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

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ABSTRACT

Melanocortin 1 receptor (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 rat pheochromocytoma cell line 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.

1. 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 non-melamona skin cancer risk [2]. Upon stimulation by α melanocyte stimulating hormone (αMSH) 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 rat pheochromocytoma cell line (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 red hair colour (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 (wtMC1R) 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

Abbreviations: GPCR, G protein-coupled receptor; MC1R, melanocortin 1 receptor; NDP-MSH, norleucine[4] D-phenylalanine[7]-melanocyte-stimulating hormone; RHC, red hair colour; MAPK, mitogen-activated protein kinase; PC12, rat pheochromocytoma cell line; wtMC1R, wild-type MC1R

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not trigger ERK activation. These results open unexpected perspectives on the functional coupling of MC1R to ERK signalling in melanocytes.

2. Materials and methods

2.1. 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].

2.2. Functional assays

Cells grown in 12-well plates were transfected, serum-deprived for 12–24 h and stimulated as required with the αMSH analogue norleucine^4 D-phenylalanine^7-melanocyte-stimulating hormone [Nle^4, D-Phe^7] αMSH (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 0.1 N HCl preheated at 70 °C, and scrapped. The mix was freeze-dried, washed with 100 μl H_2O 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 μl PBS supplemented with PMSF 100 ng/ml, 1% Igepal and 1% phosphatase inhibitor mix from Calbiochem. Samples were centrifuged (105 000×*g*, 30 min) and a volume of supernatant containing 30 μg 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 www.rsb.info.nih.gov/ij).

3. Results and discussion

3.1. 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

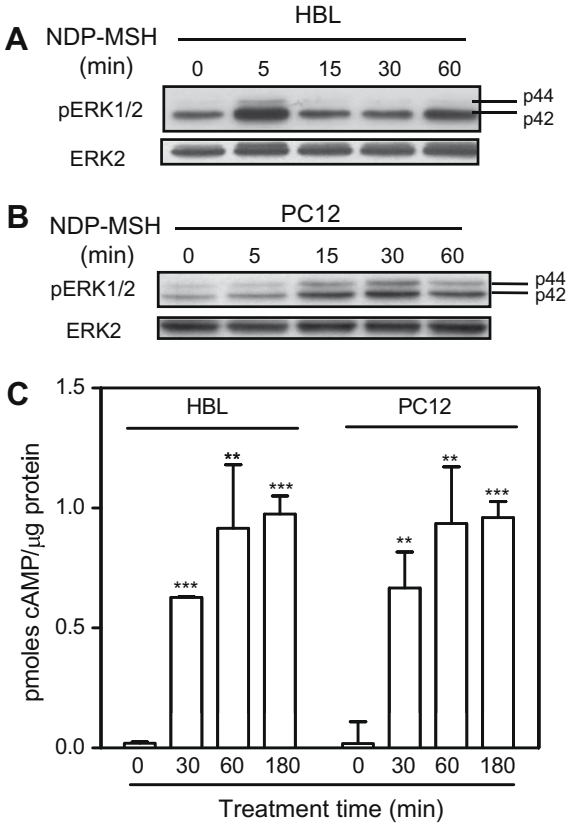

Fig. 1. MC1R-mediated ERK activation in melanocytic and heterologous cells. (A) HBL human melanoma cells were serum-deprived and stimulated with 10^{-7} M NDP-MSH for the times shown. Cell lysates were Western blotted for pERK1/2 and total ERK2 as loading control. (B) PC12 cells transfected to express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

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.

3.2. 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.

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

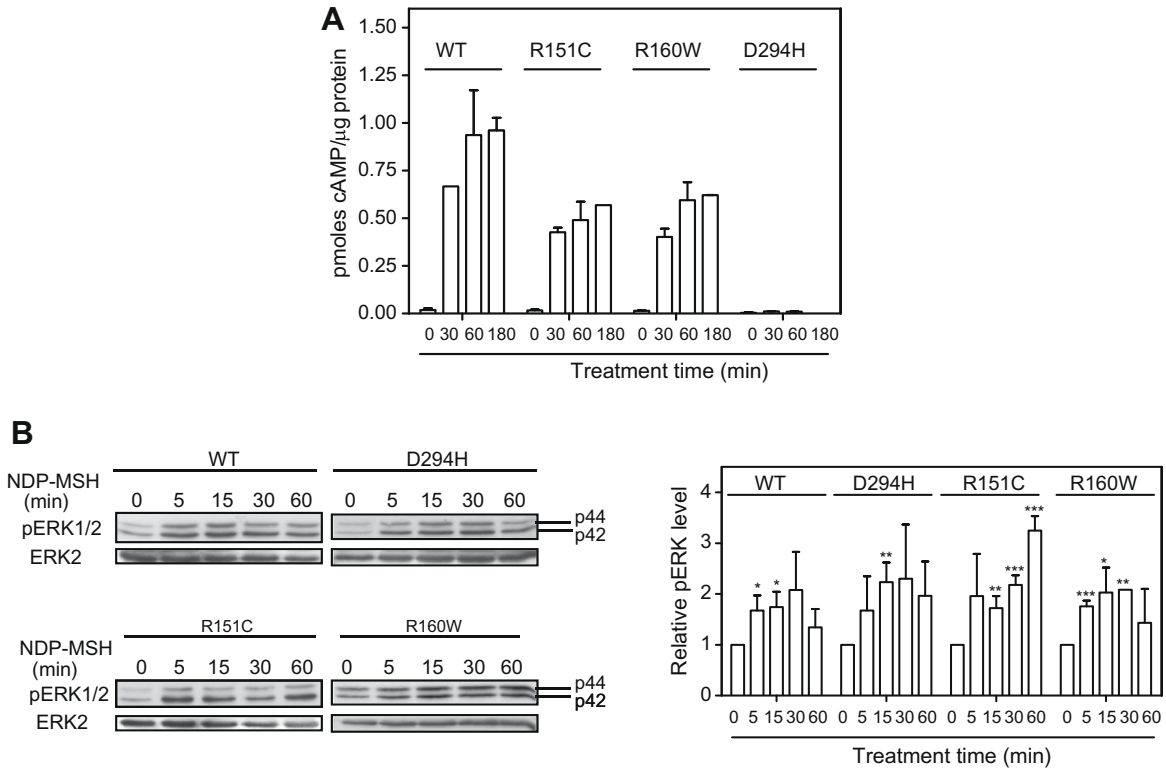

Fig. 2. Efficient signalling of RHC variants to the ERKs. PC12 cells transfected with wtMC1R or the RHC variants R151C, R160W or D294H were challenged with 10^{-7} M NDP-MSH. (A) Kinetics of cAMP generation upon stimulation with 10^{-7} M NDP-MSH. For all time points, cAMP increases over untreated controls 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 (left), and quantification of three experiments (means ± S.D., right) are shown. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0001$.

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 ± 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.

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

Treatment of B16 mouse melanoma cells with αMSH 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 three 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 μM, 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

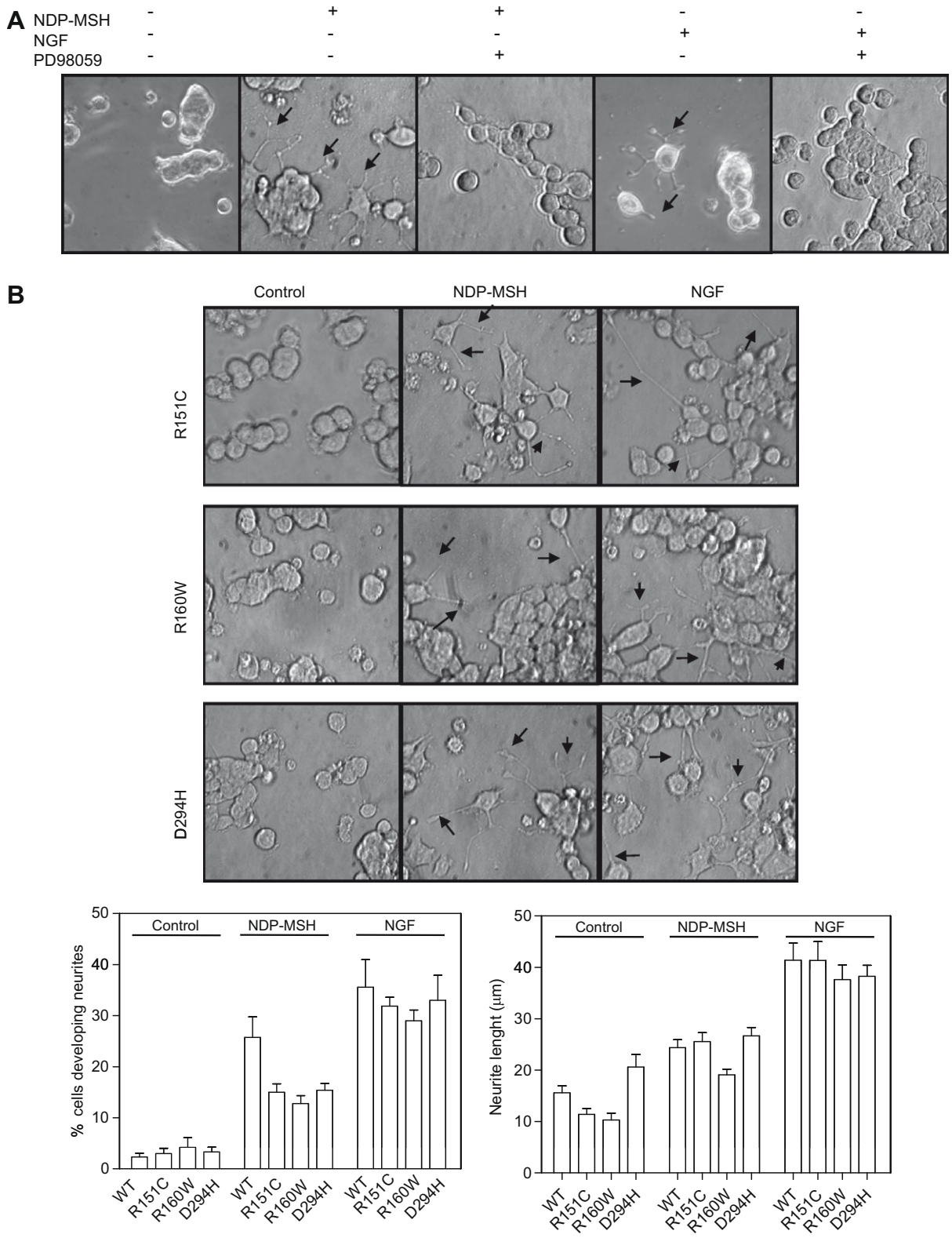

Fig. 3. MC1R-dependent neurite outgrowth in PC12 cells. (A) Cells expressing wtMC1R were treated with NDP-MSH (10^{-7} M) or NGF (100 ng/ml) for 48 h, with or without PD98059 (50 μM). (B) Neurite outgrowth in cells expressing variant MC1R. Cells expressing the variants indicated on the left of each row 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 means ± S.E.M.).

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 Gs protein responsible for coupling to cAMP. Experiments aiming at the identification of this effector have excluded the involvement of PKC or

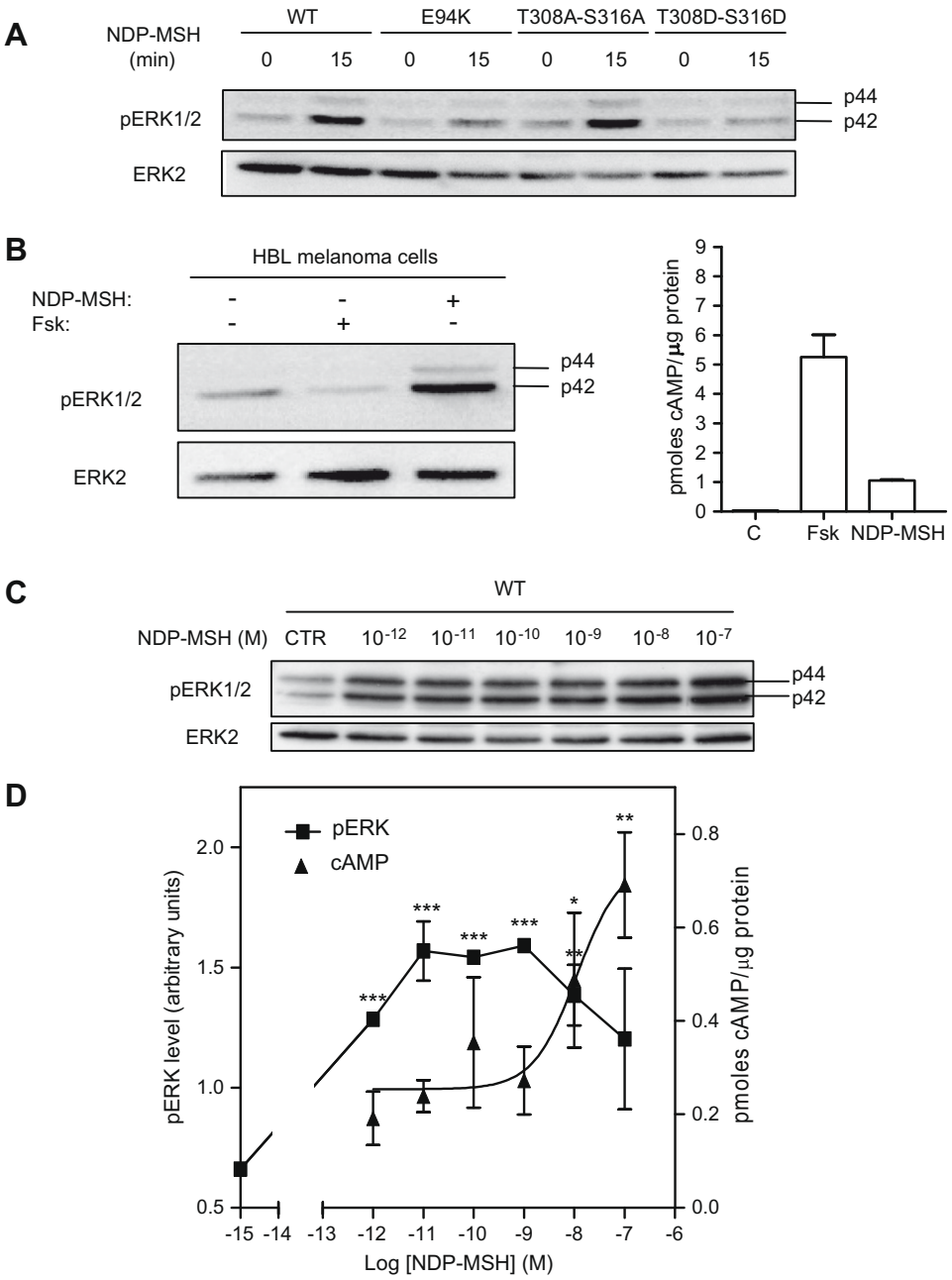

Fig. 4. (A) Agonist-induced ERK activation by MC1R mutants with altered signalling to cAMP and/or internalization. PC12 cells were transfected with a constitutively active MC1R variant (E94K), or variants with decreased signalling to cAMP and impaired (T308A-S316A) or enhanced (T308D-S316D) internalization. Cells were stimulated (10^{-7} M NDP-MSH, 15 min) and Western blotted for pERK. A blot representative of three 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 μM, 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 three 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 means ± S.D. ($n = 4$). $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0001$.

calcium fluxes (not shown). Moreover, the different dose–response curves for ERK activation and Gαs-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.

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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References

[1] Garcia-Borron, J.C., Sanchez-Laorden, B.L. and Jimenez-Cervantes, C. (2005) Melanocortin-1 receptor structure and functional regulation. Pigment Cell Res. 18, 393–410.
[2] Rees, J.L. (2004) The genetics of sun sensitivity in humans. Am. J. Hum. Genet. 75, 739–751.
[3] Slominski, A., Wortsman, J., Luger, T., Paus, R. and Solomon, S. (2000) Corticotropin releasing hormone and proopiomelanocortin involvement in the cutaneous response to stress. Physiol. Rev. 80, 979–1020.
[4] Busca, R. and Ballotti, R. (2000) Cyclic AMP a key messenger in the regulation of skin pigmentation. Pigment Cell Res. 13, 60–69.
[5] Slominski, A., Tobin, D.J., Shibahara, S. and Wortsman, J. (2004) Melanin pigmentation in mammalian skin and its hormonal regulation. Physiol. Rev. 84, 1155–1228.
[6] Busca, R., Abbe, P., Mantoux, F., Aberdam, E., Peyssonnaux, C., Eychene, A., Ortonne, J.P. and Ballotti, R. (2000) Ras mediates the cAMP-dependent activation of extracellular signal-regulated kinases (ERKs) in melanocytes. EMBO J. 19, 2900–2910.
[7] Wellbrock, C., Karasarides, M. and Marais, R. (2004) The RAF proteins take centre stage. Nat. Rev. Mol. Cell. Biol. 5, 875–885.
[8] Lefkowitz, R.J. and Shenoy, S.K. (2005) Transduction of receptor signals by beta-arrestins. Science 308, 512–517.
[9] Marinissen, M.J. and Gutkind, J.S. (2001) G-protein-coupled receptors and signaling networks: emerging paradigms. Trends Pharmacol. Sci. 22, 368–376.
[10] Dumaz, N. and Marais, R. (2005) Integrating signals between cAMP and the RAS/RAF/MEK/ERK signalling pathways. FEBS J. 272, 3491–3504.
[11] Perez Oliva, A.B., Fernendez, L.P., Detorre, C., Herraiz, C., Martinez-Escribano, J.A., Benitez, J., Lozano Teruel, J.A., Garcia-Borron, J.C., Jimenez-Cervantes, C. and Ribas, G. (2009) Identification and functional analysis of novel variants of the human melanocortin 1 receptor found in melanoma patients. Hum. Mutat. 30, 811–822.
[12] Box, N.F., Wyeth, J.R., O'Gorman, L.E., Martin, N.G. and Sturm, R.A. (1997) Characterization of melanocyte stimulating hormone receptor variant alleles in twins with red hair. Hum. Mol. Genet. 6, 1891–1897.
[13] Duffy, D.L., Box, N.F., Chen, W., Palmer, J.S., Montgomery, G.W., James, M.R., Hayward, N.K., Martin, N.G. and Sturm, R.A. (2004) Interactive effects of MC1R and OCA2 on melanoma risk phenotypes. Hum. Mol. Genet. 13, 447–461.
[14] Healy, E., Flannagan, N., Ray, A., Todd, C., Jackson, I.J., Matthews, J.N., Birch-Machin, M.A. and Rees, J.L. (2000) Melanocortin-1-receptor gene and sun sensitivity in individuals without red hair. Lancet 355, 1072–1073.
[15] Newton, R.A., Roberts, D.W., Leonard, J.H. and Sturm, R.A. (2007) Human melanocytes expressing MC1R variant alleles show impaired activation of multiple signaling pathways. Peptides 28, 2387–2396.
[16] Ringholm, A., Klovins, J., Rudzish, R., Phillips, S., Rees, J.L. and Schioth, H.B. (2004) Pharmacological characterization of loss of function mutations of the human melanocortin 1 receptor that are associated with red hair. J. Invest. Dermatol. 123, 917–923.
[17] Sanchez-Laorden, B.L., Jimenez-Cervantes, C. and Garcia-Borron, J.C. (2007) Regulation of human melanocortin 1 receptor signaling and trafficking by Thr-308 and Ser-316 and its alteration in variant alleles associated with red hair and skin cancer. J. Biol. Chem. 282, 3241–3251.
[18] Sanchez, M.J., Olivares, S.C., Ghanem, G., Haycock, J., Lozano Teruel, J.A., Garcia-Borron, J.C. and Jimenez-Cervantes, C. (2002) Loss-of-function variants of the human melanocortin-1 receptor gene in melanoma cells define structural determinants of receptor function. Eur. J. Biochem. 269, 6133–6141.
[19] Kiermayer, S., Biondi, R.M., Imig, J., Plotz, G., Haupenthal, J., Zeuzem, S. and Piiper, A. (2005) Epac activation converts cAMP from a proliferative into a differentiation signal in PC12 cells. Mol. Biol. Cell 16, 5639–5648.
[20] Stork, P.J. and Schmitt, J.M. (2002) Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. Trends Cell Biol. 12, 258–266.
[21] Beaumont, K.A., Shekar, S.N., Newton, R.A., James, M.R., Stow, J.L., Duffy, D.L. and Sturm, R.A. (2007) Receptor function, dominant negative activity and phenotype correlations for MC1R variant alleles. Hum. Mol. Genet. 16, 2249–2260.
[22] Santos, S.D., Verveer, P.J. and Bastiaens, P.I. (2007) Growth factor-induced MAPK network topology shapes Erk response determining PC-12 cell fate. Nat. Cell Biol. 9, 324–330.