.

ERK activation in NHMs expressing variant MC1R.

It has been reported that activation of the ERK pathway in mouse melanoma cells stimulated with α MSH is mediated by cAMP (31;32). Concerning human MC1R, it has been shown that the melanoma-associated RHC mutants R151C, R160W and D294H are hypomorphic in signalling via the cAMP pathway, either in melanocytic (44;45) or in heterologous cells (43-45), but they are as effective as WT in triggering ERK activation when expressed in heterologous PC12 cells (48). Since this result could be affected by receptor overexpression or depend on the cell type, we tested variant MC1R signalling to ERK in NHM cultures of defined *MC1R* genotype, at physiological levels of endogenous receptor expression and hormone concentration. No cultures homozygous for any of the RHC variants were found, but we identified two compound heterozygotes derived from Caucasian donors (R160W/D294H, culture 830c, and R151C/R160W, culture 1307c). These cultures do not respond to α MSH with detectable increases in intracellular cAMP or tyrosinase activity as opposed to a control culture (1377b) WT for MC1R (49;50). For these control cells, preliminary experiments showed a detectable ERK activation, as shown by increased levels of phosphorylated ERK (pERK) comparable for α MSH and its synthetic analogue NDP-MSH (not shown). In 1377b (WT for MC1R) or 830c (R160W/D294H) NHMs the ERKs were activated after a 15 min stimulation with 10^{-9} M α MSH (Fig. 1A). ERK activation was at least as intense in 830c mutant cells as in WT cells (Fig. 1B). A second heterozygote NHM culture (1307c, MC1R genotype R151C/R160W) yielded similar results with higher pERK levels than WT cells after hormonal stimulation (Fig. 1A and B). Therefore, activation of the ERKs by physiological concentrations of MC1R agonists was comparable in NHMs expressing endogenous WT or variant MC1R. Notably, the potent adenylyl cyclase stimulator FSK did not activate the ERKs in 1307 cells (Fig. 1A and B), nor in several other NHM cultures of different MC1R genotype tested to date (not

shown) whereas endothelin 1 treatment caused the expected strong stimulation of ERK phosphorylation

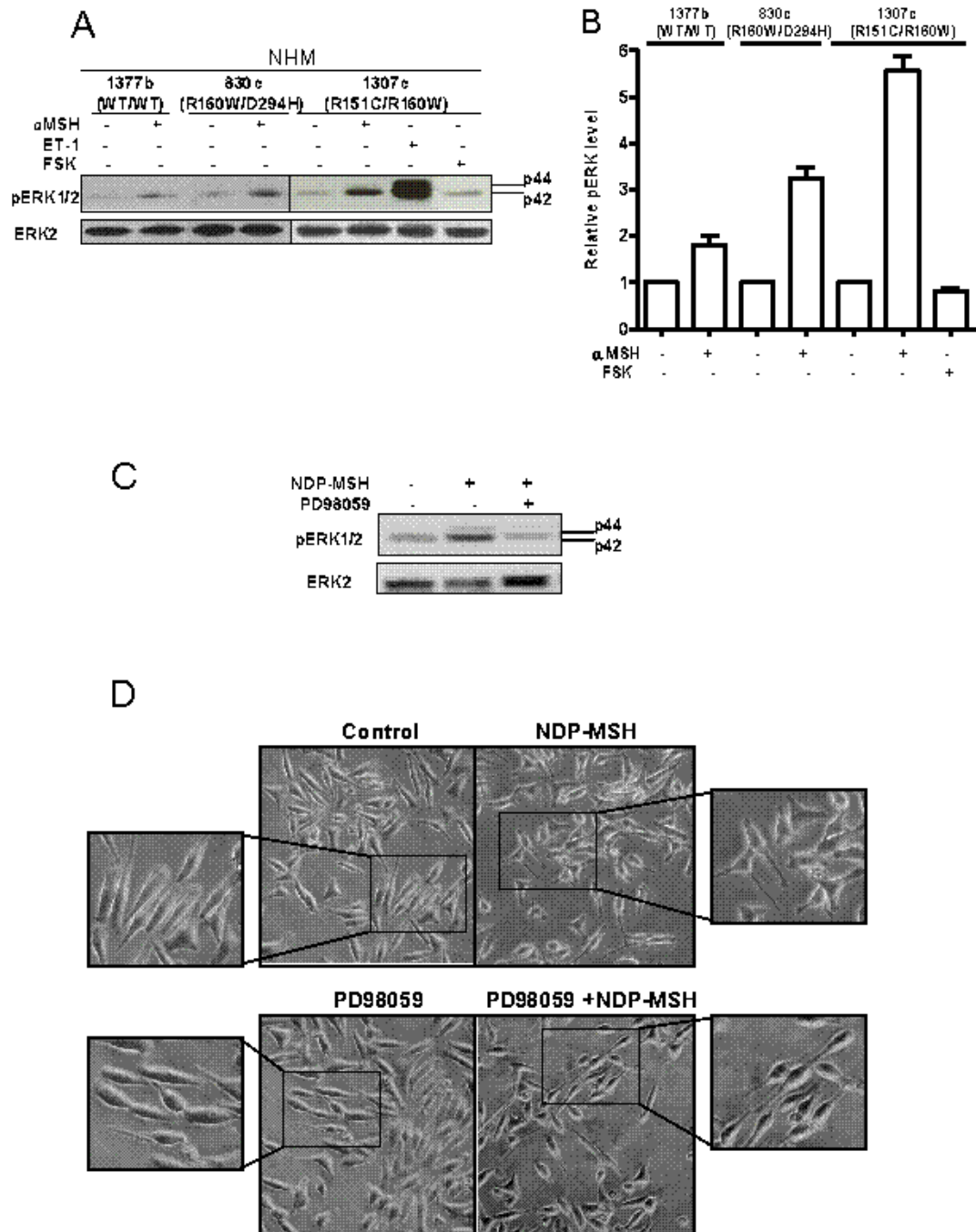


Fig. 1. Melanocortin signalling to ERK in normal human melanocytes (NHM) and melanoma cells of defined MC1R genotype. A, NHM expressing WT MC1R (1377b culture) or variant heterozygotes for the R160W and D294H alleles (830c culture) or the R151C and R160W variants (culture 1307c) were stimulated for 15 min with α MSH (10^{-9} M), 10^{-9} M endothelin 1 (ET-1), or 10^{-5} M FSK, as indicated. Cell extracts were analyzed for pERK by Western blot, and for total ERK2 as loading control. A representative blot out of two independent experiments is shown. B, Quantification of two independent experiments, normalized to the intensity of the

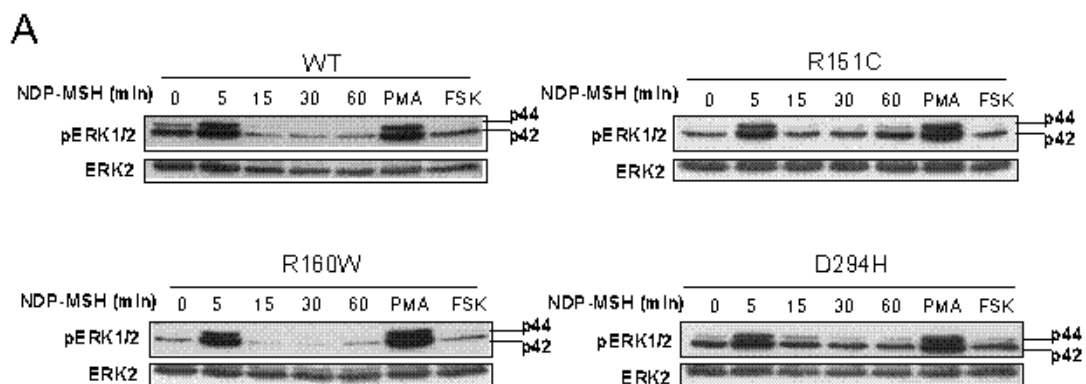
pERK signal in control cells. Results are given as mean \pm range. C, ERK activation by NDP-MSH in HBL human melanoma cells. Cells were serum-starved for 3h, then treated with NDP-MSH (10^{-7} M, 5 min) with or without the MEK inhibitor PD98059 (50 μ M). Cell lysates were analyzed for ERK1/2 phosphorylation and total ERK2 as loading control. D, ERK-dependent morphological changes induced by NDP-MSH in HBL cells. Cells grown in 6 well plates were treated with NDP-MSH for 48h in the presence or absence of PD98059, and photographed. The boxed areas in the central micrographs are enlarged and shown at a higher magnification.

The results presented above strongly suggest that in human melanocytic cells MC1R signals to the ERK module independently on cAMP. Since NHMs are extremely difficult to grow in the absence of potentially interfering cytokines and growth factors, we looked for a suitable human melanoma cell line to study the mechanism of ERK activation by MC1R. The tyrosinase-positive pigmented HBL melanoma cells are WT for the MC1R (47;51), as well as for NRAS and BRAF (our unpublished data). HBL cells stimulated with NDP-MSH showed increased ERK phosphorylation that was blocked by the MEK inhibitor PD98059 (Fig. 1C). A MC-induced change in cell shape with increased dendricity was equally abolished by PD98059 (Fig. 1D). Consistent with their WT MC1R genotype and with previous results (47), HBL cells responded to NDP-MSH with a strong time-dependent increase in intracellular cAMP with maximal levels between 15 and 30 min after addition of the MC agonist to the cultures (not shown). Given these normal and robust responses, HBL cells were subsequently used as models.

MC1R signalling to ERK in human melanoma cells is independent on cAMP.

The RHC variants R151C, R160W and D294H have been shown to act as partial dominant-negative mutants for WT MC1R signalling through the cAMP pathway (41;44;45). Therefore, it was of interest to analyze their effects on MC signalling to the ERKs in HBL cells. Cells were stably transfected with WT, R151C, R160W or D294H, and one representative clone for each variant expressing near-physiological levels of the receptor was selected (46) and analyzed. Cells expressing WT MC1R and challenged with NDP-MSH showed a marked increase in pERK, comparable with the response to the PKC activator PMA used as a positive control (Fig. 2A). pERK levels were maximal 5 min after stimulation and then returned to control levels. FSK failed to trigger detectable ERK activation. Clones expressing variant MC1R behaved similarly in terms of the strength and kinetics of ERK activation (Fig. 2A). Conversely, MC-induced cAMP production was strongly repressed in HBL cells expressing variant MC1R (Fig. 2B), consistent with a dominant negative behaviour of the mutants on

signalling through the cAMP pathway (41;44;45). Overall, these data showed that: i) MC1R activated the ERK module even at the low levels of cell surface expression found in parental HBL melanoma cells (45;46), ii) the RHC variants expressed in melanoma cells did not exert dominant negative effects on WT MC1R signalling to ERK, but apparently contributed to agonist-dependent ERK phosphorylation, and iii) a positive effect of cAMP on MC1R-dependent ERK activation was unlikely. On the other hand, the R151C and R160W receptor forms are strongly retained in intracellular compartments with cell surface expression levels approximately 5 times lower than WT (44;46), a feature that accounts for their decreased functional coupling to the cAMP pathway and for their dominant negative behaviour. Thus, the comparable stimulation of the ERK shown in Fig. 2A was surprising. We addressed this point by comparing the dose-response curves for agonist-induced stimulation of the cAMP and ERK pathways in HBL cells expressing physiological levels of the MC1R (~ 0.3 femtomoles of NDP-MSH binding sites/ μ g protein) (44;46). The saturation curve for ERK activation was strongly left-shifted relative to cAMP production, with EC50 values approximately two orders of magnitude lower (Fig. 2C and D). Similar results have been previously reported in a heterologous cellular model (48) although in this case the strong overexpression of the receptor protein did not allow for an adequate interpretation of the data. Therefore, much lower levels of hormone-receptor complexes were required to achieve maximal ERK stimulation compared with cAMP production, thus accounting for the smaller effects of RHC mutations on signalling to the ERK pathway. This suggests that the plasma membrane levels of MC1R, which are limiting for stimulation of the cAMP pathway (52) are not the limiting factor for ERK activation.



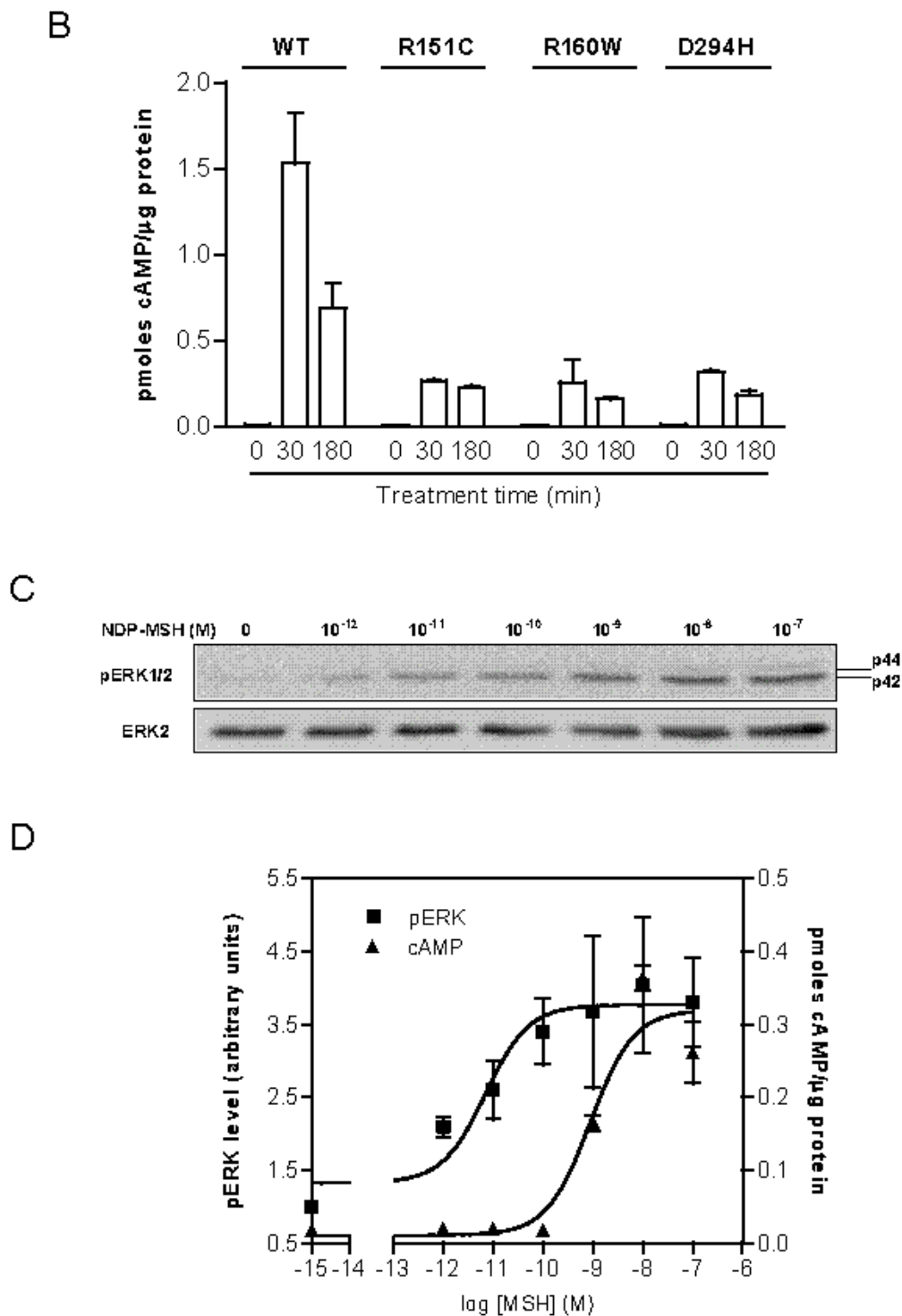
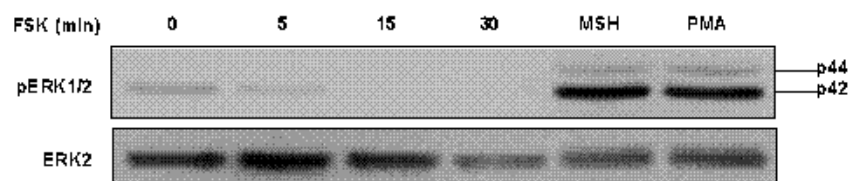


Fig. 2. ERK activation by NDP-MSH in human melanoma cells expressing variant MC1R. A, Clones of HBL cells stably expressing the MC1R forms indicated on top of each blot were stimulated with NDP-MSH (10^{-7} M) for the times shown, PMA (0.1 ng/ μ l, 15 min) or FSK (10^{-5} M, 15 min). Cell extracts were blotted for pERK. The experiment was repeated at least 3 times with similar results. B, Agonist-induced cAMP levels following stimulation with NDP-MSH in HBL

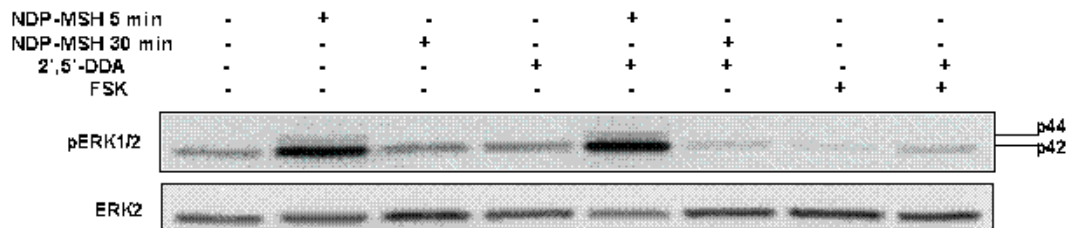
cells expressing WT or variant MC1R. Cells were serum-deprived for 3h before agonist challenge for 30 or 180 min. Results are given as mean \pm sem, $n \geq 4$. C, Dose dependence of NDP-MSH induced ERK phosphorylation in HBL cells expressing endogenous WT MC1R. Cells were serum deprived and challenged with the indicated concentrations of NDP-MSH for 5 min before estimation of pERK levels by Western blot. A representative blot out of 3 is shown. D, Comparison of the changes in pERK levels (squares, left axis) and cAMP accumulation (triangles, right axis) in HBL cells treated with the indicated concentrations of NDP-MSH. Results are the mean \pm s.e.m ($n \geq 3$).

However, since functional coupling of MC1R to the ERK pathway is currently attributed to cAMP (31;32), we performed additional experiments to address this issue. First, we incubated HBL cells with FSK for times ranging from 5 to 30 min, in order to exclude that failure to detect cAMP-dependent ERK activation could be due to inadequate selection of the time window (Fig. 3A). FSK did not activate the ERKs at the time points shown or at longer times (1 or 3 h, not shown), and even caused a small reduction in the levels of phosphorylated ERK. Although this inhibitory effect was difficult to quantify owing to the already low basal levels of ERK phosphorylation, it seemed reproducible (see for instance Fig. 3B). These data are in line with the inhibition of ERK signalling by cAMP in most cell types (53;54) and with reports of suppression of ERK activity following stimulation of cAMP production with FSK in RAS-mutated human melanoma cells (55). Next we compared the levels of cAMP and active ERKs in cells stimulated with NDP-MSH or FSK in the presence or absence of the specific adenylyl cyclase inhibitor 2', 5'-dideoxyadenosine (DDA). As shown in Fig. 3B and C, DDA had no effect on MC-dependent ERK activation, but effectively abolished MC or FSK-induced production of cAMP. Overall, these results show that cAMP is neither sufficient nor necessary to trigger ERK activation in human melanocytic cells.

A



B



C

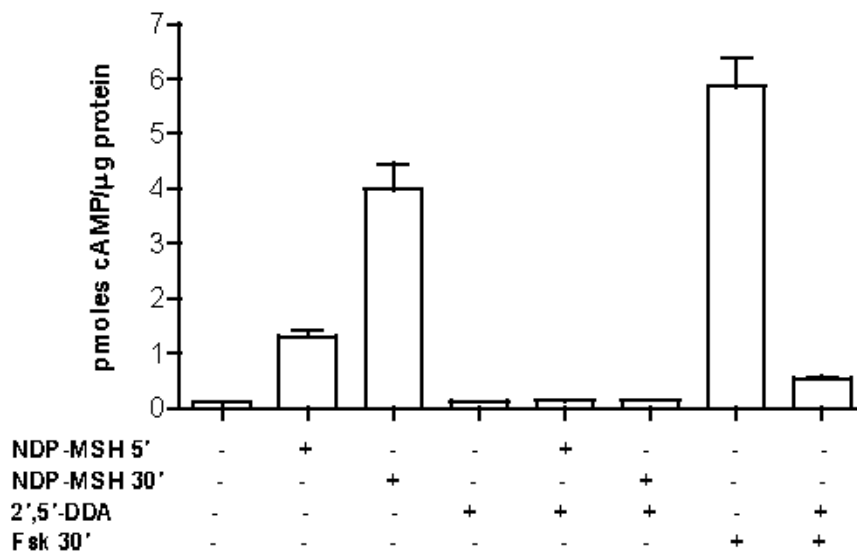


Fig. 3. ERK activation in HBL cells is independent on cAMP. A, Inability of FSK to induce ERK phosphorylation. Serum-starved HBL cells were incubated with FSK (10^{-5} M) for the times indicated, or with the positive controls NDP-MSH (10^{-7} M, 5 min) or PMA (0.1 ng/ μ l, 15 min). Cell lysates were analyzed for pERK or ERK2 as loading control. B, The adenylyl cyclase inhibitor DDA does not block MC1R-induced ERK activation. HBL cells were challenged with NDP-MSH (10^{-7} M, 5 or 30 min) or FSK (10^{-5} M, 30 min), with or without DDA (2.5 mM, 30 min). ERK phosphorylation was detected by Western blot. C, HBL cells were treated as above and intracellular cAMP levels were determined. Results are given as mean \pm sem, $n \geq 4$.

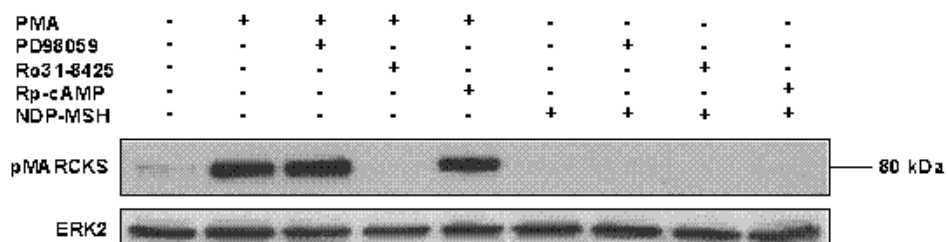
Functional coupling of MC1R to the ERK module involves transactivation of a RTK.

Regulation of ERK signalling by GPCRs is complex and dependent on the cellular context. In addition to the cAMP pathway, the PKC cascade links several GPCRs to the ERKs, either by direct PKC-dependent phosphorylation of members of the ERK module, or indirectly via activation of non-RTKs such as Src (27;28;30). ERK activation can also depend on G $\beta\gamma$ complexes dissociated from the G α subunit upon

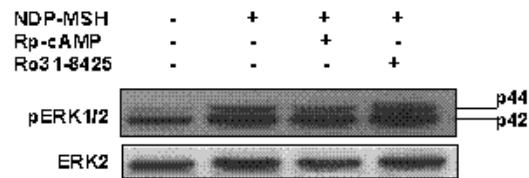
activation of heterotrimeric G proteins (56), or on the formation of signalling complexes recruited by the GPCRs upon phosphorylation by GRKs followed by binding of β -arrestins, which act as scaffolds for proteins of the ERK module (26). Moreover, several GPCRs trigger ERK signalling via transactivation of RTKs such as the epidermal growth factor receptor (EGFR) by incompletely defined mechanisms (27;28;30). We tested these possible modes of coupling using HBL cells as a model for cells of the melanocytic lineage.

To study the role of the diacylglycerol/ Ca^{2+} -activated PKC, we analyzed both PKC activation by NDP-MSH and the effects of the specific PKC inhibitor Ro31-8425 on MC-dependent ERK activation. PKC activity was estimated by detection of phosphorylation of the myristoylated alanine-rich PKC substrate (MARCKS), a major and ubiquitous PKC substrate (57). No evidence of PKC activation was obtained in cells treated with NDP-MSH, as shown by lack of detectable MARCKS phosphorylation as opposed to rapid and strong phosphorylation in cells stimulated with PMA (Fig. 4A). This response was specific for PKC activation, as it was abolished by preincubation with Ro31-8425 but was not sensitive to the PKA inhibitor Rp-cAMP or the MEK inhibitor PD98059. Moreover, Rp-cAMP and Ro31-8425 failed to decrease MC-mediated activation of the ERKs (Fig. 4B). In addition, since PKC activation by GPCRs is dependent on transient increases in cytosolic Ca^{2+} , we checked the effects of nifedipine, a dihydropyridine Ca^{2+} channel blocker, and (\pm)-Bay K8644, a L-type Ca^{2+} channel activator. Neither of these agents had any significant effect on basal or MC-induced pERK levels (Fig. 4C). Overall, these data show that ERK activation in melanocytic cells stimulated with NDP-MSH is independent on Ca^{2+} fluxes or PKC. Moreover, they strongly suggest that MC1R does not activate PKC in melanocytic cells, as opposed to other receptors of the MCR family (58;59).

A



B



C

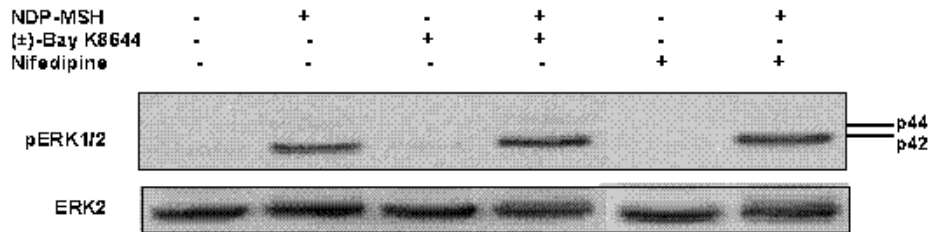
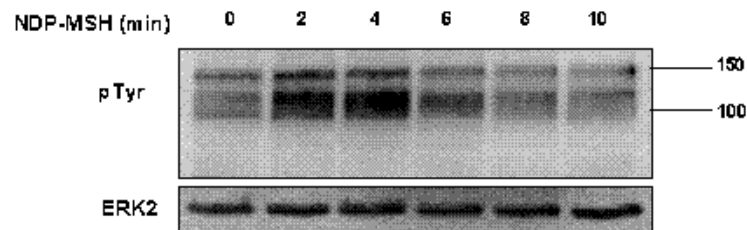


Fig. 4. MC1R-mediated ERK activation in HBL cells is independent on PKC, calcium fluxes or PKA. A, HBL cells were preincubated for 1 h in the presence or absence of the protein kinase inhibitors PD98059 (50 μ M), Ro31-8425 (25 nM) or Rp-cAMP (20 μ M) and then challenged with either NDP-MSH (10^{-7} M, 5 min) or PMA (0.1 ng/ μ l, 15 min). Cell lysates were analyzed by Western blot for detection of phosphorylation of a PKC substrate (MARCKS, upper blot) and total ERK2 as loading control. B, HBL cells were challenged with NDP-MSH (10^{-7} M, 5 min) with or without preincubation (1 h) with Ro31-8425 or Rp-cAMP. Cell extracts were blotted for pERK. C, HBL cells were challenged with NDP-MSH (10^{-7} M, 5 min) with or without (\pm)-Bay K8644 (1 μ M, 1 h) or nifedipine (10 μ g/ml, 1 h). ERK phosphorylation was analyzed by Western blot.

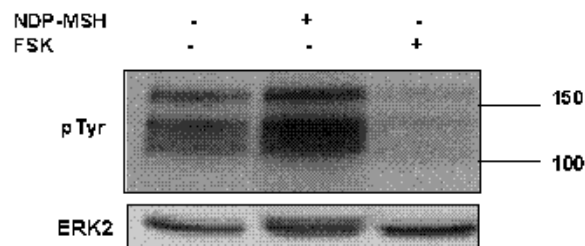
Given that we did not find evidence of cAMP, PKA, PKC or Ca^{2+} involvement in MC-induced ERK activation, we considered the possibility of transactivation of a RTK. When HBL cells were challenged with NDP-MSH, a rapid and transient increase in tyrosine (Tyr) phosphorylation was detected by Western blot, which was maximal slightly sooner than maximal pERK levels were reached (Fig. 5A). Conversely, FSK did not mediate a similar stimulation of Tyr phosphorylation (Fig. 5B). Therefore, NDP-MSH stimulated Tyr kinase activity in HBL cells independently on cAMP, with kinetics compatible with a role in activation of the ERKs. We next tested the effect of AG 1478 (tyrphostin), a strong and fairly specific EGFR inhibitor (nanomolar IC_{50} values) that also inhibits other RTKs at much higher concentrations. Preincubation of HBL cells with AG 1478 effectively inhibited basal and NDP-MSH-induced ERK phosphorylation only at micromolar concentrations (Fig. 5C), and occurred upstream of NRAS since AG 1478 (50 μ M) had no effect on ERK activation by a constitutively active NRAS mutant (Fig. 5C). Therefore, treatment of HBL cells with NDP-MSH activated a Tyr kinase located

upstream of NRAS and sensitive to relatively high concentrations of AG1478, thus most likely different from EGFR family members. Since transactivation of RTKs downstream of GPCRs is best established for the EGFR (30), we wished to confirm lack of involvement of this receptor by means of a more specific inhibitor of the EGFR family. We used PD153035, a selective compound that inhibits EGFR with an IC₅₀ as low as 25 pM (60). Following preincubation of HBL cells with PD153035 at a 0.1 μM concentration, i.e. several orders of magnitude above its IC₅₀ for the EGFR we did not observe any inhibition of either basal, NDP-MSH-stimulated or mutant NRAS-dependent ERK phosphorylation in HBL cells (Fig. 5 D). Thus, EGFR is not the RTK transactivated upon MC1R stimulation in HBL cells.

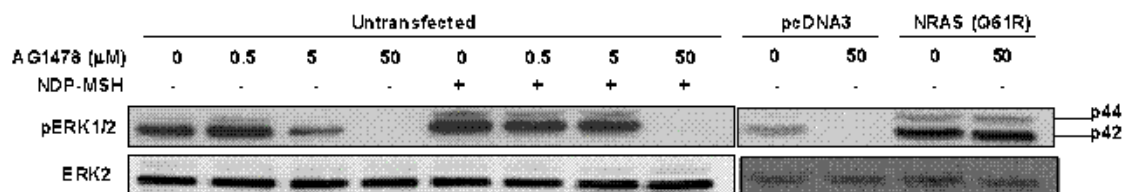
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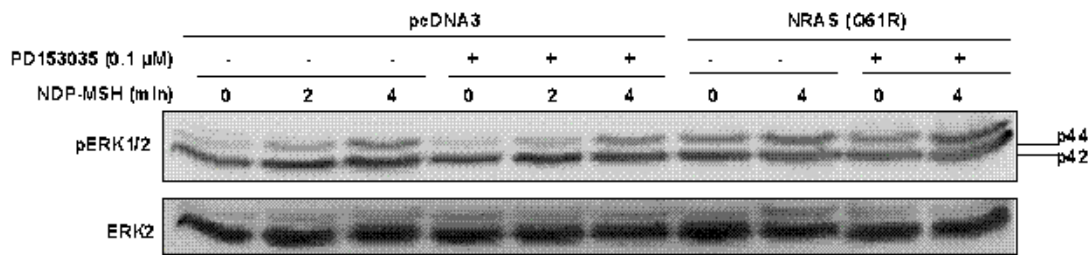


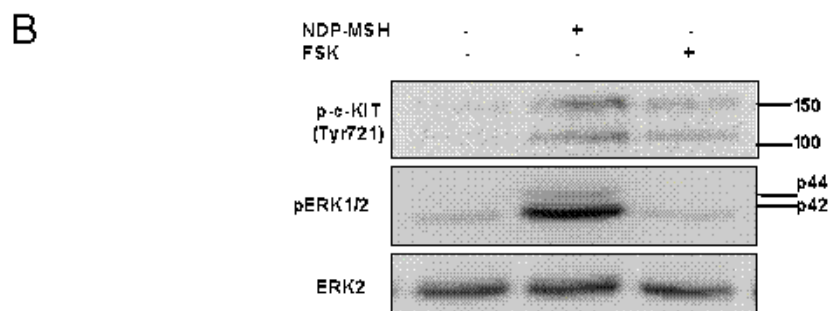
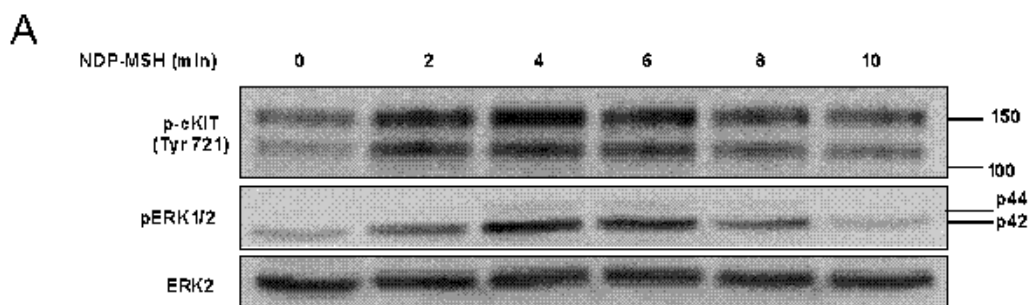
Fig. 5. Activation of Tyr phosphorylation by NDP-MSH. A, Serum-deprived HBL cells were challenged with NDP-MSH for the times shown and Tyr phosphorylation was detected by Western blot. B, Failure of cAMP to induce Tyr phosphorylation in melanoma cells. HBL cells were incubated with NDP-MSH or FSK and probed for pTyr by Western blot. C, RTK inhibition completely blocks ERK activation by NDP-MSH. Control, untransfected HBL cells were preincubated with increasing concentrations of AG1478 for 45 min, then treated with NDP-MSH. Cell extracts were analyzed for ERK phosphorylation. As a control for specificity, cells transiently transfected with empty vector or the NRAS constitutively active mutant Q61R, as indicated, were incubated with a fixed 50 μ M concentration of AG1478 for 1 h and pERK was detected by Western blot. D, NDP-MSH-induced Tyr phosphorylation is not mediated by the EGFR. HBL cells transfected with empty vector or with the Q61R NRAS mutant were pretreated or not with the specific EGFR inhibitor PD153035 (0.1 μ M, 45 min), and then challenged with 10^{-7} M NDP-MSH for the times shown. pERK levels were estimated by Western blot. Identical results were obtained in two independent experiments.

Src and c-KIT link MC1R signalling to ERK activation

The next series of experiments aimed at the characterization of the putative RTK transactivated by MC1R. We focused on c-KIT, a RTK expressed in cells of the melanocytic lineage (19;61). C-KIT is the receptor for STEEL/SCF, a cytokine that regulates melanoblast proliferation, differentiation, migration, and survival (19). Loss-of-function (LOF) dominant alleles of c-KIT yield a white coat in mice (19), and LOF mutations in humans are causally associated with piebaldism and cochlear deafness (21;22). Moreover, oral administration of the specific inhibitor of c-KIT RTK activity ISCK03 causes reversible and dose-dependent hair depigmentation in C57BL/6 mice and topical application of this drug decreases UV-induced pigmentation and epidermal melanin in Brownish guinea pigs *in vivo* (62).

Activation of c-KIT by SCF triggers autophosphorylation on several cytoplasmic Tyr residues, with phosphorylation at Tyr721 allowing binding and activation of PI3 kinase (61;63). NDP-MSH induced the transient phosphorylation of c-KIT Tyr721 in HBL cells (Fig. 6A), indicative of rapid transactivation of c-KIT. Similar results were

obtained for HBL cells expressing the RHC mutants (not shown), but no significant increases in c-KIT phosphorylation were observed in HBL cells treated with FSK (Fig. 6B). ISCK03 is a phenyl-imidazolosulfonamide compound that selectively inhibits c-KIT activity at low micromolar concentrations without any effect on HGF-induced ERK phosphorylation in 501mel melanoma cells (62). Preincubation of HBL cells with ISCK03 (5 μ M) effectively blocked NDP-MSH-induced ERK phosphorylation without effect on mutant NRAS-induced ERK activity (Fig. 6C). Given that the pharmacology of ISCK03 is relatively poorly established, two better characterized inhibitors of c-KIT were employed: GTP-14564 and Sunitinib (SU11248) (Fig. 6D). GTP-14564 has been reported to inhibit c-KIT at low micromolar concentrations. At the working concentration used in Fig. 6D (1.0 μ M) the compound is extremely selective, as it has no effect on most Tyr and Ser/Thr kinases such as EGFR, KDR or HER2 ($IC_{50} \geq 10 \mu$ M), Src, PKA, AKT, MEK or ERK (64). Sunitinib is a highly potent inhibitor of c-KIT ($K_i \sim 4$ nM) used as a chemotherapeutic agent (65), and was employed at a 10 nM concentration. Both compounds inhibited ERK activation by NDP-MSH in HBL cells (Fig. 6D) but had no effect on ERK phosphorylation in cells expressing a constitutively active NRAS mutant (not shown). Although Sunitinib is not fully specific for c-KIT and also targets vascular endothelial growth factor receptor and platelet-derived growth factor receptor with similar potency, taken together the results obtained with the 3 pharmacological inhibitors strongly suggested that c-KIT is the RTK transactivated upon stimulation of the MC1R.



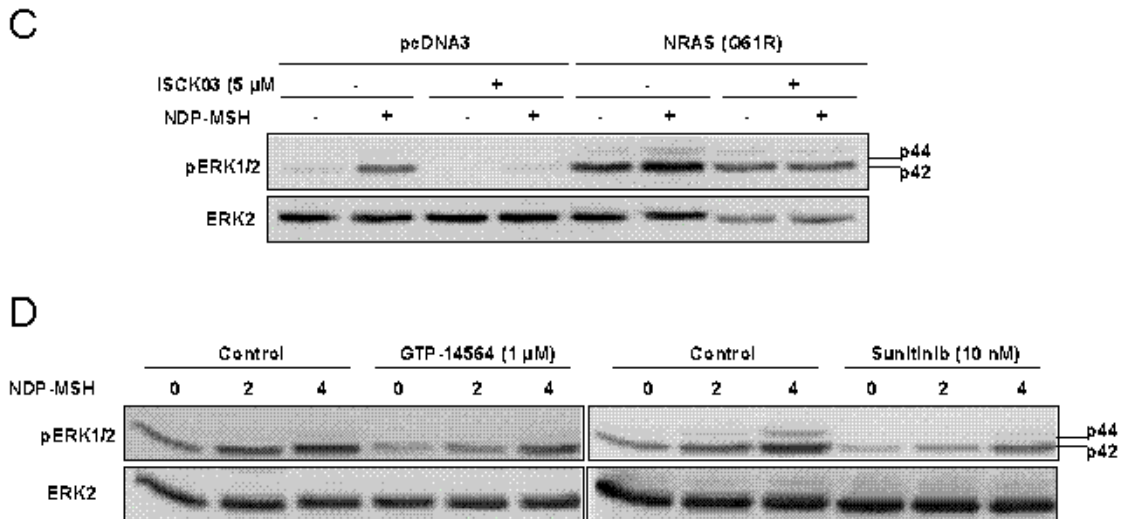
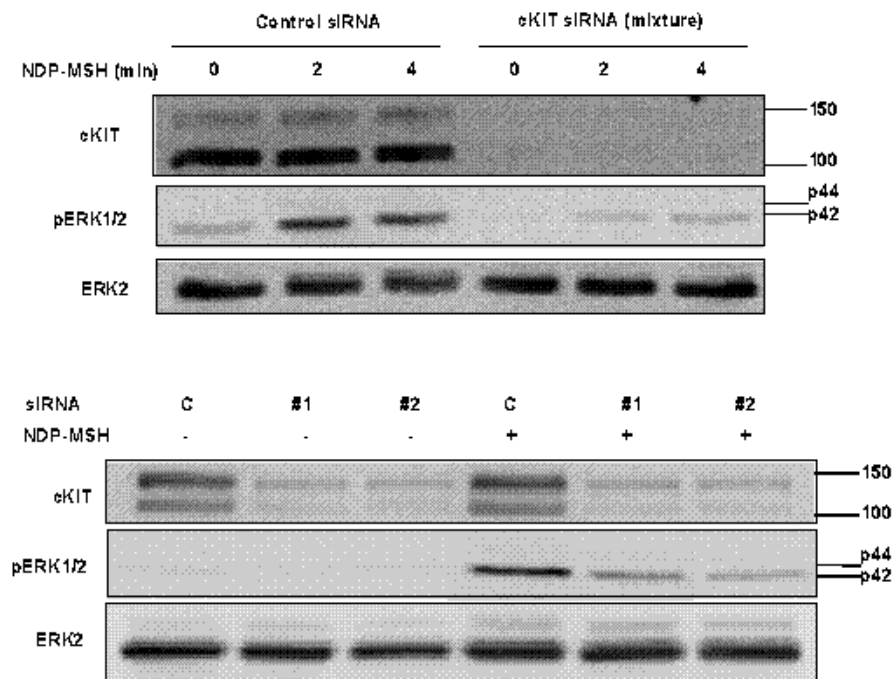


Fig. 6. cAMP-independent transactivation of c-KIT in NDP-MSH-stimulated HBL cells. A, Kinetics of c-KIT activation by NDP-MSH. Serum-starved HBL cells were stimulated with NDP-MSH for the times shown and analyzed for c-KIT phosphorylation at Tyr721 by Western blot. B, Lack of c-KIT activation by FSK. Cells were treated with FSK (10^{-5} M, 30 min) or NDP-MSH (10^{-7} M, 4 min) as positive control and blotted for c-KIT phosphorylation at Tyr721 (upper), ERK activation (middle) or total ERK (lower, loading control). C, The c-KIT inhibitor ISCK03 blocks ERK activation by NDP-MSH. Cells were transiently transfected with the empty vector (pcDNA3) or with a constitutively active NRAS mutant (Q61R), preincubated with ISCK03 (1 μ M, 1 h), then challenged with NDP-MSH for 4 min. Cell extracts were analysed by Western for pERK. D, Impaired ERK activation by NDP-MSH in HBL cells treated with the c-KIT inhibitors GTP-14564 and Sunitinib. HBL cells were pretreated for 1 h with the indicated concentrations of the c-KIT inhibitors, then challenged with NDP-MSH (10^{-7} M, 2 or 4 min) before estimation of pERK levels by Western blot.

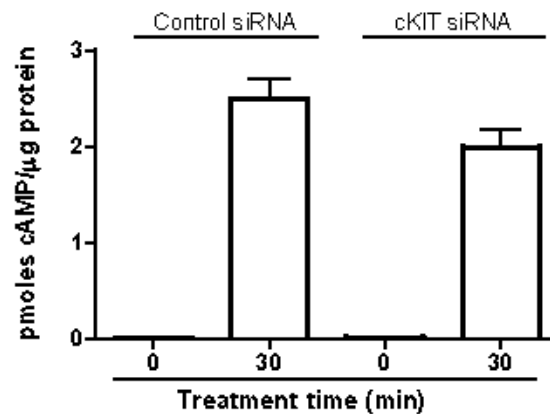
We confirmed these pharmacological evidences of c-KIT involvement in ERK activation downstream of MC1R by a series of more conclusive molecular approaches. Depletion of c-KIT with a mixture of 4 pre-designed siRNA oligonucleotides abolished MC-dependent ERK activation (Fig. 7A). This result was confirmed with 2 independent siRNA oligonucleotides employed individually. Moreover, the specificity of this inhibitory action and the absence of off-target effects were suggested by comparable stimulation of cAMP synthesis by NDP-MSH in the presence of c-KIT-directed or control siRNA (Fig. 7B). To further demonstrate that c-KIT is required for positive functional coupling of MC1R to the ERKs we performed functional reconstitution experiments in HEK293 cells transfected with MC1R or c-KIT, alone or in combination. c-KIT expression was analyzed by Western blot that showed two major bands of ~ 110 and 145 kDa, most likely corresponding to the "de novo" incompletely processed form and the mature protein, respectively. Treatment with NDP-MSH failed to achieve ERK stimulation in

cells expressing MC1R alone, but the MC caused a reproducible increase in ERK phosphorylation in cells expressing both MC1R and c-KIT, of similar intensity as the one produced by stimulation with the c-KIT ligand SCF (Fig. 7C). As a further control, cells transfected to express c-KIT alone showed increased pERK levels after stimulation with SCF but not with NDP-MSH (not shown). Of note, a NDP-MSH-induced time-dependent phosphorylation of c-KIT was also detected in cells expressing MC1R and c-KIT (Fig.7C, lower blot).

A



B



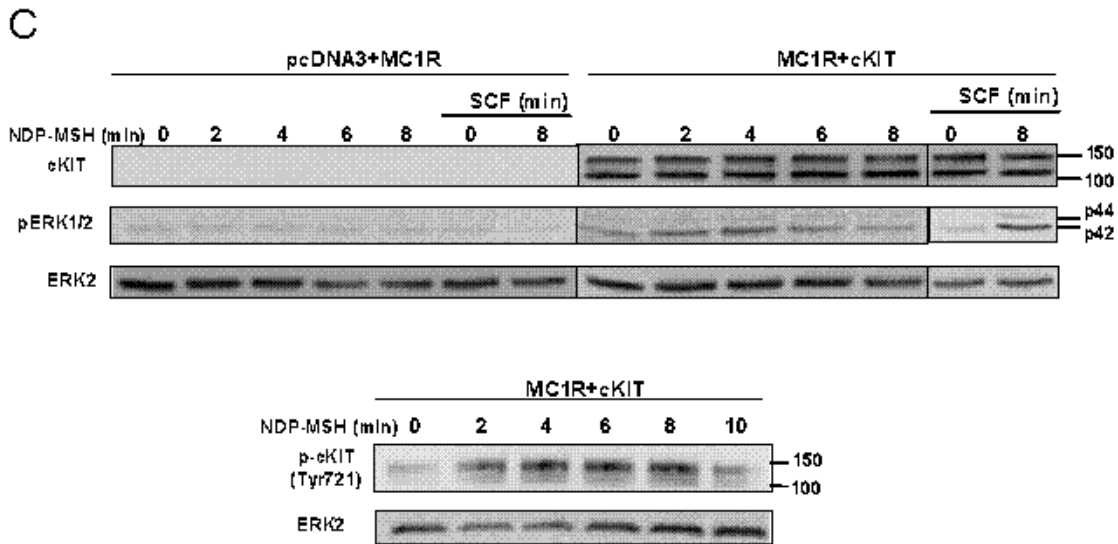


Fig. 7. Relationship of c-KIT expression and MC-dependent ERK phosphorylation. A, Effect of c-KIT silencing on ERK phosphorylation induced by NDP-MSH. In the upper series of blots, cells were transfected with a mixture of 4 c-KIT-directed siRNA or a scrambled sequence as a negative control, and stimulated with NDP-MSH for the times shown. Efficient silencing was verified by analyzing total c-KIT levels (upper blot), and ERK activation was assessed by estimation of pERK levels (middle). Total ERK2 was used as loading control (lower). In the lower series, cells transfected with control siRNA (C) or with 2 unrelated individual c-KIT-directed siRNAs (#1 and #2), were stimulated with NDP-MSH (10^{-7} M, 4 min), and analyzed for c-KIT, pERK and total ERK as above. B, Lack of effect of c-KIT silencing by siRNA on MC-dependent cAMP production. Cells treated with the same siRNA mix as in the upper blot were analyzed for intracellular cAMP by radioimmunoassay (results are the mean \pm sem, $n \geq 4$). C, Reconstitution of c-KIT and ERK activation by NDP-MSH in HEK293 cells transfected with MC1R and c-KIT. HEK293 cells were transfected to express MC1R alone (blot labelled pcDNA + MC1R), or MC1R and c-KIT simultaneously. In the upper series of blots, cells were serum-deprived and stimulated with NDP-MSH (10^{-7} M) or SCF (50 ng/ml) for the times shown. ERK phosphorylation, total c-KIT expression (labelled c-KIT), c-KIT phosphorylation at Tyr721 (labelled p-cKIT) and total ERK2 were detected by Western blot.

Melanoma progression is often associated with loss of c-KIT expression (24;25). If ERK activation by MC1R is indeed dependent on transactivation of c-KIT, then loss of c-KIT expression in melanoma cells should uncouple MC1R from the ERKs. We analyzed a panel of human melanoma cell lines for c-KIT expression. Only cell lines WT for NRAS or BRAF were considered, in order to avoid interferences due to mutations in these upstream components of the ERK pathway. Four such c-KIT-positive and 5 c-KIT-negative cell lines were identified (Fig. 8A), according to the presence or absence of a majority band of \sim 145 kDa in Western blots, consistent with the electrophoretic mobility reported for the mature, glycosylated form of the protein

(66). These cell lines were tested for MC-induced ERK activation. The ERKs were activated by NDP-MSH in c-KIT-positive cell lines whereas c-KIT-negative cells were unresponsive (Fig. 8B and C). Of note, neither c-KIT-positive nor c-KIT-negative cells increased ERK phosphorylation when stimulated with FSK (not shown).

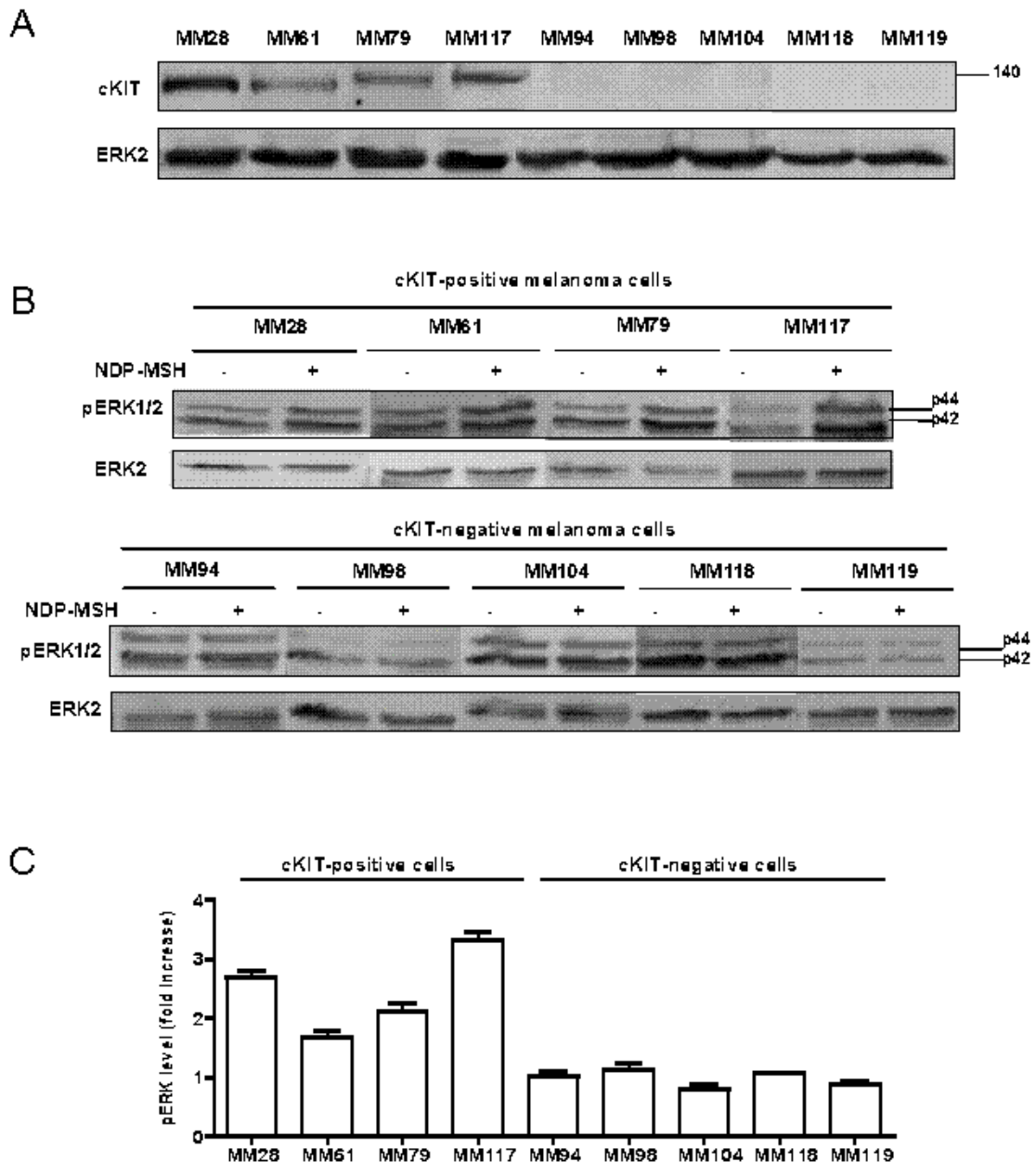


Fig. 8 Functional coupling of MC1R to the ERK pathway in human melanoma cell lines is dependent on c-KIT expression. A, Expression of c-KIT in human melanoma cell lines. Cell extracts from LOCE-MM28, 61, 79, 94, 98, 104, 117, 118 and 119 human melanoma cells were analyzed for c-KIT expression by Western blot. B, Effects of NDP-MSH on pERK levels in c-KIT positive and negative human melanoma cells. After serum deprivation, cultures of the indicated cell lines were stimulated with NDP-MSH and ERK phosphorylation was compared by Western blot. C, Quantification of ERK activation in c-KIT positive and negative human melanoma cells.

Two independent blots as shown in panel B were quantified and the intensity of the pERK signal was normalized to the intensity of the band in control cells, after correction for loading. Results are given as fold increase in pERK (mean \pm range).

Overall, the results presented thus far prove that MC1R stimulation triggers activation of the ERKs through the cAMP-independent transactivation of c-KIT. Moreover, our data formally exclude cAMP and PKC-related events as the mechanisms relaying MC binding to the MC1R to ERK activation. In an attempt to identify components of this signalling pathway downstream of MC1R and upstream of c-KIT, we focused on Src, a non-RTK that has been shown to play key roles in RTK transactivation (30;56;67-69). Src kinase activity is controlled by phosphorylation/dephosphorylation, with a major role for changes in the phosphorylation status of a specific Tyr residue located near the C terminus (Tyr530 in human Src). When phosphorylated, this residue binds intramolecularly to a SH2 domain, leading to the stabilization of a compact and inactive conformation. Regulated dephosphorylation of Tyr530 disrupts this intramolecular interaction and is sufficient to convert Src to an open and catalytically active state (68-70), which can be further activated by phosphorylation of other residues, mainly Tyr416. Accordingly, the functional status of Src can be estimated from the degree of phosphorylation of the C-terminal Tyr530 by means of phosphospecific antibodies. Stimulation with NDP-MSH of HBL melanoma cells or heterologous HEK293 cells transfected to express both MC1R and c-KIT caused a rapid and transient dephosphorylation of Src Tyr530 (Fig. 9A). Thus, MC signalling resulted in activation of Src. Importantly, FSK failed to attenuate the phosphorylation of Tyr530 in Src (Fig. 9B), even though SCF triggered ERK activation (see below). These data suggest that Src is activated downstream of MC1R in HBL melanoma cells, and that its activation by NDP-MSH is cAMP-independent.

A role for Src in transmitting the MC signal to the ERKs was next analyzed with a set of 3 different pharmacological inhibitors. The pyrazolopyrimidine derivatives N-PP1 and PP-2 are potent and selective inhibitors of the Src family of non-RTKs (71) with a submicromolar IC₅₀ for Src inhibition, and potencies about one order of magnitude lower for a few other kinases such as p38 MAPK (72). These compounds were used at final concentrations ranging from 0.1 to 10 μ M. The clinically relevant Dasatinib was described as a potent pan-Src inhibitor with IC₅₀ values near 1 nM (73), but it has also been shown to inhibit several RTKs including c-KIT at higher concentrations, with IC₅₀ around 100 nM (74). Accordingly, Dasatinib was used at a fixed concentration of 10 nM, expected to block Src-dependent responses with minor

effects on the activity of c-KIT and other RTKs. Preincubation of HBL cells with Na-PP1 or PP-2 before stimulation with NDP-MSH dramatically inhibited MC-induced ERK phosphorylation in a dose-dependent manner (Fig. 9C), with a higher potency for PP-2 consistent with the reported pharmacological data for these compounds (72). ERK activation was completely blocked by 1 μ M PP-2, but was already attenuated at nanomolar concentrations of the inhibitor. Although the specificity of PP-2 is not absolute, those doses should be selective for Src (71). Moreover, Dasatinib at a 10 nM concentration completely abolished ERK activation by NDP-MSH (Fig. 9C). This concentration is most likely sufficient to completely inhibit Src activity with marginal effects on c-KIT, according to the reported IC50. Failure of Na-PP-1, PP-2 or Dasatinib to block mutant RAS-induced ERK phosphorylation at comparable doses placed their targets upstream of NRAS and further suggested selectivity of the compound (Fig. 9C).

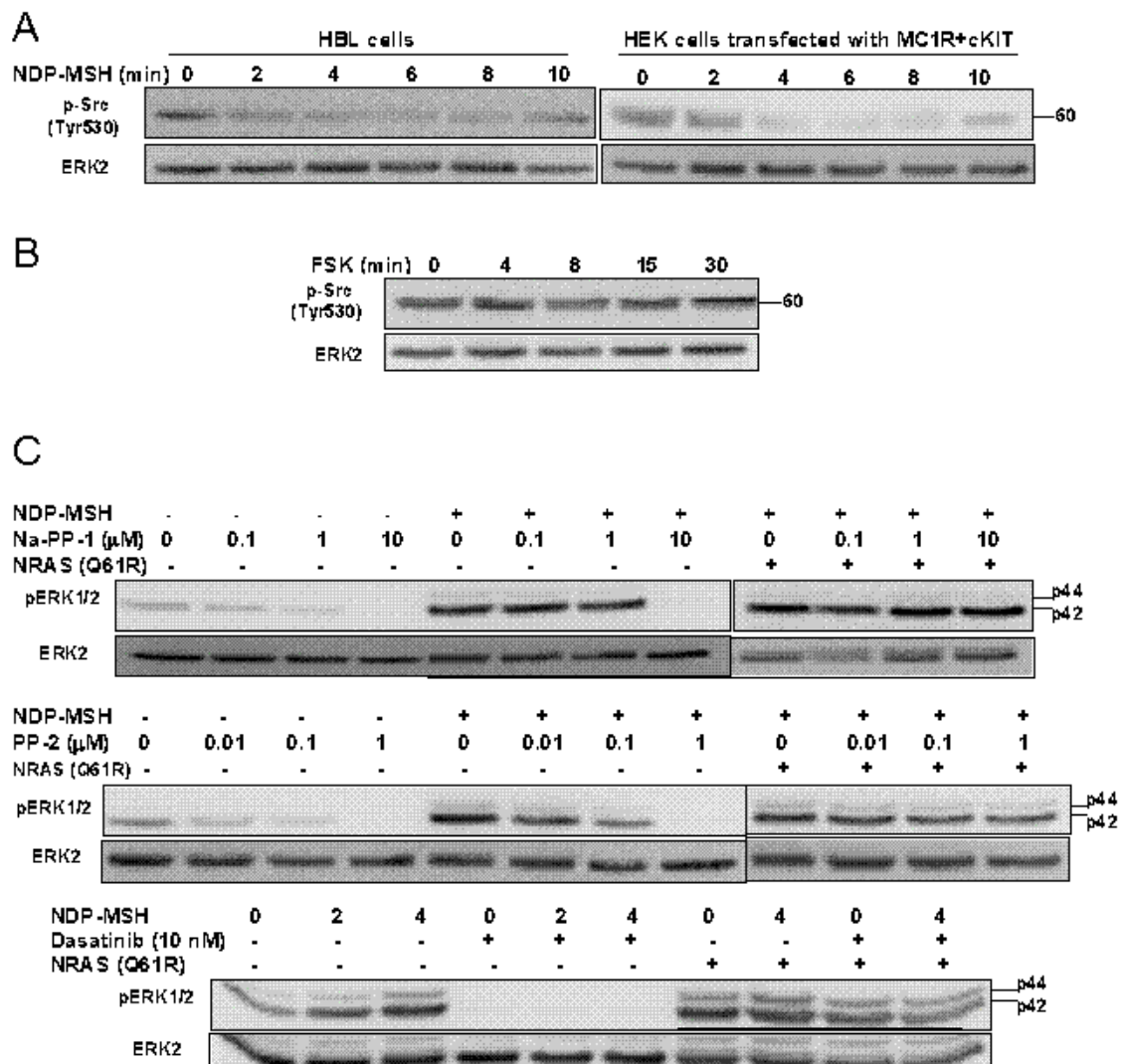


Fig. 9 Activation of Src by MC1R signalling and possible role in downstream activation of the ERK pathway. A, Kinetics of Src activation in HBL cells (left) or HEK293 cells transfected with

MC1R and c-KIT (right) following stimulation with 10^{-7} M NDP-MSH (left). Src activation was assessed by decreased inhibitory Tyr530 phosphorylation. B, Lack of Src activation by FSK. HBL cells were treated with 10^{-5} M FSK for the times shown and tested for Src activation as in panel A. C, Effect of the Src inhibitors Na-PP-1 (upper), PP-2 (middle) and Dasatinib (lower) on ERK activation mediated by NDP-MSH or by the constitutively active Q61R mutant of NRAS. HBL cells transfected with empty pcDNA3 or with a construct corresponding to the Q61R NRAS mutant were preincubated with the Src inhibitors at the concentrations shown, and then challenged with NDP-MSH (10^{-7} M, 5 min). ERK activation was estimated by Western blot.

To locate the position of Src in the signalling pathway linking MC1R to the ERKs and to further test a model of MC1R-dependent ERK activation based on c-KIT transactivation mediated by Src, we analyzed the effects of c-KIT stimulation with SCF on Src activity. SCF did not induce the dephosphorylation of the inhibitory phospho-Tyr530 eventhough the c-KIT ligand triggered ERK activation (Fig. 10A). Finally, we confirmed the previous results obtained with an antibody specific for the phosphorylated form of c-KIT Tyr721 by means of a different antibody that recognizes phosphoTyr703. Phosphorylation of Tyr703 has been shown to provide a docking site for the adaptor protein Grb2 that may link c-KIT activation to the RAS/RAF/MEK/ERK pathway (61;75). As shown in Fig. 10B, NDP-MSH triggered the phosphorylation of c-KIT Tyr703. In the presence of the Src inhibitor PP-2 at a 1 μ M concentration, NDP-MSH-induced Tyr703 phosphorylation was blocked, with a concomitant inhibition of ERK phosphorylation. Conversely, the SCF-dependent phosphorylation of Tyr703 was much less sensitive to PP-2, suggesting that the inhibitor targets a component of the signalling pathway located upstream of c-KIT. Moreover, PP-2 had no effect on SCF-dependent ERK phosphorylation even at high concentrations previously found to block completely MC1R signalling to the ERKs (Fig 10B).

Overall, the data presented above showed that Src was activated by NDP-MSH independently of cAMP, and suggest that pharmacological inhibition of Src kinase activity blocked MC1R-mediated activation of c-KIT and of the ERKs. Moreover, given that Src is not activated by SCF, and that PP-2 has a small or negligible effect on SCF-mediated c-KIT and ERK activation, it would appear that the non-RTK is located upstream of c-KIT in the pathway linking MC1R and the ERKs (Fig. 10C).

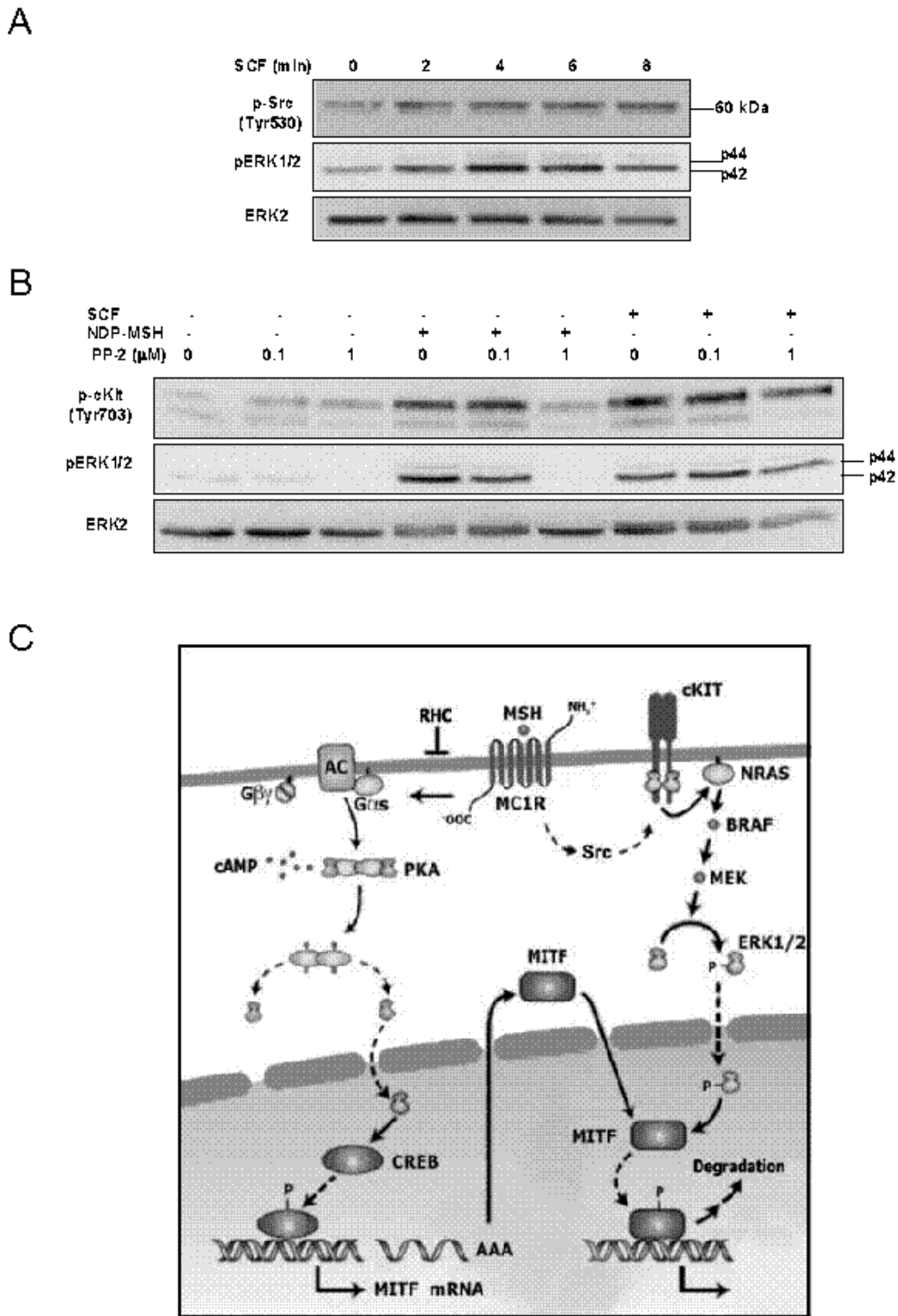


Fig. 10. Src is most likely located upstream of c-KIT. A, Inability of the c-KIT ligand SCF to attenuate inhibitory Tyr530 phosphorylation in HBL melanoma cells. Serum starved cells were stimulated with SCF (50 ng/ml) for the times shown and analyzed for Tyr530 phosphorylation by Western blot. B, Effect of PP-2 on c-KIT phosphorylation and ERK activation following stimulation with NDP-MSH or SCF. Control HBL cells or cells pretreated with the concentrations of PP-2 shown were challenged with 10^{-7} M NDP-MSH or 50 ng/ml SCF (4 min), then lysed and tested for activatory c-KIT phosphorylation at Tyr703, and pERK levels. C, A model for

signalling from MC1R. Two different signalling pathways originate from the MC1R, the cAMP (left branch) and the ERK pathways (right branch). Both pathways converge at the level of MITF. cAMP increases MITF gene expression via PKA and CREB, and ERK-dependent phosphorylation of MITF increases its transcriptional activity but targets the protein for degradation. Whereas functional coupling to the cAMP cascade is strongly impaired by the RHC mutations, ERK activation is triggered by transactivation of c-KIT via Src and is comparably efficient for variant and WT MC1R.

DISCUSSION.

It has been reported that treatment of mouse melanoma cells with MC1R ligands or with cAMP-elevating agents such as FSK and the phosphodiesterase inhibitor IBMX leads to stimulation of the ERKs, and accordingly it has been assumed that human MC1R signalling to the ERKs is also dependent on cAMP (32). However, we have presented here extensive and conclusive evidence disproving a positive role for cAMP in MC1R-mediated activation of the ERKs in human melanocytic cells. This evidence includes: a) efficient activation of the ERKs by MC1R mutant alleles with impaired or absent cAMP signalling in NHM and human melanoma cells at physiological levels of receptor expression and hormone concentration, b) different dose-response curves for ERK activation and cAMP production at physiological MC1R levels, c) failure of the adenylyl cyclase activator FSK to activate the ERKs, d) inability of the cAMP antagonist Rp-cAMP to block ERK activation in human melanoma cells expressing WT MC1R, and e) lack of effect of the adenylyl cyclase inhibitor DDA on MC1R-dependent ERK activation in spite of complete inhibition of MC-stimulated cAMP production. This combination of complementary genetic and pharmacological approaches unequivocally showed that cAMP is neither sufficient nor necessary for positive functional coupling of MC1R to the ERK pathway. Moreover, we found a reproducible inhibition of basal levels of phosphorylated ERK following treatment of HBL cells with FSK, extending previous reports of cAMP-dependent suppression of ERK activity in RAS-mutated human melanoma cells (55) to at least one cell line WT for NRAS and BRAF. Overall, these data called for a re-evaluation of current paradigms on the crosstalk between the cAMP and ERK pathways in human melanocytic cells (53;54;76), and, accordingly, we analyzed possible cAMP-independent mechanisms accounting for ERK activation in MC-stimulated human melanocytic cells. We found that in HBL cells, signalling from MC1R to the ERKs is mediated by the transactivation of c-KIT. Again, this conclusion was reached on the basis of both pharmacological and molecular approaches that showed: a) a rapid increase of tyrosine phosphorylation and c-KIT activation in cells treated with NDP-

MSH but not with FSK, b) efficient blockade of ERK activation in MC-stimulated cells by 3 c-KIT inhibitors (ISCK03, GTP-14564 and Sunitinib), but not by EGFR specific inhibitors (PD 1153035 and low concentrations of AG 1478) under conditions that fail to block downstream signalling initiated at the level of NRAS c) ablation of MC-induced ERK activation following silencing of c-KIT expression with siRNA, d) ERK activation following MC stimulation of c-KIT-positive human melanoma cells but not of cell lines lacking c-KIT expression and e) reconstitution of c-KIT activation by MCs and of a positive pERK response to the MC1R ligand in HEK cells simultaneously transfected with MC1R and c-KIT constructs, but not with either one of the individual constructs alone.

The precise mechanisms coupling MC1R activation by MC hormones and c-KIT transactivation are currently under study in our laboratory. Our data strongly suggest that the non-RTK Src plays a key role upstream of c-KIT (Figs. 9 and 10). Indeed, we found that Src was rapidly activated by treatment of HBL cells with NDP-MSH, but not with FSK or with the c-KIT ligand SCF. Similar results were obtained in HEK cells transfected to express MC1R and c-KIT. Moreover, the Src inhibitors Na-PP1, Dasatinib and PP-2 blocked ERK activation in MC-stimulated cells. Since concerns have been raised about the selectivity pharmacological Src inhibitors (67;71;77) we took special care to ascertain sufficient specificity and found that under our experimental conditions Na-PP1 and PP-2 had no effect on either c-KIT or downstream kinases as shown by inability to block ERK phosphorylation in cells stimulated with the c-KIT ligand SCF, or expressing a constitutively active NRAS mutant. Dasatinib was equally unable to block mutant NRAS-driven ERK phosphorylation. Moreover, our data clearly located Src upstream of c-KIT and NRAS, since: a) Src was not activated by direct stimulation of c-KIT with SCF, b) incubation of HBL cells with the Src inhibitor PP-2 under conditions that completely block the pERK response to NDP-MSH had no effect on either SCF-induced c-KIT autophosphorylation at Tyr703 or ERK activation, and c) under identical conditions, PP-2 abolished NDP-MSH-mediated phosphorylation of c-KIT Tyr703. Overall, these results strongly support the involvement of Src in MC-signalling to the ERKs although this point should be confirmed by complementary molecular approaches.

The molecular events linking MC1R to Src on one hand, and Src to c-KIT on the other remain unknown. Concerning the latter, we have found expression of the c-KIT ligand SCF in HBL and other human melanoma cells (unpublished observations), and accordingly one attractive possibility would be the MC-induced proteolytic activation of a latent form of this growth factor, with release of the active form on the extracellular side of the cell membrane. Such a proteolytic ectodomain shedding mechanism has

been demonstrated for the transactivation of several RTKs by GPCR signalling in various cell types (28;30). However, preliminary experiments showed that the broad spectrum matrix metalloprotease inhibitor GM1001 had no effect on MC1R-dependent ERK activation (results not shown). Accordingly, other transactivation mechanisms appear more likely particularly in the light of the extremely complex interplay of Src family kinases and RTKs (67) and of reports of direct and selective phosphorylation of c-KIT and other RTKs by Src family kinases (78-80).

Since the first report of EGFR activation by a GPCR (81), transactivation has been shown for various RTKs such as FGFR1, HER2/ErbB2/neu, IGF1R, Trk, PDGFR and VEGFR. Therefore, transactivation seems a rather general phenomenon that may underlie the mitogenic effects of signalling from certain GPCRs (30). However, transactivation of c-KIT by MC1R signalling offers several unique aspects. On one hand, it is the first report of activation of c-KIT by signalling from a GPCR. On the other, we have found compelling evidence that whereas c-KIT transactivation and hence ERK activation are comparable for WT-MC1R and the RHC mutants, the RHC forms display a clear loss-of-function phenotype in signalling to the cAMP cascade (41-45). Accordingly, the RHC alleles correspond to imbalanced signalling variants rather than to *bona fide* hypomorphic proteins. This observation can be interpreted in terms of the different dose-response curves for stimulation of the ERK and cAMP pathways, which show that the former is fully activated at much smaller concentrations of active hormone-receptor complexes. Thus, the MC1R/c-KIT system described here (Fig. 10C) shows that different signalling pathways originating from a single GPCR can be differentially altered by naturally occurring mutations, with phenotypic consequences.

Another unique aspect of c-KIT transactivation by MC1R signalling consists of the convergence of the cAMP and the ERK pathways triggered by the MC1R at the level of MITF, a master regulator of melanocyte biology (3;4;7). cAMP stimulates *MITF* gene expression and the ERKs phosphorylate the MITF protein resulting in increased transcriptional activity but decreased intracellular stability (82). Accordingly, the cellular responses to the MCs might be fine-tuned by an atypical feedback loop whereby the cAMP-dependent transcriptional induction of MITF would be counterbalanced by ERK-dependent downregulation of MITF protein following its activation. Some evidence in support of a regulatory loop involving cAMP and the ERKs has been reported for mouse melanocytic cells (83). In human melanocytes harbouring RHC mutant MC1R alleles, the function of this feedback loop would be altered due to the differential impact of the mutations on signalling to cAMP production or ERK activation (48). It is tempting to speculate that loss of positive cAMP-dependent effects and retention of negative

ERK-dependent regulatory influences would cooperate to maintain low levels of MITF in variant human melanocytes, thus decreasing their melanogenic activity.

Our finding that MC1R recruits c-KIT to activate the ERKs independently on cAMP also accounts for the involvement of NRAS in MC-mediated ERK signalling previously reported by others (32). Moreover, it might provide a molecular basis for the observation that treatment of melanocytes with α MSH activates the PI3 kinase-AKT pathway (84), a process that may participate in the protective action of the MCs against ultraviolet radiation-induced cellular damage. Indeed, by transactivating a RTK the MC1R would trigger antiapoptotic and survival pathways.

Human *MC1R* is a well established melanoma susceptibility gene (85) and the RHC alleles are strongly associated with increased risk for melanoma and other skin cancers. It will be interesting to analyze whether imbalanced signalling to the cAMP and ERK pathways, with impairment of cAMP and photoprotection-related activity but retention of proliferation-promoting signalling contributes to the association of variant RHC alleles such as R151C, R160W and D294H with skin cancer. In any case, the identification of the cAMP-independent, c-KIT-dependent transactivation of the ERK pathway by the MCs has unexpected and wide implications for our understanding of the functional connections of pathways critical for the regulation of melanocyte proliferation and differentiation.

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CAPÍTULO 4

Relaciones funcionales de las vías del AMPc y de las proteínas quinasas activadas por mitógenos ERK1 y ERK2 mediadas por el receptor de MC 1 en células de melanoma humano.

RESUMEN.

El MC1R, uno de los principales determinantes del fototipo de la piel, es un receptor acoplado a la proteína Gs (GPCR) que regula la producción de pigmento en los melanocitos. La estimulación del MC1R por la hormona estimulante del melanocito α (α MSH) o péptidos relacionados denominados melanocortinas (MCs) provoca la síntesis de AMPc que conduce a la activación de la enzima limitante melanogénica tirosinasa. Además, la señalización por MC activa la cascada de las MAP quinasas ERK1 y ERK2, que controla la proliferación y diferenciación. Previamente hemos demostrado que en células melanocíticas humanas la activación de ERK por MC ocurre a través de la transactivación del receptor c-KIT independientemente del AMPc, lo que sugiere que el acoplamiento del receptor a estas dos vías implica diferentes efectores y, posiblemente, mecanismos reguladores distintos. Con el fin de analizar la regulación diferencial de la señalización del MC1R a las ERK y al AMPc, hemos evaluado el impacto de mutaciones naturales y artificiales dentro del MC1R en la activación de las ERKs, la producción de AMPc y la unión del agonista. Varios mutantes que no consiguen activar la producción de AMPc fueron capaces de activar las ERKs de manera eficiente, pero no encontramos ningún mutante natural que conservara el acoplamiento funcional al AMPc siendo incapaz de activar las ERKs. Esto sugiere que el acoplamiento funcional a la vía de ERK tiene requisitos menos estrictos. La sobreexpresión de miembros de la maquinaria de desensibilización responsables del desacoplamiento del MC1R de la vía del AMPc no tuvo ningún efecto sobre la activación de ERK dependiente de MC. Además, la activación de ERK no se vio afectada por la mahogunin ring finger 1, una proteína que inhibe la señalización del MC1R a la vía del AMPc mediante la competencia por el acoplamiento a la proteína Gs. Finalmente, examinamos el entrecruzamiento de las vías del AMPc y de las

ERKs en las células de melanoma humano HBL (genotipo silvestre para MC1R, NRAS y BRAF). La activación de ERK por los mutantes constitutivamente activos NRAS o BRAF y la inhibición con PD98059 no tuvo apenas efecto sobre la síntesis de AMPc estimulada por MC. Por otra parte, niveles elevados de AMPc bloquearon eficientemente la activación de ERK mediada por MC1R y esta inhibición tuvo lugar aguas arriba de c-KIT. Por tanto, el AMPc se acopla negativamente a la vía de las ERK en células melanocíticas humanas en lugar de positivamente, como se sugirió por analogía con los melanocitos de ratón. Por último, hemos estudiado el estado funcional de las vías del AMPc y de las ERK en un panel de 26 líneas celulares de melanoma humano de genotipo MC1R, NRAS y BRAF definido. En estas líneas, el acoplamiento funcional del MC1R a la vía del AMPc se encuentra inhibido muy frecuentemente. Esta alteración no se explica únicamente por la presencia de mutaciones en MC1R y no es específica de líneas mutadas en NRAS.

Functional relationships of the MC 1 receptor-activated cAMP and mitogen-activated protein kinase ERK1 and ERK2 pathways in human melanoma cells.

ABSTRACT.

MC1R, a major determinant of skin phototype, is a G protein-coupled receptor (GPCR) that regulates pigment production in melanocytes. MC1R stimulation by α melanocyte stimulating hormone or related peptides termed melanocortins (MCs) triggers cAMP synthesis leading to activation of the rate-limiting melanogenic enzyme tyrosinase. MC signalling also activates the MAP kinase module leading to ERK1 and ERK2, which control proliferation and differentiation. We showed that in human melanocytic cells ERK activation by MCs is achieved by transactivation of the c-KIT receptor independently on cAMP, suggesting that MC1R coupling to these two pathways involves different effectors and, possibly, different regulatory mechanisms. In an attempt to analyze the differential regulation of MC1R signalling to the ERKs and the cAMP pathway, we evaluated the impact of natural and artificial mutations within the MC1R on activation of the ERKs, cAMP production or agonist binding. Several mutants that failed to activate cAMP production were able to achieve efficient ERK activation, but we did not find any natural mutants preserving functional coupling to cAMP but unable to activate the ERKs. This suggests less stringent requirements for functional coupling to the ERK pathway. Overexpression of members of the GPCR desensitization machinery responsible for uncoupling MC1R from the cAMP pathway had no effect on MC-dependent ERK activation. Additionally, ERK activation was insensitive to mahogunin ring finger 1, a protein that inhibits MC1R signalling to the cAMP pathway by interfering with coupling to Gs. Finally, we examined the crosstalk of the cAMP and ERK pathways in HBL human melanoma cells (WT for MC1R, NRAS and BRAF). ERK activation by constitutively active NRAS or BRAF mutants, and inhibition with PD98059 had little effect on MC-stimulated cAMP synthesis. On the other hand, high cAMP levels efficiently blocked MC1R-mediated ERK activation and this inhibition occurred upstream of c-KIT. Thus, cAMP couples negatively to the ERK pathway in human melanocytic cells rather than positively, as suggested by analogy with mouse melanocytes. Finally, we studied the functional status of the cAMP and ERK pathways in a panel of 26 human melanoma cell lines of defined MC1R, NRAS or BRAF genotype. In these cell lines, functional coupling of the MC1R to the cAMP pathway was very frequently inhibited.

This impairment is not always accounted for by mutations in the MC1R and is not exclusive of NRAS-mutant cells.

INTRODUCTION.

The cAMP and the mitogen-activated protein kinase (MAPK) extracellular signal-regulated (ERK) ERK1 and ERK2 pathways regulate key aspects of melanocyte biology. cAMP induces melanocyte differentiation by stimulating responses such as melanin synthesis (1), and ERK signalling is crucial for the control of both proliferation (2) and melanogenesis (3). Both pathways converge in the cell nucleus, through the activation of the cyclic AMP response element binding protein (CREB) (4) and the microphthalmia-associated transcription factor (MITF), a master regulator of melanocyte development, differentiation and proliferation (5). cAMP activates CREB via PKA, thereby increasing MITF expression in both normal human melanocytes and in B16 melanoma cells (6), and the ERKs activate p90^{RSK}, a protein kinase which phosphorylates CREB at Ser133(4) and phosphorylate directly MITF at Ser73, thereby increasing its transcriptional activity, but decreasing its intracellular stability.

cAMP synthesis in melanocytes is strongly stimulated by α melanocyte stimulating hormone (α MSH) via the MC 1 receptor (MC1R), a G protein-coupled receptor (GPCR) that regulates the amount and type of melanin pigments and is a major determinant of skin phototype, sensitivity to ultraviolet radiation and skin cancer risk (7). Conversely, cAMP levels are negatively regulated by cAMP-phosphodiesterases, a family of enzymes which degrade cAMP into AMP (8). MC1R signalling also results in activation of the ERK1/2 pathway, both in mouse (3) and in human melanocytes and melanoma cells (9). ERK1 and ERK2 are serine-threonine kinases, which are activated by dual phosphorylation at Tyr and Thr residues by the MAPK kinases MEK1 and MEK2. MEK are phosphorylated and activated by the RAF kinase proteins, ARAF, BRAF and CRAF. These RAF isoforms are activated by complex and still incompletely known mechanisms involving the active membrane-bound small G protein RAS (10).

The human *MC1R* gene is extremely polymorphic (11) and several variant alleles are associated with red hair and fair skin (the RHC phenotype) and increased risk for melanoma and nonmelanoma skin cancer. Three frequent and penetrant RHC alleles, R151C, R160W, and D294H with reduced functional coupling to the cAMP cascade (12;13) retain full capacity to activate the ERKs (9;14). We have shown that the different effect of the RHC mutations on signalling to the cAMP and ERK pathways is due to the recruitment of different effectors to activate each

pathway (14). Indeed, in human melanocytic cells stimulated with MC1R agonists, activation of the ERKs is cAMP-independent and most likely involves the sequential action of the Src non-receptor tyrosine kinase (non-RTK), and c-KIT, a RTK crucial for melanogenesis, proliferation, migration, and survival of pigment-producing cells (15).

The observation that the ERK and cAMP pathways originating from the MC1R initially diverge at the plasma membrane level raised the possibility of a pathway-specific regulation of each one of the two branches of MC signalling. First, a differential regulation at the genotype level could be considered, given that it has been shown that major RHC mutations such as R151C, R160W and D294H disrupt cAMP signalling but not functional coupling to the ERKs. It remains to be seen how general this differential impact is by analysis of more natural and artificial MC1R mutants. Second, it is conceivable that MC1R signalling to cAMP on one hand and the ERKs on the other might be differentially controlled by general GPCR regulators such as the β -arrestins (β ARRs) (16), or more specific MC1R effectors such as MGRN1, an endogenous antagonist of functional coupling to the cAMP pathway (17). We report here a study of these possibilities. Moreover, since the cAMP and the ERK pathways establish complex and cell type-specific interactions (18;19), we also analyzed their crosstalk in human melanocytic cells.

EXPERIMENTAL PROCEDURES.

Materials.

A radioimmunoassay kit for cAMP was from Amersham Pharmacia Biotech (Little Chalfont, UK). The transfection reagent Lipofectamine 2000 and competent DH5 α cells were from Invitrogen, (Carlsbad, CA). Igepal CA-630, BSA, EDTA, PMSF, bicinchoninic acid, ampicillin, β -mercaptoethanol, SDS, anti-HA-peroxidase conjugate and PD98059 were from Sigma Chemical Company (St. Louis, MO, USA). The anti-pERK1/2 (pERK1/2), anti-ERK2 and anti-phospho-c-KIT (Tyr 721) rabbit polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-phospho-c-KIT (Tyr703) rabbit monoclonal antibody was from Cell Signaling (Boston, MA, USA). Reagents for SDS-PAGE and Western blot were from Bio-Rad (Richmond, CA, USA). The synthetic α MSH analogue [Nle⁴, D-Phe⁷] α MSH (NDP-MSH) and FSK were from Calbiochem (Darmstadt, Germany). Other reagents were from Merck (Darmstadt, Germany) or Prolabo (Barcelona, Spain).

Cell culture and treatments.

Cell culture reagents were from Gibco BRL-Life Technologies (Gaithersburg, MD). PC12 cells were grown in DMEM supplemented with 15% foetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin sulfate. HBL (LOCE-MM01) human melanoma cells were grown in MEM with antibiotics and 10% foetal bovine serum. All other human melanoma cell lines used in this study were established at the Laboratory of Oncology and Experimental Surgery (LOCE), Université Libre de Bruxelles, Belgium, and were provided by Prof G Ghanem. They were cultured in HAM F-10 medium with 10% foetal calf serum and antibiotics (100 U/ml penicillin, 100 µg/ml kanamycin sulphate, 100 µg/ml streptomycin). NDP-MSH, FSK and PD98059 were used at 10^{-7} , 10^{-5} and 5×10^{-5} M concentrations respectively.

Expression construct and transfection.

All constructs were prepared in pcDNA3 (Invitrogen). The following expression constructs have been previously described: WT-MC1R, the Flag-tagged variants C35A (20), V38M, S41F, V51A, M128T, N281S and C289R (17;21), R162P (22), L93R and E94K (23), C315A, the C-terminus-deleted mutants $\Delta 1$ -, $\Delta 3$ -, $\Delta 5$ -MC1R (24) and Δ -TM7 (20), T157A and T157D (25). Mutants V60L, V92M and I155T were obtained by site-directed mutagenesis with the QuickChange kit (Stratagene, La Jolla, CA) and WT Flag-MC1R as template. The GRK5 and GRK6 (26) and the GRK6 dominant negative mutant GRK6-K215/K216R have also been described (13). cDNAs encoding bovine GRK2 and its dominant negative mutant GRK2-K220R cloned into pcDNA3 were gifts from Prof. F. Mayor Jr. (Madrid, Spain). NRAS Q61R and BRAF V600E were amplified by PCR and cloned into pcDNA3. β -ARRs were obtained by PCR amplification of cDNA from HBL cells and were cloned into pcDNA3 (Pérez-Oliva et al, manuscript in preparation). All constructs were verified by double strand automated sequencing.

Cells grown to approximately 80% confluence were transfected with 0.3 µg plasmid DNA/well, using Opti-MEM to dilute DNA and Lipofectamine.

Functional assays.

Cells grown in 12-well plates were transfected, serum-deprived for 12-24h and stimulated as required. The medium was aspirated and the cells quickly washed with 800 µl ice-cold PBS. Cells were lysed with 200 µl/well 0.1N HCl preheated at 70° C, and scrapped. The mix was freeze-dried, washed with 100 µl H₂O and freeze-dried again. cAMP was measured with a commercial radioimmunoassay, as

per instructions. Parallel dishes were used for protein determination performed with the bicinchoninic acid method. Radioligand binding assays were done with 10^{-9} M [125 I]-NDP-MSH as previously described (27).

Western blot.

Cells were washed twice with PBS and solubilized in 75 μ l solubilization buffer (PMSF 100 ng/ml, 1% Igepal and 1% phosphatase inhibitor mix containing 200 mM imidazole, 100 mM NaF, 115 mM sodium molybdate, 100 mM sodium o-vanadate and 400 mM sodium tartrate). Samples were centrifuged (105,000xg, 30 min) and a volume of supernatant containing 30 μ g protein was mixed (2:1 ratio) with electrophoresis sample buffer (180 mM Tris-HCl pH 6.8, 15% glycerol, 9% SDS, 0.075% bromophenol blue and 7.5% β -mercaptoethanol). Electrophoresis and Western blotting were performed as described (12;13;23). Blots were probed with the required antibodies and stained with a chemiluminescent substrate (Amersham). Comparable loading was ascertained by stripping and reprobing the membranes with an anti-ERK2 antibody.

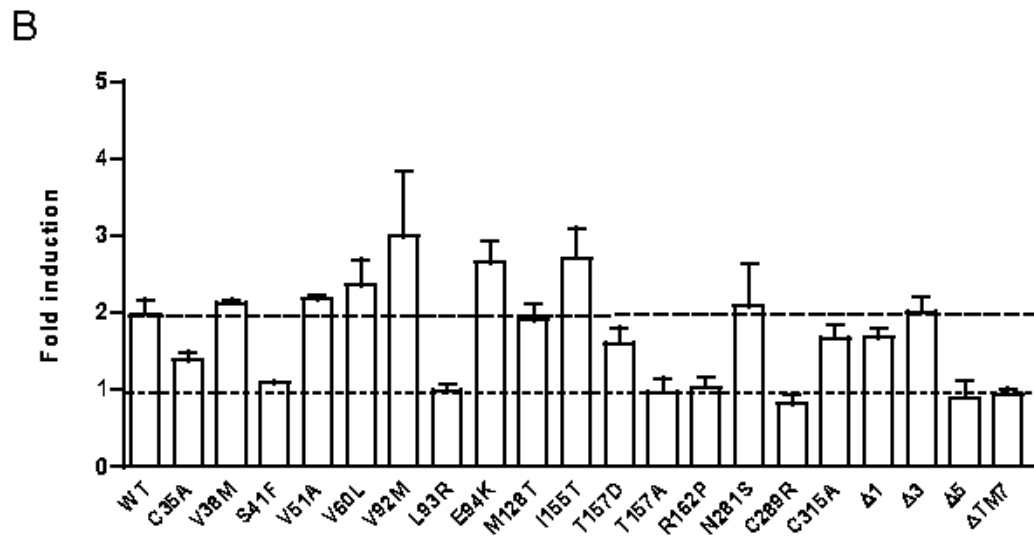
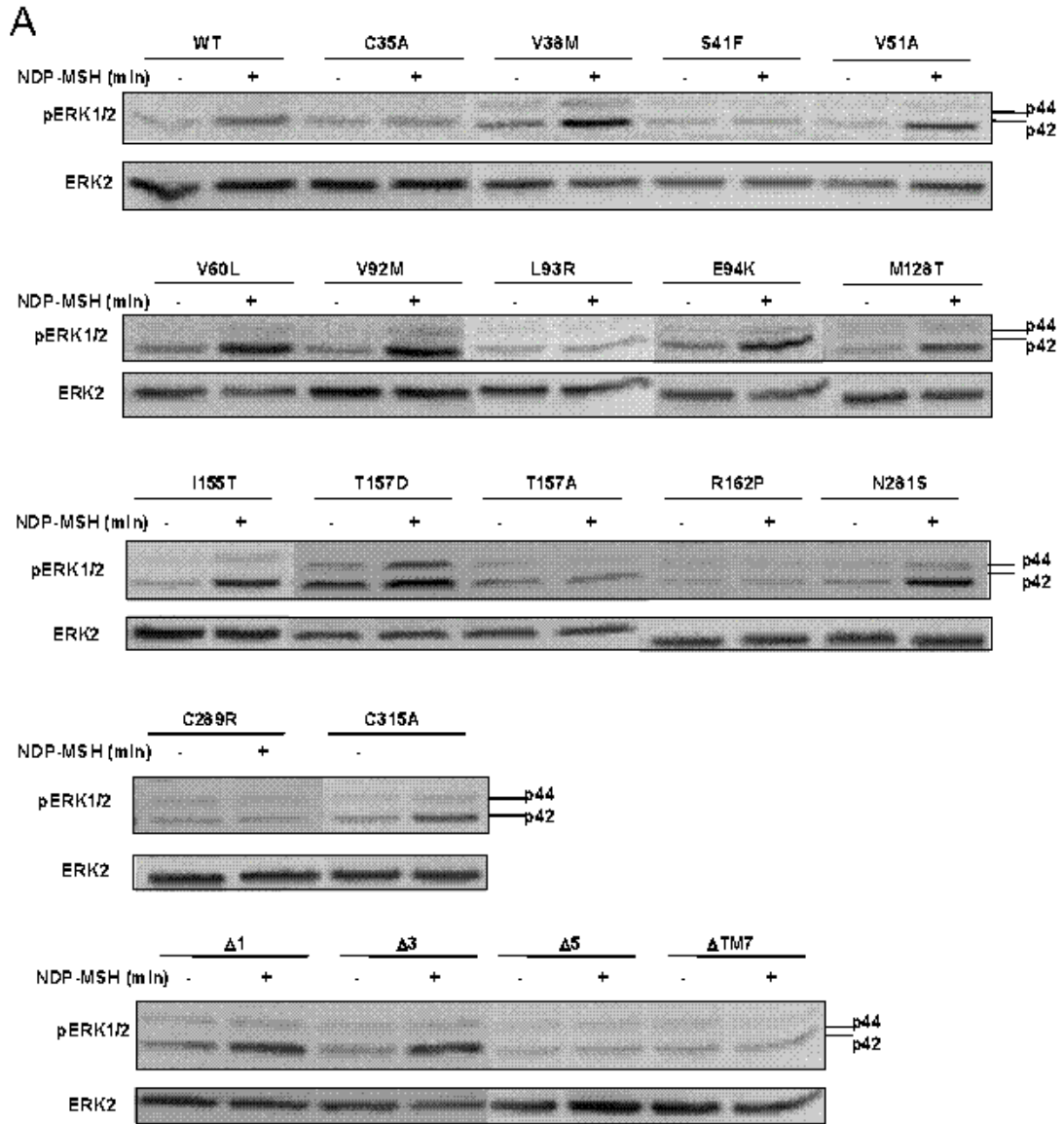
RESULTS.

Effect of MC1R mutations on signalling to the cAMP and ERK pathways.

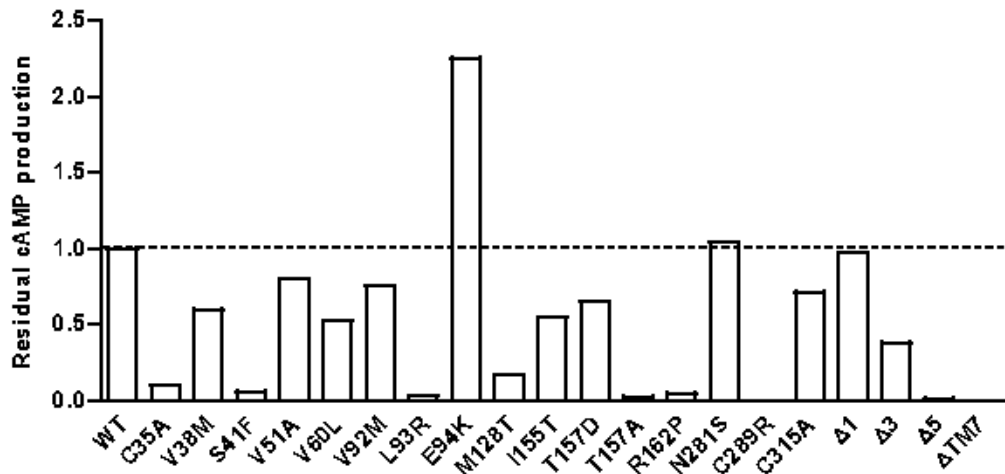
To study the regulation of MC1R signalling at the genotype level and to gain insight on differential requirements for functional coupling, we compared agonist-induced ERK phosphorylation and cAMP production in PC12 cells expressing WT and mutant MC1R. We selected a panel of 20 mutants spanning the widest range of functional alterations in signalling to the cAMP, from complete LOF to constitutive activity (13;17;20-26). PC12 cells were transfected to express the mutants, stimulated with 10^{-7} M NDP-MSH, and lysates were prepared to detect ERK activation by Western blot. Agonist-induced cAMP production and the availability of NDP-MSH binding sites on the cell surface of transfected cells were measured in parallel (Fig. 1). 8 mutants out of the 20 MC1R variants tested behaved as essentially complete LOF forms for both pathways (Fig 1, A-C). These were the C35A, S41F, L93R, T157A, R162P and C289R point mutants, and two deleted forms, $\Delta 5$ and $\Delta TM7$ lacking the last 5 C-terminal amino acids and a larger fragment comprising the seventh transmembrane helix and the complete cytosolic C-terminal tail, respectively. On the other hand, 9 mutants with different degrees of LOF in the cAMP pathway showed normal or even slightly increased functional coupling to the ERKs. These were the highly impaired M128T (residual cAMP stimulation \sim 25%),

the V38M, V60L, I155T and $\Delta 3$ alleles retaining ~ 50% signalling, and the V51A, V92M, T157D and C315A showing a slight reduction of functional coupling to cAMP synthesis (residual signalling ~ 75%). Finally, 2 mutants with cAMP coupling similar to WT (N281S and $\Delta 1$) and E94K, a constitutively and hyperactive form analogous to the *Sombre* mutation in the mouse, also behaved as WT in signalling to the ERKs.

We also compared the mutants for their ability to bind agonist when expressed in PC12 cells (Fig. 1D). Only 2 mutants showed normal cell surface expression of binding sites (V92M and N281S) and the other variants displayed variable residual binding. Those with residual binding lower than 10% relative to WT failed to activate the ERKs. Mutants with an agonist binding capacity over this threshold were at least as effective as WT in activating the ERKs, in spite of sometimes important LOF in the cAMP pathway. Conversely, no mutants were found with a higher decrease in ERK signalling compared with cAMP coupling.



C



D

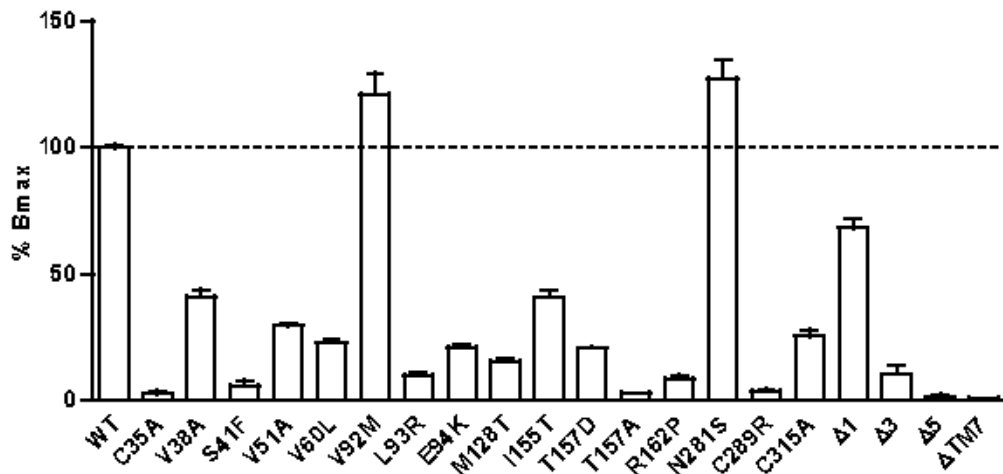


Fig. 1. MC1R-mediated ERK activation and cAMP production in PC12 cells expressing variant MC1R. A, PC12 cells were transiently transfected with natural (V38M, S41F, V51A, V60L, V92M, L93R, E94K, M128T, I155T, R162P, N281S, C289R) and artificial (C35A, T157D, T157A and C315A) MC1R point mutants and variants deleted at the C-terminus (Δ1, Δ3, Δ5 and ΔTM7). Transfected cells were serum-deprived for at least 3h and then, challenged with 10^{-7} M NDP-MSH for 15 min. Cell extracts were analyzed for ERK phosphorylation by Western blot and for total ERK2 as loading control. B, Quantification of two independent experiments each in duplicate, normalized to the intensity of the pERK signal in control cells expressing each variant. The upper and lower dashed lines indicate WT signalling and complete LOF, respectively. C, Agonist-induced cAMP production in HEK293T cells expressing the natural and artificial MC1R mutations and the deleted forms mentioned above. The dashed lines indicates WT signalling. D, Expression of 125 I-NDP-MSH binding sites. HEK293T cells were transfected with WT or mutant MC1R and incubated with

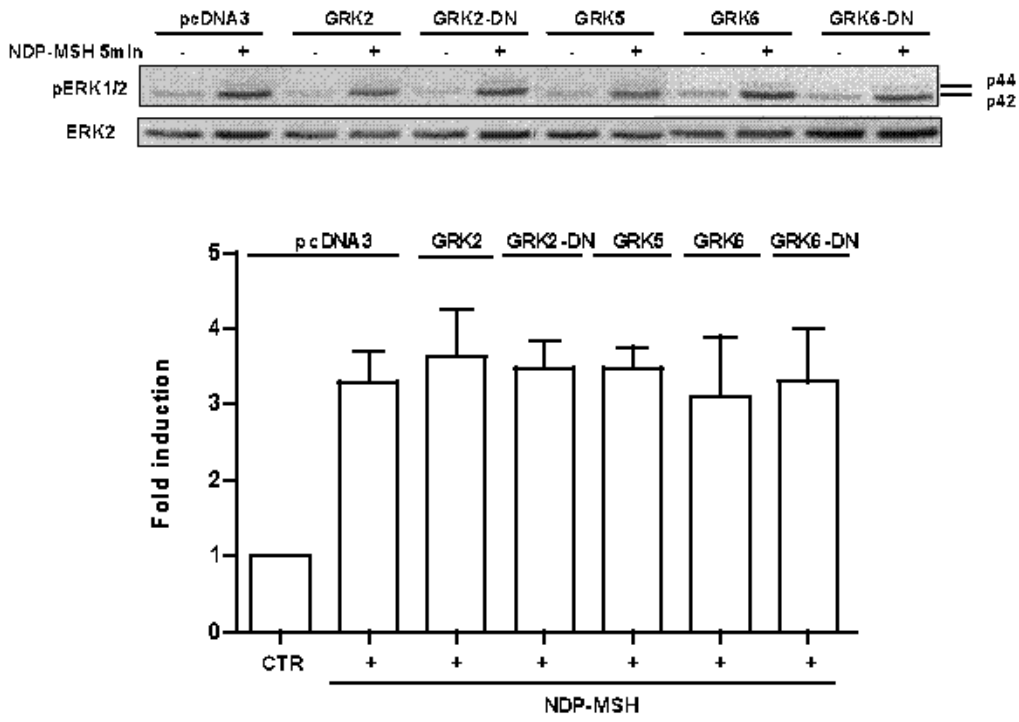
^{125}I -NDP-MSH (10^{-9} M, 10^5 cpm/well) to determine specific radioligand binding. The dashed line corresponds to a binding equivalent to WT.

The desensitization machinery terminating activation of the cAMP pathway does not modulate MC1R-dependent activation of the ERKs.

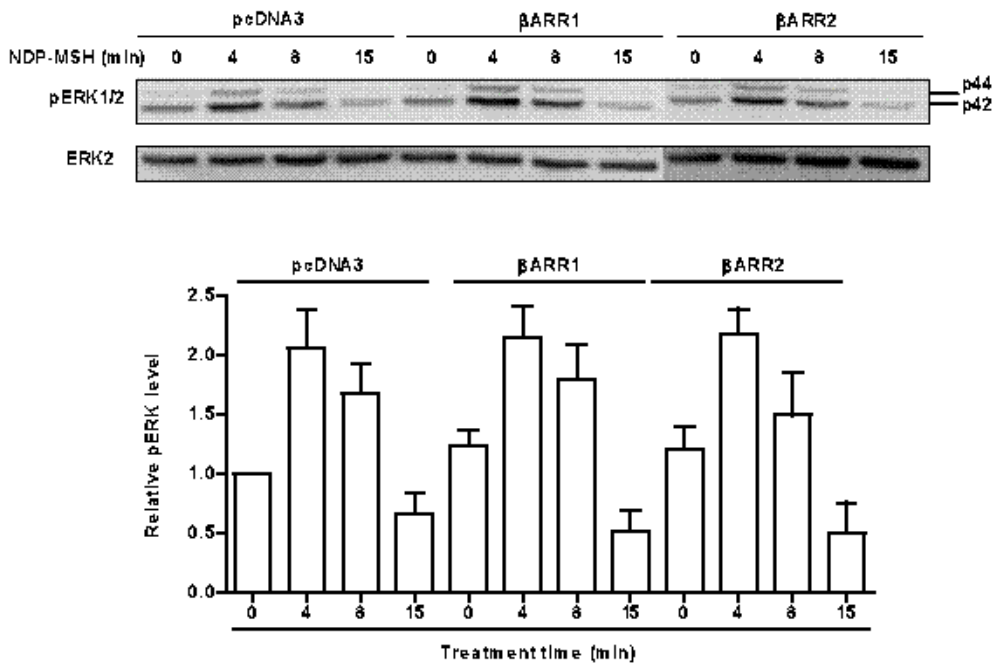
We investigated the effects of general mechanisms of GPCR signalling regulation on functional coupling of MC1R to the ERK pathway. For most GPCRs, signalling is strongly reduced within minutes of agonist exposure, in a process termed homologous desensitization. Desensitization involves the phosphorylation of the agonist-occupied receptor by specific kinases known as GPCR kinases (GRKs), followed by the recruitment to the phosphorylated receptor of members of the β -arrestin (β ARR) family of cytosolic proteins, which prevents coupling to the transducing G proteins (16;28;29). We have previously shown that MC1R desensitization is mediated by GRK2 or GRK6, two GRKs expressed in melanocytes and melanoma cells (26) and that GRK6 is also able to trigger MC1R internalization (13). We analyzed ERK activation by NDP-MSH in HBL human melanoma cells transiently transfected to express these GRKs, as well as GRK5, another member of the GRK family expressed in melanocytic cells (our unpublished observations). We also tested two dominant-negative kinase-dead mutants of GRK2 and GRK6 (GRK-DN). We found that none of the GRKs or the dominant-negative mutants had a significant effect on ERK activation following NDP-MSH treatment (Fig. 2A). Next, we investigated whether the β ARR had any effect on MC1R-dependent ERK activation. HBL cells were transfected with empty pcDNA3, β ARR1 or β ARR2 and stimulated with 10^{-7} M NDP-MSH for various times. Neither β ARR1 nor β ARR2 modified the extent or kinetics of ERK activation by NDP-MSH (Fig. 2B).

According to the data shown in Fig. 2. A and B, ERK activation by MC1R did not appear to be regulated by the general GPCR desensitization machinery, as opposed to the reported effect on functional coupling to the cAMP pathway (13;26). Thus, we analyzed whether functional coupling to the ERK module was modulated by more specific mechanisms of regulation of MC signalling. Mahogunin ring finger-1 (MGRN1) is a RING domain-containing ubiquitin ligase that acts as an accessory protein for MC signalling (30). We compared the effects of overexpression of two isoforms of mahogunin ring finger 1 (MGRN1 L (+) and MGRN1 L (-)). HBL cells expressing HA epitope-labelled MGRN1 isoforms did not show a significant modification of the levels of ERK phosphorylation following stimulation with NDP-MSH (Fig. 2C).

A



B



C

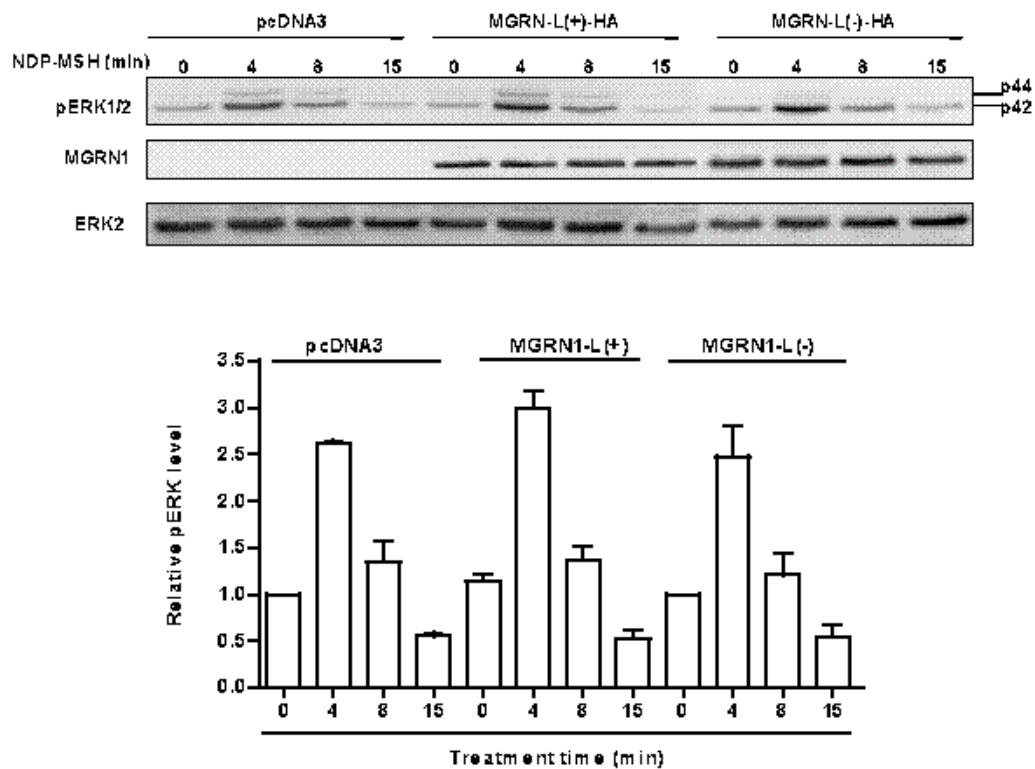


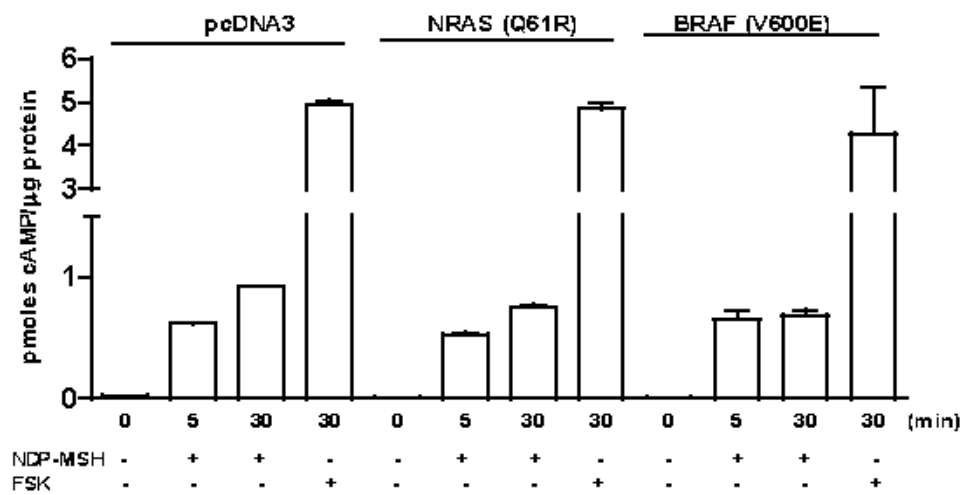
Fig. 2. MC1R-dependent ERK activation is independent on the GRK/ β ARR system or MGRN. A, HBL cells transfected with empty vector, the indicated GRKs or dominant-negative mutants were serum-deprived for 3h and stimulated with 10^{-7} M NDP-MSH for 5 min. Cell extracts were blotted for phospho-ERK1/2 and total ERK2 as loading control. The intensity of the bands was measured and normalized to the ERK2 signal. The quantification of three independent experiments is shown in the lower panel. B, HBL cells grown in 12-well plates were transfected with empty vector or with vectors encoding for β ARR1 or β ARR2, and stimulated as in panel A, for times ranging from 4 to 15 min. ERK activation was detected by Western blot and the quantification of two independent experiments, normalized with the ERK2 signal, is displayed on the lower panel. C, Cells transfected with empty vector or HA epitope-tagged MGRN1-L(+) or MGRN1-L(-) were challenged with 10^{-7} M NDP-MSH for the times shown. Cell lysates were probed for ERK1/2 phosphorylation, MGRN1 expression (with an anti-HA antibody), and for total ERK2 as loading control. The quantification of two independent experiments is also shown.

Lack of acute effects of ERK activity on MC1R coupling to the cAMP pathway.

We next examined the possible crosstalk of the ERK and cAMP pathways in HBL melanoma cells, WT for BRAF, NRAS and MC1R (14). First, we measured MC-induced cAMP production under conditions of high ERK activity. These conditions were achieved by expressing constitutively active NRAS and BRAF mutants (Q61R

and V600E, respectively). Cells transfected with the mutants were challenged with either 10^{-7} M NDP-MSH or 10^{-5} M FSK for various times and cAMP was determined. As shown in Fig. 3A, cells expressing the constitutively active upstream effectors of the ERKs responded with comparable stimulations of cAMP production. Therefore, high ERK activity did not modify adenylyl cyclase or phosphodiesterase enzymatic activity or the positive coupling of MC1R to this enzyme. Next, HBL cells were pretreated with the MEK inhibitor PD98059 to decrease basal levels of ERK signalling and block their activation before stimulation with NDP-MSH. The cAMP response to the MC1R agonist was also similar in PD98059 and control cells (Fig. 3B). Accordingly, MC-induced cAMP synthesis was largely insensitive to changes in ERK activity, thus suggesting that the ERK pathway had little impact on cAMP production.

A



B

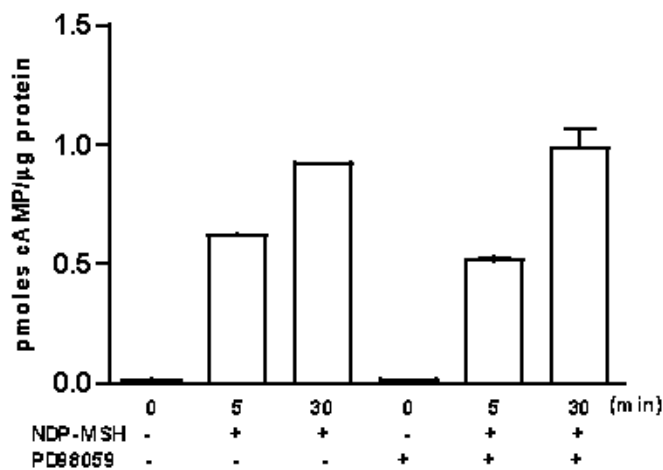


Fig. 3. Lack of effect of ERK activity on cAMP production. A, HBL cells expressing empty vector or NRAS and BRAF mutants (Q61R and V600E, respectively) were stimulated with

either 10^{-7} M NDP-MSH or 10^{-5} M FSK for the times shown. Intracellular cAMP production was measured by radioimmunoassay. B, HBL cells were challenged as above in the presence or absence of the MEK inhibitor PD98059 and intracellular cAMP levels were analyzed.

This result was somewhat surprising in the light of reports that mutant NRAS disrupts cAMP signalling at the level of cAMP metabolism in melanocytes and melanoma cells (30). Therefore, we analyzed a panel of 26 human melanoma cell lines for NRAS, BRAF and MC1R genotype, as well as for the functional response of the ERK and cAMP pathways to stimulation of MC1R (Fig. 4). This panel consisted of 15 cell lines WT for NRAS and BRAF, 9 BRAF-mutant and 2 NRAS-mutant cell lines. Concerning the MC1R, 9 lines were WT, 12 heterozygote and 5 either compound heterozygote or mutant homozygote. Serum-deprived melanoma cell lines were stimulated for 30 min with either 10^{-7} M NDP-MSH or 10^{-5} M FSK and intracellular cAMP levels were measured (Fig. 4). NRAS-mutant cells (MM052 and MM125) failed to respond to NDP-MSH in spite of a moderate increase in cAMP levels following FSK treatment. This failure could not be explained by the MC1R genotype, since MM052 cells were WT for the receptor, and MM125 were heterozygote for the V92M low penetrance RHC allele which retains significant activity, according to the data shown in Fig. 1 and with previous reports by others (31). On the other hand, 8 out of 9 BRAF-mutant cells did not respond to NDP-MSH and most of them showed a poor but reproducible response to FSK. Also in this case, this behaviour could not be readily explained by the MC1R genotype, since some of the cell lines were homozygous for the WT allele (MM029) whereas others were heterozygotes carrying WT and mutant MC1R alleles with significant residual activity such as R163Q (32;33), V60L (32;34) or V92M (31;35;36). One of the BRAF-mutant cells (MM061) responded adequately to NDP-MSH and FSK, with cAMP levels comparable with WT cells such as HBL, MM028 or MM117 cells. Significantly, these BRAF-mutant but responsive cells were heterozygote carriers of the MC1R R142H RHC allele.

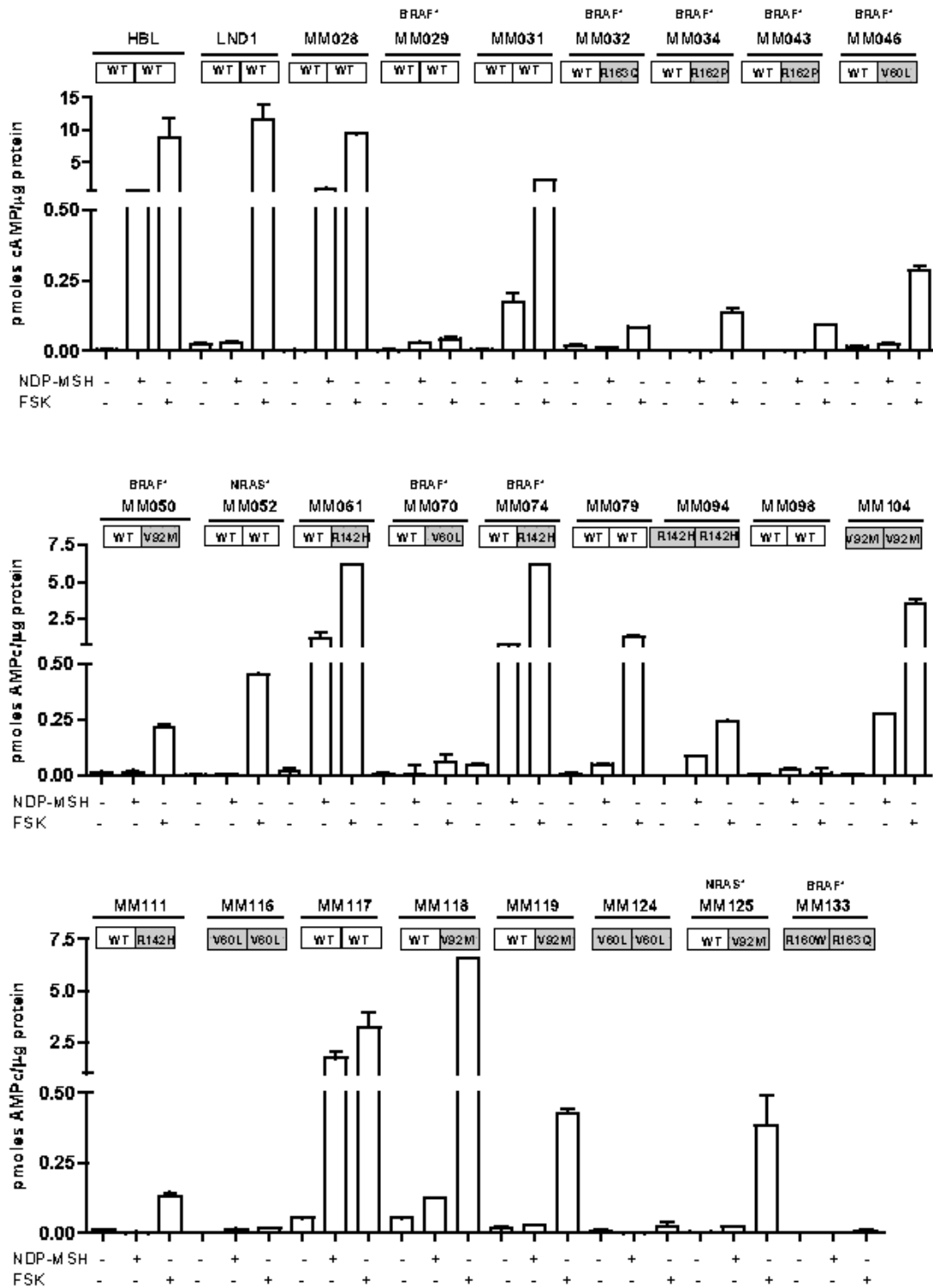
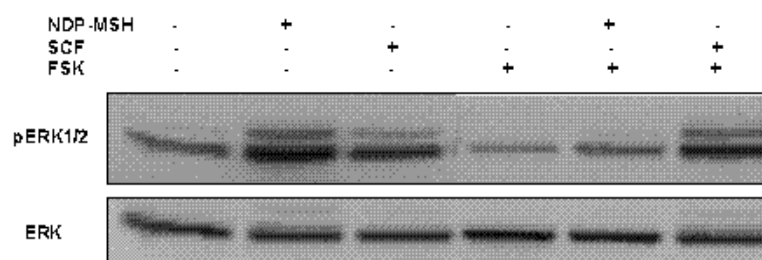


Fig. 4. Intracellular cAMP levels in response to NDP-MSH and FSK. Melanoma cell lines were serum-deprived for at least 3h and then challenged with 10^{-7} M NDP-MSH (30 min) or 10^{-5} M FSK (30 min). cAMP production was assessed by radioimmunoassay.

Inhibition of MC1R-mediated ERK activation by cAMP in human melanoma cells.

We have shown previously that stimulation of adenylyl cyclase with FSK decreased basal ERK activity in HBL human melanoma cells (14), suggesting a negative regulation of the ERK pathway by cAMP. This finding was in sharp contrast with the reported activation of ERK signalling by cAMP in mouse melanoma cells (37). Accordingly, we examined in more detail the effects of cAMP on the functional status of the ERK pathway by analyzing not only basal ERK phosphorylation, but also MC1R-dependent activation in human melanoma cells. HBL human melanoma cells were pretreated with FSK or with vehicle, and then stimulated with 10^{-7} M NDP-MSH. Since stimulation of the ERKs by MC1R signalling depends on the transactivation of the c-KIT receptor tyrosine kinase (14), we also analyzed the effects of FSK on ERK activation by the c-KIT ligand SCF. As shown in Fig. 5A, pretreatment of HBL cells with FSK not only decreased basal levels of phosphorylated ERKs, but also potently inhibited ERK stimulation by NDP-MSH. Noticeably, FSK had no effect on SCF-mediated ERK activation. These data indicate that high cAMP levels efficiently blocked MC1R-dependent activation of the ERKs in human melanoma cells, presumably upstream of the c-KIT RTK. Should this be the case, then melanoma cell lines with constitutive activation of the ERK pathway as a consequence of activatory mutations in NRAS or BRAF should not respond to the inhibitory action of FSK since ERK activation would be initiated downstream of the site of action of cAMP. We tested this hypothesis by comparing the effects of FSK on the NRAS mutant (Q61R) cell lines MM052 and MM125 and the BRAF mutant cell lines MM046 and MM074. These cell lines were selected because they all responded to FSK with moderate to even high increases in cAMP levels (see Fig. 4). Treatment of the NRAS- or BRAF-mutant cells with agonist or FSK had no effect on pERK levels (Fig. 5B), again suggesting that the site of cAMP inhibitory action was located upstream of NRAS.

A



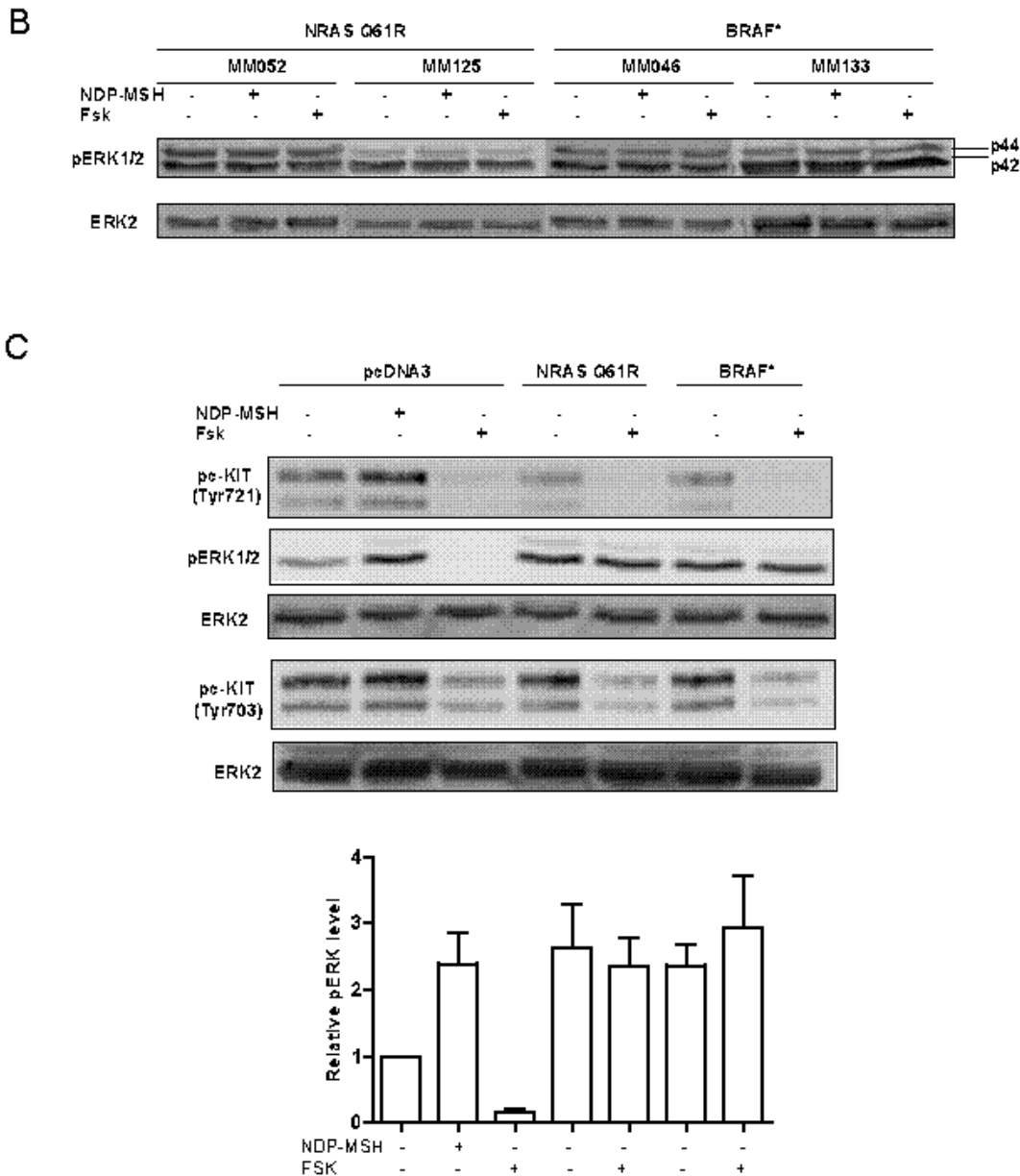


Fig. 5. Effect of FSK on ERK activation in human melanoma cells. A, HBL cells were pretreated with 10^{-5} M FSK or vehicle for 15 min, then challenged with 10^{-7} M NDP-MSH (5 min), or 50 ng/ml SCF (6 min) in the continuous presence of the adenylyl cyclase activator. Cell extracts were analyzed for pERK levels by Western blot. Total ERK2 was used as a control for comparable loading. B, The melanoma cell lines LOCE-MM052 and 125, carrying the Q61R activatory mutation in NRAS and MM046 and 050, with the V600E mutation in BRAF, were challenged with 10^{-7} M NDP-MSH for 5 min or 10^{-5} M FSK, 15 min. pERK level was detected by Western blot and total ERK2 as loading control. C, HBL cells expressing empty vector pcDNA3 or constitutively active mutants NRAS (Q61R) or BRAF (V600E) were treated with FSK and blotted for c-KIT phosphorylation at tyrosine721 or tyrosine703, ERK activation or total ERK as indicated.

Finally, we tested the effects of FSK on HBL cells transiently transfected with the constitutively active NRAS and BRAF mutants. We also analyzed the activation of c-KIT by comparing the levels of phosphorylation at two functionally important Tyr residues, before and after agonist or FSK treatment. These residues were Tyr721, and Tyr703 whose phosphorylation provides a docking site for binding and activation of phosphatidylinositol 3 kinase (38;39), and for the adaptor protein Grb2 that may link c-KIT activation to the RAS/RAF/MEK/ERK pathway (39;40), respectively. Expression of mutant NRAS or BRAF increased ERK activity, but had no effect on the level of phosphorylation of c-KIT (Fig. 5C). FSK treatment did not block ERK activation by the constitutively active effectors, consistent with the results previously shown for human melanoma cells expressing endogenously the oncogenic mutants (Fig. 5B). Conversely, FSK strongly inhibited c-KIT activity, as shown by decreased phosphorylation at Tyr721 and Tyr703.

DISCUSSION

Stimulation of MC1R by MC agonists has been shown to activate two major signalling pathways: the canonical cAMP pathway and the ERK module (1;9;14). The ERK pathway is typically triggered in melanocytes by growth factors such as the c-KIT ligand SCF or basic fibroblast growth factor (41) to regulate proliferation. The cAMP pathway activated by the MC1R upon binding of MC ligands (11) is weakly mitogenic for melanocytes and is closely associated with differentiation (30;42). In mouse melanoma cells, these pathways seem positively connected, since early studies suggested that ERK signalling relied on the cAMP-dependent activation of NRAS by an undefined PKA- and Epac-independent mechanism (37). However, in human melanocytic cells MC signalling to the cAMP and ERK pathways might diverge immediately downstream MC1R, since ERK activation apparently follows a cAMP-independent pathway involving a different set of effectors including c-KIT (9;14). Accordingly, MC1R signalling to cAMP on one hand, and the ERKs, on the other, might be differentially regulated. Within melanocytic cells, several layers of differential MC1R regulation could be considered, including: i) the expression of variant alleles, ii) the modulation of the interaction of MC1R with downstream effectors and signalling partners in each module and iii) the crosstalk between the pathways.

In order to analyze the influence of MC1R genotype on signalling to the cAMP and ERK pathways, we selected a panel of 20 mutations, of which 12 correspond to natural variants (17;21-23) and the other 8 to artificial mutants

previously obtained in our laboratory (20;24;25). These mutations were scattered throughout the MC1R molecule, and together with the previously reported R151C, R160W and D294H they form a representative repertoire of 23 MC1R variants that allows for probing most of its relevant structural domains. The variants were transiently expressed in heterologous PC12 cells, and tested for their ability to activate the ERKs when challenged with NDP-MSH. We chose the PC12 model since we have previously shown that signalling from the RHC alleles R151C, R160W and D294H expressed in PC12 cells increases ERK phosphorylation following stimulation with NDP-MSH as effectively as WT (9;14), despite their reduced ability to stimulate cAMP production (9;14). Accordingly, the PC12 model recapitulates the behaviour of RHC variants expressed in melanocytic cells (1;9;13). We found that functional coupling to the ERK pathway, with around 65% of the mutants retaining WT signalling, was much more robust than association with the cAMP pathway, with around 80% of the mutants showing varying degrees of functional impairment. Moreover, we did not find a single mutant retaining significant coupling to cAMP, but unable to trigger the ERK module. Concerning the properties of the mutants that did not activate the ERKs, they were all characterized by a very low binding of agonist (less than 10% of the WT values after transfection in PC12 cells). This dramatically reduced binding was due either to aberrant trafficking with reduced cell surface expression for C35A, S41F, L93R, T157A or R162P (17;20-23;25), or to normal cell surface expression but very low binding affinity for C289R (17). On the other hand, mutants with significantly impaired but detectable expression on the cell surface above a threshold of approximately 10% of WT values always retained signalling to the ERKs but often displayed a LOF phenotype in functional coupling to the cAMP pathway. This suggested that a reduced number of MC1R-agonist complexes could be sufficient to saturate the transduction machinery connecting the MC1R to the ERK module, whereas a higher density of complexes would be required to achieve maximal stimulation of the cAMP pathway. Hence, for the WT receptor in a normal melanocytic environment, the number of spare receptors would be much higher for coupling to the ERKs as compared with the cAMP pathway, consistent with the dramatic left-shift of the dose response curves for MC-induced signalling to the ERKs compared with cAMP (9;14).

On the other hand, the duration and intensity of agonist-induced GPCR signalling through the cAMP pathway are regulated by general mechanisms involving the coordinated action of two families of regulatory proteins, the β ARRs and the GRKs. After continuous exposure to the agonist, GPCRs are phosphorylated by the GRKs (26;29). This phosphorylation promotes recruitment of

β ARRs, thereby uncoupling the GPCR and the G protein and triggers the internalization of the agonist-receptor complex. This cascade of events terminates G protein-dependent signalling in a process known as homologous desensitization (29;43). However, β ARRs can also serve as adaptors or scaffolds for various molecules involved in signalling functions, such as members of the ERK pathway and in fact they are positively involved in activation of the ERKs by a number of GPCRs (28;29). Thus, we investigated the possible role of the β ARR-GRK system on MC1R mediated ERK activation. We found that neither GRKs, nor arrestins decreased MC signalling to the ERKs, under conditions that desensitize signalling to the cAMP cascade and cause MC1R internalization (13;26). Moreover, in addition to the general regulatory mechanisms acting on most GPCRs, other specific processes contribute to the regulation of MC signalling. The ubiquitin E3 ligase MGRN1 has been shown to decrease MC1R signalling to the cAMP pathway by competitive displacement of $G_{\alpha s}$ (44), a mechanism reminiscent of the mode of action of β ARRs. We found that expression of MGRN1 had no effect on ERK activation. This was consistent with the lack of effect of β ARRs and with our previous report that signalling from the MC1R to the ERKs is not dependent on G_s , but rather on c-KIT.

On the other hand, the cAMP and ERK pathways establish a complex, cell type-specific and often bidirectional crosstalk that contributes to fine-tune the cellular responses to environmental cues. In most cell types, high cAMP levels inhibit ERK activity by a number of mechanisms. One of these mechanisms involves the inhibition of CRAF by PKA, either directly or via Akt. Other mechanisms rely on the activation of Rap1, a GTPase that blocks the ERK pathway by preventing activation of CRAF by RAS (18;19), or of the MAP kinase phosphatases (MKPs), a small family of dual specificity tyrosine and serine/threonine phosphatases that target the MAPKs (45;46), and are transcriptionally activated by cAMP (47;48). Conversely, ERK activity can impact on the cAMP pathway at several levels, including regulation of phosphodiesterase activity (49;50), or interference with the functional coupling of activated receptors with G protein via regulation of GRK activity (51;52).

In contrast with previous reports that high cAMP levels activate ERK signalling in mouse melanoma cells (37), we have shown here that in HBL human melanoma cells WT for MC1R, NRAS and BRAF FSK effectively blocks ERK activation downstream of the MC1R in addition to decreasing the levels of phosphorylated ERKs in resting cells. We have not yet attempted to identify the target(s) of this strong inhibitory effect. Nevertheless, our data clearly indicate that it is located upstream of NRAS or BRAF, as shown by lack of effect of FSK on downstream signalling from constitutively active mutants of these ERK effectors, in

two experimental settings: transient expression of the oncogenic mutants in HBL cells or continuous expression of the endogenous mutant proteins in human melanoma cells. This may account for high proliferation rates in human melanomas with hyperactive forms of NRAS or BRAF but WT MC1R. Importantly, FSK inhibition of MC1R coupling to the ERKs also appeared located upstream of c-KIT, since pretreatment with FSK prior to challenging HBL cells with the MC1R agonist NDP-MSH not only blocked c-KIT activation but even decreased phosphorylation of specific tyrosine residues important for receptor function below the levels observed in resting cells. Moreover, FSK did not block ERK activation by the physiological c-KIT agonist, SCF. It is important to point out that our observation that cAMP inhibited MC1R signalling to the ERKs upstream of c-KIT, NRAS and BRAF, is fully compatible with the co-mitogenic effect of cAMP in melanocytes. Indeed, melanocytic cells should be able to activate the ERK mitogenic pathway in response to canonical growth factors such as SCF and probably basic fibroblast growth factor even under conditions leading to increased cAMP levels.

We also considered the effects of the ERKs on the functional coupling of MC1R to the cAMP pathway. We found that acute modulation of ERK activity did not modify cAMP levels following treatment of HBL cells with FSK, or with NDP-MSH. This showed that the ERKs did not target key components of cAMP metabolism for rapid ERK-dependent phosphorylation and functional regulation. However, these data did not exclude the possibility that long-term, continuous activation of the ERKs could lead to modulation of cAMP metabolism as reported by others (30), through slower onset mechanisms of action like modulation of gene expression. We addressed this possibility by analyzing a panel of 26 human melanoma cell lines of defined MC1R, NRAS and BRAF genotype for their cAMP levels following stimulation with FSK or NDP-MSH. We found that 2 out of 2 melanoma cell lines mutant in NRAS showed absent responsiveness to NDP-MSH but still responded to FSK with a significant cAMP production, in spite of an MC1R genotype expected to allow for a normal response to MC agonists. Accordingly, at least part of the functional defect in these cells should be located upstream of adenylyl cyclase, hence at the level of MC1R availability on the cell surface or MC1R functional coupling to the cyclase. On the other hand, 8 out of 9 melanoma cell lines mutant for BRAF were equally unresponsive to NDP-MSH in terms of increased cAMP levels, thus showing that disruption of cAMP signalling in human melanoma is not exclusive of NRAS mutant melanomas. In addition, one BRAF mutant cell line (MM074) responded normally to both NDP-MSH and FSK, and several other mutant cell lines (MM032, MM034, MM043, MM046 and MM050) responded weakly but detectably to

FSK. This showed that a normal cAMP response to the MCs could be supported by mutant melanoma cells with a hyperactive ERK pathway, and again suggested that disruption of cAMP metabolism occurs upstream of the cyclase.

The lack of effect of acute modulation of ERK activity on cAMP metabolism in melanoma cells WT for MC1R, NRAS or BRAF suggests that chronic, rather than acute ERK activation is responsible for the frequent cAMP-deficient phenotype in melanoma cells mutant in NRAS or BRAF. Nevertheless, other explanations cannot be ruled out, and it could be that absent or impaired cAMP signalling is the cause rather than the consequence of BRAF or NRAS mutations, in keeping with reports that a mutant MC1R genotype is associated with increased risk of mutation in BRAF (53;54). Alternatively, mutations in members of the ERK pathway on one hand, and impaired cAMP signalling on the other might be independent and mechanistically unrelated events that nevertheless cooperate in melanomagenesis, thus increasing the likelihood of their co-existence in melanoma. In keeping with this possibility, lack of a cAMP response to agonists and/or FSK was occasionally observed even for cell lines WT for MC1R, NRAS and BRAF (MM079, MM098). This shows that a genotypic analysis of MC1R is not a sufficiently accurate index of the functional status of the cAMP responsiveness to MC ligands in melanoma.

In any case, we have shown that functional coupling of the MC1R to the ERK and cAMP pathways is differentially regulated by the MC1R genotype, and at the level of specific interactions with signalling partners. Thus, MC1R-mediated MC signalling to the ERKs might be significant in a number of situations where the MC-dependent activation of the cAMP pathway would be very low or absent (Fig. 6). These situations would include inherent properties of given populations of melanocytes such specific variant MC1R or MGRN1 genotypes, or external factors such as low MC concentrations or continuous exposure to high levels of agonist.

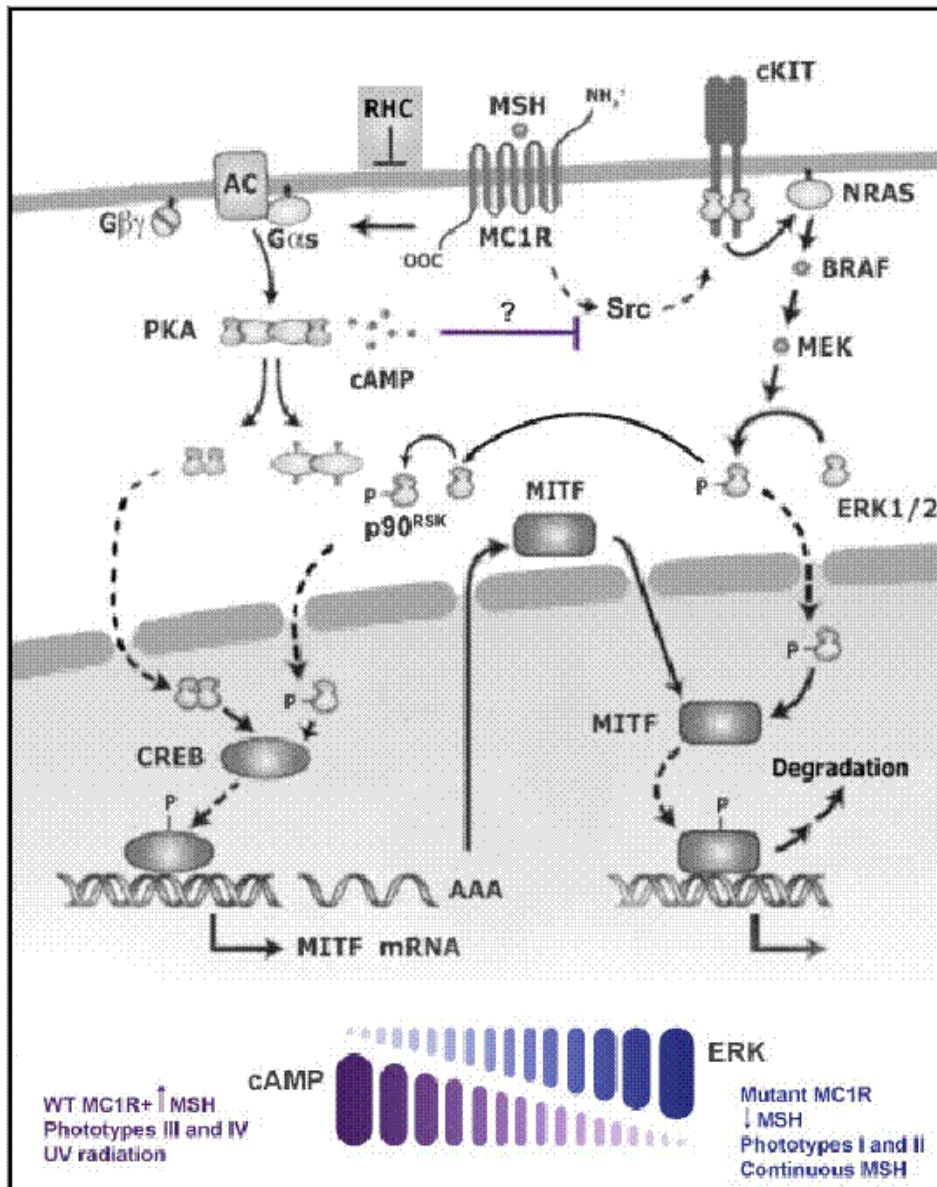


Fig. 6. MC1R-mediated cAMP and MAPK ERK1 and ERK2 pathways in human melanoma cells.

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CONCLUSIONES.

CONCLUSIONES:

1. El MC1R está N-glicosilado en los residuos Asn15 y Asn29, probablemente por adición de un glicano rico en manosa y otro de tipo mixto, más grande y de mayor complejidad, respectivamente.
2. La presencia del carbohidrato en el MC1R no es imprescindible para el tráfico anterógrado del receptor ni para su acoplamiento funcional, pero aumenta su expresión en la superficie celular.
3. En células melanocíticas humanas, la estimulación de la vía de señalización de las ERKs mediada por el MC1R es independiente del AMPc e implica la transactivación del RTK c-KIT.
4. Src se activa aguas abajo del MC1R pero aguas arriba de c-KIT en la vía de señalización no canónica que conduce a la activación de las ERKs.
5. La activación de la vía de las ERKs requiere niveles mucho más bajos de agonista que la síntesis de AMPc.
6. Muchas de las variantes alélicas del MC1R con pérdida del acoplamiento funcional a la vía canónica de síntesis de AMPc, son capaces de activar la vía no-canónica de las ERKs. Entre estas variantes destacan los alelos RHC de alta penetrancia R151C, R160W y D294H.
7. Todas las variantes del MC1R con pérdida de función en la vía de las ERKs son deficientes en la producción de AMPc, muy poco frecuentes y se caracterizan por una muy baja capacidad de unión de agonista.
8. La activación de las ERKs mediada por MC1R no está regulada positiva ni negativamente por la maquinaria de la desensibilización homóloga del receptor en cuanto a su acoplamiento a la cascada del AMPc.
9. Altos niveles de AMPc inhiben la activación de ERK mediada por MC1R y esta inhibición ocurre aguas arriba de c-KIT.

10. Cambios agudos en el nivel de actividad de las ERK no modifican la inducción de la síntesis de AMPc en respuesta a la activación del MC1R por sus agonistas, o de la adenilato ciclasa por FSK.

11. En los melanomas humanos es frecuente la pérdida de señalización de MC1R a la cascada del AMPc, y este defecto se observa incluso en líneas celulares de melanoma con genotipo MC1R silvestre y sin mutaciones en NRAS o BRAF.

SUMMARY.



PhD THESIS SUMMARY

SIGNALLING FROM MELANOMA-ASSOCIATED MELANOCORTIN 1 RECEPTOR ALLELIC VARIANTS

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INTRODUCTION

The melanocortins (MCs) are structurally related peptides originally characterized as regulators of cutaneous pigmentation and cortisol production (1). MCs are expressed in the pituitary and peripheral locations including the skin. The natural MCs, ACTH and the melanocyte-stimulating hormones (α -, β -, and γ -MSH), derive from the common precursor proopiomelanocortin (POMC) (2). Physiologic actions of MCs are mediated by a five-member subfamily of G-protein coupled receptors (GPCR), the melanocortin receptors (MCRs), which trigger the G_s-protein-dependent activation of the cyclic AMP (cAMP) signalling pathway (3). Two of these members, MC1R and MC5R, are expressed in human epidermis. MC1R is preferentially expressed in melanocytes and melanoma cells (4), where it regulates the amount and type of pigment production and is a major determinant of skin phototype, sensitivity to ultraviolet radiation and melanoma and non-melanoma skin cancer risk (5). α -MSH stimulates adenylyl cyclase via activation of MC1R and leads to elevation of intracellular cAMP. This results in increased transcription of microphthalmia transcription factor (MITF), which in turn leads to transcriptional activation of the rate-limiting melanogenic enzyme tyrosinase and increased production of dark, photoprotective eumelanins (2;6). As a consequence, cAMP signalling in melanocytes is mainly related with differentiation, although it also has a detectable mitogenic action (7).

The mitogen-activated protein kinase (MAPK) module leading to the Ser/Thr kinases ERK1 and ERK2 is a major intracellular signalling pathway controlling key cellular decisions such as proliferation or differentiation (8). This signalling cascade is most often initiated by binding of growth factors to cell surface tyrosine kinase receptors (RTKs), followed by sequential activation of RAS, then members of the RAF family of kinases, the MAPK kinase MEK and finally, ERK1 and ERK2 (9). Active ERK phosphorylates multiple cytoplasmic and cytoskeletal proteins (10), including several subfamilies of MAPK-activated protein kinases, MKs (MSKs, MNKs, RSKs). Additionally, active ERK1/2 translocate to the nucleus and regulate the activity of transcription factors such as c-Myc, Elk1 and ATF2, influencing gene transcription and thereby controlling cellular proliferation (11;12). The crucial role of the RAS/RAF/MEK/ERK pathway in the regulation of melanocyte proliferation is demonstrated by the occurrence of *BRAF* mutations in 50-70% of human melanomas (9;13-15). Moreover, *NRAS* activating mutations are another prevalent genetic alteration in melanoma found in 20-30% of lesions (14;16;17). Since *NRAS*

and *BRAF* mutations are mutually exclusive in melanoma (18), they collectively account for the observed ERK hyperactivation in over 75% of these tumours.

Within melanocytes, the ERK signalling pathway is activated, among other receptors, by c-KIT, a RTK crucial for melanogenesis, proliferation, migration, and survival of the pigment-producing cells, again demonstrating the importance of ERK signalling in melanocytes (19). c-KIT is allelic with the *W* (*white spotting*) locus in mice and its ligand, SCF, is the product of the *S*/locus. Mutation in either of these loci results in a pleiotropic phenotype with white spotting of the fur due to absence of melanocytes, lack of mast cells and defects in haematopoiesis and germ cell development (20). c-KIT mutations have also been identified in human piebaldism (21;22) and at low frequency in human melanomas (23), and c-KIT expression is often lost in these tumors (24;25).

In addition to this classical RTK pathway, the RAS/RAF/MEK/ERK module is also regulated by GPCRs (26-29). In this case, ERK activation is usually dependent on the activity of second messenger regulated protein kinases such as PKC, non-receptor tyrosine kinases (non-RTKs) such as Src or on the generation of intracellular signalling complexes formed by binding of β -arrestins or other scaffolds to the activated GPCRs. Moreover, GPCRs can stimulate cytosolic or membrane-bound metalloproteases which cleave the membrane anchoring domain of RTK ligands to release the active agonist, thus promoting receptor activation (29-31).

In mouse melanoma cells, MC1R activation by MCs is positively coupled to cAMP and ERK signalling. Based on data from the B16 mouse melanoma model it has been proposed that ERK activation in human melanocytic cells is due to cAMP-dependent but PKA-independent activation of NRAS and BRAF, although the mechanism of NRAS activation is still unknown (32;33). Moreover, a direct verification of this hypothesis was missing when we undertook this work.

Human *MC1R* is extremely polymorphic and more than 100 non conservative natural mutations have been reported (34). Several relatively frequent alleles are associated with red hair and fair skin (the RHC phenotype) (5;35-37) and increased risk for melanoma and other skin cancers (38;39). Accordingly, the study of the structural and functional characteristics of these mutants is of wide biomedical interest. Three frequent and penetrant melanoma-associated alleles, R151C, R160W, and D294H, are hypomorphic variants with reduced functional coupling to the cAMP cascade (40-45). Since cAMP was reported to trigger ERK activation in melanocytes, signalling to the ERKs might also be impaired or absent for these variants. However, this possibility has never been investigated. In addition, the natural mutations could conceivably have a differential impact on the cAMP and

ERK pathways, thus causing an imbalanced response to the MC. Again, this hypothesis has not been tested.

On the other hand, many GPCRs undergo N-glycosylation during intracellular traffic from the ER to the plasma membrane (46). This post-translational modification may play a critical role in high affinity ligand binding, cell surface expression and/or functional coupling (47-53). MC1R contains two potential N-glycosylation sequins, ¹⁵NST¹⁷ and ²⁹NQT³¹, located at the extracellular N-terminus. Previous studies have demonstrated that MC1R is expressed as a glycoprotein both in heterologous systems (43) and in melanoma cells (34;54). However, these studies did not address the occupancy of each specific glycosylation sequon within the extracellular N-terminus of the receptor, or the functional consequences of glycosylation. Moreover, MC1R glycosylation pattern was highly unusual in that WT MC1R was sensitive to endoglycosidase H (EndoH) digestion, which only cleaves core high-mannose N-glycan chains and hybrid-type chains. This feature was surprising for a fully active WT protein, and apparently excludes the use of this endoglycosidase as a tool to study the processing of mutant MC1R. Accordingly, no data are available on the possibility of alterations in the glycosylation pattern of MC1R mutants that may underlie aberrant signalling properties.

AIMS

In an attempt to better characterize the functional and phenotypic effects of natural mutations in the human MC1R gene, we considered the following specific aims:

- 1- Analysis of the glycosylation pattern of WT MC1R and several natural variants associated with cutaneous phenotypes and increased skin cancer.
- 2- Functional characterization of the carbohydrate in MC1R.
- 3- Analysis of the effect of frequent and penetrant melanoma-associated MC1R mutations, such as R151C, R160W and D294H, on MC1R-dependent ERK activation.
- 4- Study of the mechanism of human MC1R-mediated ERK activation.

- 5- Assessment of the impact of a wide panel of MC1R mutations on signalling to the ERK and cAMP pathways.
- 6- Examination of the possible crosstalk between RAS/RAF/MEK/ERK and cAMP signalling pathways mediated by MC1R.

RESULTS AND DISCUSSION

1. **N-glycosylation of the human melanocortin 1 receptor. Occupancy of glycosylation sequons and functional role.**

As previously reported (34;43;54), we confirmed that MC1R expressed in HEK cells and in HBL melanoma cells migrated as a doublet comprising a glycosylated band of $M_r \sim 34$ kDa and a non-glycosylated protein of apparent $M_r \sim 29$ kDa. Both EndoH and PNGaseF fully digested the upper 34 kDa band but the mobility of the 29 kDa form remained unchanged, and identical to the one of the glycosylation-null mutant. To analyze the MC1R for sequon occupancy, functional effects of glycosylation and changes in the glycosylation pattern of mutant forms, we used site-directed mutagenesis to ablate the putative glycosylation sites (Asn15 and Asn29), alone or in combination. We found that both sequons are occupied by structurally inequivalent sugar chains. Moreover, comparison of the electrophoretic mobility and apparent M_r of the mutants showed that the oligosaccharide bound to the Asn29 was bulkier than the one attached to Asn15. This suggested a different processing for the *N*-oligosaccharide bound to each residue. For the bulkier *N*-glycan in Asn29, EndoH sensitivity excluded processing to a complex type oligosaccharide, and its contribution to the overall M_r of the protein was consistent with a hybrid type structure. On the other hand, the small size and EndoH sensitivity of the glycan in Asn15 was compatible with a core high mannose-type structure (46). Then, we assessed the effect of glycosylation on MC1R cell surface expression and on binding affinity by performing radioligand binding studies using ^{125}I -labelled NDP-MSH in HEK cells transfected with WT MC1R or the glycosylation mutants. We found a decreased agonist binding to cells expressing N29Q or the glycosylation-null mutant, most probably due to lower cell surface expression, rather than impaired affinity. Moreover, we also tested the effect of two drugs on MC1R ligand binding: the nucleoside antibiotic tunicamycin (TNM), which blocks the initial transfer of the dolichol phosphate-bound oligosaccharide chain to Asn acceptors in

proteins by inhibition of ER phosphosugar transferases and 1-deoxymannojirimycin (DMJ), which inhibits Golgi-resident mannosidases, preventing trimming and processing of the initial carbohydrate chain to hybrid or complex type oligosaccharides. HBL cells treated with either TNM or DMJ bound significantly lower amounts of the MC1R radioligand, but the electroforetic pattern of MC1R remained unchanged after DMJ treatment. Additionally, we examined cAMP production in cells expressing MC1R glycosylation mutants and in HBL cells treated with DMJ or TNM. The cAMP response was strongly decreased for N29Q, T31A and N15Q-N29Q and cAMP levels were also significantly inhibited by the drugs in HBL cells. We looked for alterations in forward or retrograde transport of MC1R glycosylation mutants by comparing their subcellular distribution by confocal microscopy. We found that the labelling pattern of N29Q and N15Q-N29Q was indicative of association with the ER, whereas staining for N15Q was consistent with normal forward trafficking and cell surface expression. We also checked whether MC1R glycosylation had any effect on receptor internalization. We found that N29Q and N15Q-N29Q showed a significant increase in MC1R internalization, which suggested that the stability of these forms on the cell surface was impaired. A similar increase in the rate of internalization was observed for HBL cells treated with DMJ. We compared the co-localization of MC1R forms with markers of endocytic vesicles (55). Consistent with their higher internalization rate, mutants N29Q and N15Q-N29Q displayed a significant co-localization with the endocytic markers Rab5 and Rab7 (56), as opposed to WT and the N15Q mutant. However, defective glycosylation and the resulting increase in internalization had no effect on the intracellular stability of the protein, as degradation of MC1R was not accelerated by incubation with DMJ in HBL cells, thus suggesting its recycling to the cell surface. Finally, we compared the glycosylation pattern of a panel of natural and artificial MC1R mutants expressed in HEK cells. We showed that the efficiency of N-glycosylation did not display a simple correlation with cell surface expression or forward trafficking of MC1R variants, in that hypoglycosylation was found for mutants with a strong intracellular retention as well as mutants with a normal cell surface expression. In summary, we demonstrated that MC1R is N-glycosylated at residues Asn15 and Asn29, with structurally and functionally different glycan chains. We also showed that N-glycosylation is not necessary for high affinity agonist binding or functional coupling. Conversely, glycosylation had a strong effect on the availability of MC1R molecules on the plasma membrane, most likely by a combination of improved forward trafficking and decreased internalization. Finally, we found that MC1R mutants exhibited different degrees of glycosylation which did

not show a simple correlation with their degree of functional impairment or their intracellular trafficking.

2. Melanocortin 1 receptor mutations impact differentially on signalling to the cAMP and the ERK mitogen-activated protein kinase pathways.

ERK activation by cAMP has been reported in a few cell types including PC12 cells and mouse melanoma cells (8). In order to study MC1R-mediated ERK activation, we selected a human melanoma cell line, HBL (WT for *NRAS*, *BRAF* and *MC1R*) and PC12 cells. In HBL cells, pERK signals increased rapidly and transiently upon treatment with the synthetic melanocortin analogue NDP-MSH, with maximal activation 5 min after agonist challenge. PC12 cells expressing MC1R also showed an increase in ERK phosphorylation and maximal values were achieved 15-20 min after NDP-MSH stimulation. In both cell types, cAMP levels also increased strongly after agonist treatment. Then, we analyzed ERK activation by the melanoma-associated MC1R variants. The R151C, R160W and D294H variants expressed in PC12 cells activated the ERKs as effectively as WT MC1R with similar kinetics and phosphorylation extent, in spite of a strong impairment of cAMP production. To further demonstrate WT MC1R and RHC variant signalling to the ERKs, we studied NDP-MSH-induced neurite outgrowth in PC12 cells, since sustained ERK activation in these cells is associated with their differentiation with neurite outgrowth (57;58). NDP-MSH induced neurite outgrowth in cells expressing WT MC1R or the RHC variants. This differential effect of the RHC mutations on signalling to cAMP or the ERKs indicated that ERK activation by MC1R might be independent on cAMP production. Consistent with independence on cAMP, treatment of HBL human melanoma cells with the potent adenylyl cyclase activator forskolin (FSK) strongly increased intracellular cAMP levels, but failed to activate the ERKs. This suggested that coupling of MC1R activation to cAMP or to ERKs were independent events, and provided a remarkable example of differential impact of point mutations on two signalling pathways. To further explore the relationship between cAMP and ERK signalling, we analyzed the functional coupling of 3 artificial variants with altered signalling to cAMP. The internalization-resistant T308A-S316A mutant (44) with impaired signalling to cAMP was as efficient as WT MC1R in triggering ERK phosphorylation following stimulation with NDP-MSH. Neither the constitutively active in cAMP signalling E94K variant (59) nor the constitutively internalized T308D-S316D mutant (44) were more potent than WT MC1R in activating the ERKs. These data suggested that MC1R-mediated ERK activation was independent on

receptor internalization, and did not involve cAMP synthesis. Moreover, NDP-MSH dose-response curves in PC12 cells overexpressing the MC1R studies showed that ERK phosphorylation was triggered at much lower agonist concentrations than cAMP synthesis, suggesting that melanocytes might display a graded spectrum of responses to melanocortin ligands of MC1R, with ERK-dependent processes occurring at lower agonist levels than cAMP-dependent events. Accordingly, our data showed that melanocytes harbouring variant MC1R might display an imbalanced functional response to MCs, with normal signalling to the ERKs but reduced coupling to cAMP.

3. Signalling from the Human Melanocortin 1 Receptor to ERK1 and ERK2 Mitogen-Activated Protein Kinases Involves Transactivation of cKIT.

We have shown that human MC1R variants associated with red hair, fair skin (RHC phenotype) and increased skin cancer risk display reduced cAMP signalling, but activate ERKs as efficiently as WT in heterologous PC12 cells (60), suggesting independent signalling to ERKs and cAMP in human melanocytes. We tested variant MC1R signalling to ERK in normal human melanocytes (NHM) cultures of defined *MC1R* genotype and in human melanoma HBL cells. We showed that MC1R signalling activated the ERK pathway in NHM and melanoma cells expressing physiological levels of endogenous RHC variants. ERK activation was comparable for WT and mutant MC1R and was independent on cAMP, as it was neither triggered by stimulation of cAMP synthesis with FSK, nor blocked by the adenylyl cyclase inhibitor 2',5'-dideoxyadenosine. Overall, these results show that cAMP is neither sufficient nor necessary to trigger ERK activation in human melanocytic cells. Accordingly, we analyzed possible cAMP-independent mechanisms accounting for ERK activation in MC-stimulated human melanocytic cells. We found that ERK activation in melanocytic cells stimulated with NDP-MSH is independent on PKA, Ca²⁺ fluxes or PKC. Then, we considered the possibility of transactivation of a RTK in MC-induced ERK activation. We found that in HBL cells, signalling from MC1R to the ERKs is mediated by the transactivation of c-KIT. This conclusion was reached on the basis of both pharmacological and molecular approaches that showed: a) a rapid increase of tyrosine phosphorylation and c-KIT activation in cells treated with NDP-MSH but not with FSK, b) efficient blockade of ERK activation in MC-stimulated cells by 3 c-KIT inhibitors (ISCK03, GTP-14564 and Sunitinib), but not by EGFR specific inhibitors (PD 1153035 and low concentrations of AG 1478) under conditions that fail to block downstream signalling

initiated at the level of NRAS c) ablation of MC-induced ERK activation following silencing of c-KIT expression with siRNA, d) ERK activation following MC stimulation of c-KIT-positive human melanoma cells but not of cell lines lacking c-KIT expression and e) reconstitution of c-KIT activation by MCs and of a positive pERK response to the MC1R ligand in HEK cells simultaneously transfected with MC1R and c-KIT constructs, but not with either one of the individual constructs alone. In an attempt to identify components of this signalling pathway downstream of MC1R and upstream of cKIT, we focused on Src, a non-RTK that has been shown to play key roles in RTK transactivation (31;61;62). We found that Src was rapidly activated by treatment of HBL cells with NDP-MSH, but not with FSK or with the c-KIT ligand SCF. Similar results were obtained in HEK cells transfected to express MC1R and c-KIT. Moreover, the Src inhibitors Na-PP1, Dasatinib and PP-2 blocked ERK activation in MC-stimulated cells. Overall, our data located Src upstream of c-KIT and NRAS, since: a) Src was not activated by direct stimulation of c-KIT with SCF, b) incubation of HBL cells with the Src inhibitor PP-2 under conditions that completely block the pERK response to NDP-MSH had no effect on either SCF-induced c-KIT autophosphorylation at Tyr703 or ERK activation, and c) under identical conditions, PP-2 abolished NDP-MSH-mediated phosphorylation of c-KIT Tyr703. These results strongly support the involvement of Src in MC-signalling to the ERKs, although this point should be confirmed by complementary molecular approaches.

4. Functional relationships of the melanocortin 1 receptor-activated cAMP and mitogen-activated protein kinase ERK1 and ERK2 pathways in human melanoma cells.

The observation that the ERK and cAMP pathways mediated by MC1R initially diverge at the plasma membrane level raised the possibility of a pathway-specific regulation of each one of the signalling modules. Several layers of MC1R regulation can be considered, including a genotypic level through the expression of variant alleles, protein-protein interactions modulating the association of MC1R with signalling partners and crosstalk between cAMP and ERK pathways. To study the regulation of MC1R signalling at the genotype level and to gain insight on differential requirements for functional coupling, we compared agonist-induced ERK phosphorylation and cAMP production in PC12 cells expressing WT and a panel of 20 mutants MC1R with different functional alterations in signalling to the cAMP, from complete LOF to gain-of-function and constitutive activity (34;44;45;59;63-67). The

C35A, S41F, L93R, T157A, R162P and C289R point mutants, and two deleted forms, $\Delta 5$ and $\Delta TM7$, lacking the last 5 C-terminal amino acids and a larger fragment comprising the seventh transmembrane helix and the complete cytosolic C-terminal tail respectively, behaved as essentially complete LOF forms for both pathways. On the other hand, 9 mutants with different degrees of LOF in the cAMP pathway showed normal or even slightly increased functional coupling to the ERKs, whereas the other 2 mutants with cAMP coupling similar to WT (N281S and $\Delta 1$) and E94K, a constitutively and hyperactive form also behaved as WT in signalling to the ERKs. We also compared the mutants for their ability to bind agonist when expressed in PC12 cells. Variants with residual binding lower than 10% relative to WT failed to activate the ERKs. Mutants with an agonist binding capacity over this threshold were at least as effective as WT in activating the ERKs, in spite of sometimes important LOF in the cAMP pathway. Conversely, no mutants were found with a higher decrease in ERK signalling compared with cAMP coupling. Next, we investigated the effects of general mechanisms of GPCR signalling regulation on functional coupling of MC1R to the ERK pathway. One of these mechanisms is GPCR homologous desensitization. After continuous exposure to the agonist, GPCRs are phosphorylated by the GRKs. This phosphorylation promotes the interaction of proteins of the arrestin family with the receptor, which uncouples the GPCR from the G protein, triggers the internalization of the agonist-receptor complex and terminates G protein-dependent signals. We found that neither GRKs, nor arrestins decreased melanocortin signalling to the ERKs, under conditions that desensitize signalling to the cAMP cascade and cause MC1R internalization. Additionally, we tested the effects of MGRN1 (Mahogunin ring finger-1) (68) on ERK activation. MGRN1 is a E3 ubiquitin ligase that decreases MC1R signalling to the cAMP by a mechanism that involves physical association with the receptor with competitive displacement of the Gs protein. HBL cells overexpressing MGRN1 isoforms did not show a significant modification of the levels of ERK phosphorylation following stimulation with NDP-MSH. We checked the possible crosstalk of the ERK and cAMP pathways in HBL melanoma cells under conditions of high or low ERK activity. These conditions were achieved in cells expressing constitutively active NRAS and BRAF mutants (Q61R and V600E, respectively) or in the presence of the MEK inhibitor, PD98059. We found that melanocortin-induced cAMP synthesis was largely insensitive to the level of ERK activity, thus suggesting that the ERK pathway had little impact on cAMP production. In contrast, other reports have demonstrated that mutant NRAS disrupts cAMP signalling at the level of cAMP metabolism in melanocytes and melanoma cells (69). Therefore, we analyzed a panel of 26 human melanoma cell lines for

NRAS, BRAF and MC1R genotype, as well as for the functional response of the ERK and cAMP pathways to stimulation of MC1R. We found that NRAS-mutant cells showed absent and decreased responsiveness to NDP-MSH and the adenylyl cyclase activator forskolin (FSK), respectively, in terms of cAMP production. On the other hand, 8 out of 9 BRAF-mutant cells were equally impaired in MC signalling to the cAMP pathway, showing that disruption of cAMP signalling in human melanoma is not exclusive of NRAS mutations but may be a characteristic of cells with ERK hyperactivity. Finally, we examined the effects of cAMP on the ERK pathway in human melanoma cells. We have previously shown that FSK decreased basal ERK activity in HBL human melanoma cells (70). Conversely, other studies have reported that cAMP activated ERK signalling in mouse melanoma cells (8). Thus, we analyzed human melanoma cells not only for basal ERK phosphorylation, but also for MC1R-dependent activation. Our data indicated that high cAMP levels efficiently blocked MC1R-dependent activation of the ERKs in human melanoma cells, presumably upstream of the c-KIT RTK, since FSK had no effect on SCF-mediated ERK activation. We also compared the effects of FSK on ERK phosphorylation in NRAS- or BRAF-mutant cells. None of the cell lines tested were responsive to FSK stimulation, again suggesting that the site of cAMP inhibitory action was located upstream of NRAS. Additionally, we tested the effects of FSK on HBL cells transiently transfected with the constitutively active NRAS and BRAF mutants. FSK treatment did not block ERK activation by the constitutively active effectors, but strongly inhibited cKIT activity, as shown by decreased phosphorylation at Tyr721 and Tyr703. Taken together our results show that cAMP blocks MC1R functional coupling to the ERK pathway at a site located upstream of c-KIT, NRAS and BRAF. This allows for a normal response to the c-KIT ligand even in the presence of high levels of cAMP, consistent with the co-mitogenic action of this second messenger.

CONCLUSIONS

1. MC1R is N-glycosylated at both residues Asn15 and Asn29, most probably by addition of a core high-mannose N-glycan chain and a bulkier hybrid-type chain, respectively.
2. N-glycosylation is not an essential requirement for forward trafficking of the receptor to the plasma membrane or its functional coupling, but increases its cell surface expression.

3. In human melanocytic cells, non-canonical signalling from MC1R to the ERKs is cAMP-independent and relies on the transactivation of c-KIT.
4. Src is likely activated downstream of the MC1R but upstream of c-KIT in the non-canonical signalling pathway leading to ERK activation.
5. ERK activation is achieved at much lower agonist concentrations than cAMP synthesis.
6. Many of the frequent allelic variants with strongly impaired functional coupling to the cAMP canonical pathway are still able to activate the non-canonical ERK pathway as effectively as WT. Among these variants are R151C, R160W and D294H.
7. MC1R variants with complete LOF in the ERK pathway are always cAMP-deficient, very rare and have extremely low agonist binding.
8. The homologous desensitization machinery is not involved in the regulation of MC1R signalling to the ERKs.
9. High cAMP levels block MC1R-mediated ERK activation in human melanoma cells and this inhibition occurs upstream of c-KIT.
10. Acute modulation of ERK activity does not modify cAMP levels in response to MC-induced MC1R activation or adenylyl cyclase stimulation by FSK.
11. In human melanomas, loss of MC1R signalling to the cAMP cascade is frequent and this impairment is observed even for cell lines WT for MC1R, NRAS and BRAF.

PUBLICATIONS

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70. **Herraiz C, Journe F, Abdel-Malek Z, Ghanem G, Jimenez-Cervantes C, Garcia-Borrón JC** 2010 Signaling from the Human Melanocortin 1 Receptor to ERK1 and ERK2 Mitogen-Activated Protein Kinases Involves Transactivation of cKIT. *Mol Endocrinol*

APÉNDICE.

1. TAMPONES.

a) Generales.

PBS 1X (pH 7.2):

NaCl	137 mM
KCl	2.7 mM
Na ₂ HPO ₄	10.0 mM
KH ₂ PO ₄	1.0 mM

Tampón de solubilización celular I:

PBS	1x
PMSF	100 ng/ml
Igepal-CA630	1%
Inhibidores de fosfatasas	1%

Tampón de solubilización celular II:

Tris (pH 8.0)	50 mM
Igepal-CA630	1%
EDTA	1 mM
PMSF	0.1 mM
Iodoacetamida	10 mM

Tampón Tris/EDTA para AMPc:

Tris/HCl	50 mM	pH 7.4
EDTA	4 mM	

Tampón de desglucosilación:

Na ₂ HPO ₄ /NaH ₂ PO ₄	50 mM	pH 7.0
EDTA	10 mM	
SDS	0.1%	

Tampón de lavado ácido:

Glicina	50 mM
NaCl	150 mM

b) **ADN.**

Tampón de carga de muestras de ADN (5x):

Ficoll 400	20%	
Azul de bromofenol	0.05%	
Azul xileno	0.05%	
EDTA	50 mM	pH 7.2

Tampón de recorrido TAE:

Tris	40 mM
EDTA	1.0 mM
Ácido acético	30 mM (0.175% v/v)

2. MEDIOS DE CULTIVO DE CÉLULAS EUCARIOTAS.

Abajo se especifica la composición de los medios empleados en mg/l, tal y como aparece en el catálogo de medios de cultivo de GIBCO (Invitrogen) y de Sigma-Aldrich.

Para tripsinizar las células se empleó tripsina (0.1 mg/ml) y EDTA (0.2 mg/ml) en PBS.

Componentes	DMEM	Ham's F10	MCDB 153
Sales inorgánicas			
CaCl ₂ 2H ₂ O	264.0	44.0	3.33
Ca(NO ₃) ₂ 4H ₂ O			
CuSO ₄ 5H ₂ O		0.0025	0.00275
FeNO ₃ ·9H ₂ O	0.1		
FeSO ₄ 7H ₂ O		0.834	1.39
KCl	400.0	285.0	111.83
MgCl ₂ 6H ₂ O			57.13
MgSO ₄ 7H ₂ O	200.0	153.0	
NaCl	6400.0	6900.0	7599.0
NaHCO ₃	3700.0	1200.0	
NaH ₂ PO ₄ 2H ₂ O	140.0		
Na ₂ HPO ₄ anhidro		154.5	284.088
ZnSO ₄ 7H ₂ O		0.03	0.144
Aminoácidos			
L-Ala	35.6	9	8.91
L-Arg	84.0	211	210.7
L-Asn	60.0	15	15
L-Asp	53.0	13.0	3.99
L-Cys		25.0	42.04
L-cistina	48.0		
L-Glu	59.0	14.7	14.71

Componentes	DMEM	Ham's F10	MCDB 153
Gly	30.0	7.5	7.51
L-His	42.0	23.0	16.77
L-hPro			
L-Ile	105.0	2.6	19.68
L-Leu	105.0	13.0	65.6
L-Lys	146.0	29.0	18.27
L-Met	30.0	4.5	44.8
L-Phe	66.0	5.0	4.96
L-Pro	46.0	11.5	34.53
L-Ser	42.0	10.5	63.06
L-Thr	95.0	3.6	11.91
L-Trp	16.0	0.6	3.06
L-Tyr	72.0	1.8	3.41
L-Val	94.0	3.5	35.13
Vitaminas			
Biotina		0.024	0.0146
Ác. pantoténico	4.0		
Cloruro de colina	4.0	0.7	13.96
Ác. Fólico	4.0	1.3	0.79
i-Inositol	7.2	0.5	18.02
Nicotinamida	4.0	0.6	
Ác. p-aminobenzoico			1.0
Piridoxina	4.0		0.06171
Riboflavina	0.4	0.4	0.0376
Tiamina	4.0	1	0.337
Vitamina B ₁₂		1.4	0.407
Otros componentes			
D-glucosa	4500.0	1100.0	1081.0
Rojo fenol	17.0	1.2	1.242
Hipoxantina		4.08	
Putrescina HCl			0.161
Piruvato sódico		110.0	55.0
HEPES		5958	6600.0
Timidina		0.7	0.727

3. REACTIVOS SDS-PAGE Y TRANSFERENCIA WESTERN.

a) SDS-PAGE

GELES de poliacrilamida: la mezcla acrilamida/bisacrilamida (AA/bAA) se utilizó en la proporción 30:0.8. A continuación se detalla la composición de los geles utilizados habitualmente en SDS-PAGE.

Gel separador (10% acrilamida)

H ₂ O	1.6 ml
AA/bAA	1.33 ml
Tris-HCl 1.5 M 0.4% SDS pH 8.8	1.25 ml

(NH ₄) ₂ S ₂ O ₈ 10%	40 µl
TEMED	5 µl

Gel hacinador (4% poliacrilamida)

H ₂ O	1.2 ml
AA/bAA	270 µl
Tris-HCl 0.5 M 0.4% SDS pH 6.8	0.5 ml
(NH ₄) ₂ S ₂ O ₈ 10%	20 µl
TEMED	2.5 µl

Tampón de muestra (4x):

Tris HCl	0.25 M	pH 6.8
Glicerol	20%	
SDS	8%	
Azul de bromofenol	0.08%	
β-mercaptoetanol	7.5%	

Tampón de recorrido:

Tris (base)	25 mM	
Glicina	190 mM	
SDS	0.1%	(pH aproximado 8.3)

b) Transferencia Western.

Tampón de transferencia:

Tris (base)	48 mM	pH 9.2
Glicina	39 mM	
SDS	0.04%	
Metanol	20%	

TGS 10x:

Tris	250 mM
Glicina	1.9 M
SDS	1%

TGST:

TGS	1x
Tween 20	0.05%

TBS 10x:

Tris	200 mM
NaCl	1.37 M

TBST :

TBS	1x
Tween 20	0.1%

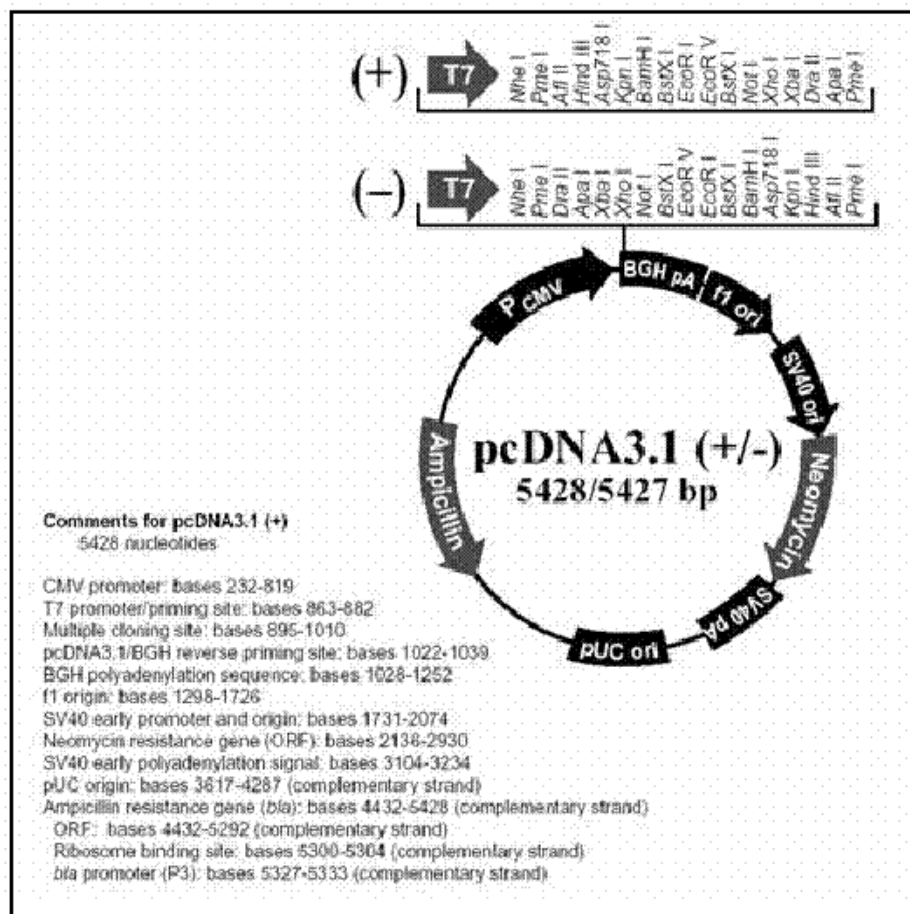
Los anticuerpos utilizados, la casa comercial, la dilución empleada y su tampón de unión están detallados en el apartado 6 de Materiales y Métodos.

4. MICROSCOPIA CONFOCAL

Las construcciones Rab1-EGFP, Rab5-EGFP y Rab7-EGFP, fueron cedidas amablemente por el Dr. Miguel Seabra (Imperial College, Londres, Reino Unido).

5. MAPAS Y SITIOS DE RESTRICCIÓN DE VECTORES EMPLEADOS.

a) **pcDNA3.1.** <http://www.invitrogen.com/content/sfs/vectors/pcdna3.1+.pdf>



6. BÚSQUEDA DE BIBLIOGRAFÍA Y SECUENCIAS.

En la búsqueda de referencias bibliográficas fueron de gran utilidad los archivos de la Hemeroteca Científica de la Universidad de Murcia y las siguientes direcciones de Internet:

Centro Nacional para la Información Biotecnológica de Bethesda (NCBI):

<http://www.ncbi.nih.gov/entrez/query.fcgi?db=PubMed>

Biblioteca de la Universidad de Murcia:

http://www.um.es/biblioteca/bibl_electr/revistas/azlist/index.html

Asimismo, las secuencias de cDNAs y proteínas se localizaron en el NCBI y en el Instituto Sanger:

<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide>

<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein>

<http://www.ensembl.org/index.html>