Genetic Association and Cellular Function of MC1R Variant Alleles in Human Pigmentation

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ABSTRACT: We have examined MC1R variant allele frequencies in the general population of South East Queensland and in a collection of adolescent dizygotic and monozygotic twins and family members to define statistical associations with hair and skin color, freckling, and mole count. Results of these studies are consistent with a linear recessive allelic model with multiplicative penetrance in the inheritance of red hair. Four alleles, D84E, R151C, R160W, and D294H, are strongly associated with red hair and fair skin with multinomial regression analysis showing odds ratios of 63, 118, 50, and 94, respectively. An additional three low-penetrance alleles V60L, V92M, and R163Q have odds ratios 6, 5, and 2 relative to the wild-type allele. To address the cellular effects of MC1R variant alleles in signal transduction, we expressed these receptors in permanently transfected HEK293 cells. Measurement of receptor activity via induction of a cAMP-responsive luciferase reporter gene found that the R151C and R160W receptors were active in the presence of NDP-MSH ligand, but at much reduced levels compared with that seen with the wild-type receptor. The ability to stimulate phosphorylation of the cAMP response element binding protein (CREB) transcription factor was also apparent in all stimulated MC1R variant allele–expressing HEK293 cell extracts as assessed by immunoblotting. In contrast, human melanoma cell lines showed wide variation in the their ability to undergo cAMP-mediated CREB phosphorylation. Culture of human melanocytes of known MC1R genotype may provide the best experimental approach to examine the functional consequences for each MC1R variant allele. With this objective, we have established more than 300 melanocyte cell strains of defined MC1R genotype.

KEYWORDS: hair color; skin color; cAMP; melanocyte; melanoma

The human melanocortin–1 receptor (MC1R) is specifically expressed on the surface of melanocytes and is a key regulator of intracellular signaling to the melanin biosynthetic pathway governing pigment formation. The MC1R locus is highly polymorphic in human populations, with variant forms of the receptor underlying the diverse range of human pigmentation phenotypes and skin phototypes.[1] Several of the

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MC1R variant alleles have been associated with the red hair and fair skin (denoted RHC; red hair color) phenotype, a condition that is caused by the synthesis of a high level of pheomelanin and that can place individuals at higher risk of skin cancer (reviewed in Sturm[2]). To begin to quantify the relative contribution of each independent MC1R variant allele to human pigmentary phenotypes, we have examined statistical associations of some common MC1R gene variants with red hair, skin color, degree of freckling, and nevus counts in a large collection of adolescent twins, parents, and siblings. Findings from these genetic studies also must be compared and contrasted with those found using functional approaches to probe changes in cellular physiological responses initiated by variant MC1R receptor proteins. Ultimately, this will give a mechanistic understanding of these pigmentary effects in melanocytes, and such approaches also have been initiated in this study.

MC1R VARIANT ALLELE PENETRANCE IN RED HAIR AND SKIN PIGMENTARY TRAITS

Previous studies examining variant MC1R alleles in relation to hair color all have been consistent in finding the RHC phenotype to be a recessive trait, although the reported influence of some of variant alleles has varied between studies (reviewed in Sturm[2]). Homozygote or compound heterozygote variant MC1R genotype carriers are generally red haired, but because this is not always the case it is likely that other loci are involved in the expressivity of the trait.[3] In addition, red hair occurs in a significant proportion of heterozygote consensus MC1R allele carriers, some alleles displaying greater influence than others. It is unlikely that each of these variant alleles represent complete loss of receptor function or that they have a simple Mendelian recessive mode of inheritance; rather, variant alleles more likely represent a linear series of differential strength alleles.

There were 2,331 family members in 645 pedigrees for whom some phenotypic data were recorded, and DNA was available for genotyping for 1,779 individuals within 460 of these pedigrees.[4] Excluding one member of each genotyped MZ twin pair, there were 1,569 individuals with complete MC1R genotype, hair color, eye color, and gender recorded. The allele frequencies and odds ratios for nine polymorphic amino acid sites within the MC1R coding region are summarized in TABLE 1. RHC variant alleles R151C, R160W, D294H are common in the Queensland population and are responsible for most of the red hair color in our community, with at least one of these three alleles found in 93% of those with red hair. With the exception of the R142H and I155T variants, which occur at relatively low frequencies, all variants could be found in at least one red-haired individual, most commonly paired with one of the other RHC alleles as a compound heterozygote. The consensus allele in combination with a variant allele was found in only 11% of redheads, and no consensus homozygotes with red hair were found.

Penetrance for red hair of each MC1R variant allele was modeled in a logistic regression analysis.[4] This indicated that the RHC alleles D84E, R151C, R160W, and D294H were highly associated with red hair and fair skin, showing odds ratios (ORs) of 63, 118, 50, and 94 relative to the consensus MC1R allele compared with low-strength alleles V60L and V92M with ORs 6 and 5. The weakest allele was R163Q, and this gave only a twofold increase in association with red hair. From these anal-

TABLE 1. MC1R variant allele frequency in South East Queensland and odds ratio for red hair and fair skin

Variant allele	Frequency[a] (%)	Red hair OR (95% CI)[b]	Fair/pale skin OR (95% CI)[b]
Consensus (+)	50.4	1	1
V60L	12.2	6.4 (2.8–14.9)	1.7 (1.3–2.1)
D84E	1.2	62.8 (17.6–223.7)	12.5 (4.8–42.8)
V92M	9.7	5.3 (2.2–12.9)	2.3 (1.8–3.1)
R142H	0.4	0.0 (0.0–∞)[c]	1.8 (0.6–5.7)
R151C	11.0	118.3 (51.5–271.7)	4.4 (3.3–5.7)
I155T	0.9	0.0 (0.0–∞)[c]	2.1 (1.0–4.3)
R160W	7.0	50.5 (22.0–115.8)	3.2 (2.4–4.4)
R163Q	4.7	2.4 (0.5–11.3)	2.0 (1.4–2.9)
D294H	2.7	94.1 (33.7–263.1)	7.5 (4.4–13.7)
r	26.7	5.1 (2.5–11.3)	1.9 (1.6–2.3)
R	21.8	63.3 (31.9–139.6)	4.2 (3.4–5.2)

[a]The total number of individuals ranged from 1,747 to 1,787 scored at the different variants.
[b]Odds ratio relative to consensus genotype (95% Confidence Interval).
[c]Insufficient numbers to test for statistical association.
r = V60L, V92M, R163Q.
R = D84E, R151C, R160W, D294H.

TABLE 2. MC1R genotype and penetrance of red hair phenotype

Allele	+	*r*	*R*
	0[a]	0.2	2.5
+	**0**		
	0:425[b]		
		1.0	11.8
r	**1.0**	**0.9**	
	4:408	1:110	
			62.1
R	**1.5**	**10.8**	**67.1**
	5:339	22:181	49:24

[a]*Top triangle matrix* is the expected frequency (%) of red-haired individuals, using a linear multiplicative inheritance model for each genotype (allele 1OR * allele 2OR from TABLE 1).
[b]*Bottom triangle matrix* gives the penetrance (in *boldface*) and below that the numbers of red/non-red–haired individuals (RHC:NRHC) of each MC1R genotype [penetrance = 100*RHC/(RHC + NRHC)].

yses, it can be concluded that the D84E, R151C, R160W, and D294H variants can be considered strong RHC alleles, which we designate "*R*." The V60L,V92M, and R163Q variants are relatively weak RHC alleles and are designated *"r.*" The penetrances of the six genotypes formed upon combining these alleles grouped as weak and strong, together with the consensus "+" allele for red–nonred hair color are shown in TABLE 2 as a matrix of genotypes. This shows that significant numbers of red-haired individuals are seen only in *R/r* and *R/R* genotypes with 10.8% and 67.1%, respectively. The frequency of red hair in those with a heterozygous *R/+* genotype is only 1.5%, and less than 1% in those with an *r/r* genotype or *r/+* genotype. Using a regression model in which these alleles act multiplicatively on expression of red hair, we compared the predicted penetrance for the various compound heterozygote and homozygote genotypes (top triangle, TABLE 2) to that observed in our sample (bottom triangle). The concordance between the observed and expected frequencies of red hair for each grouped genotype supports a multiplicative penetrance model with the RHC alleles acting in a linear recessive fashion.

Subjects with a consensus MC1R genotype showed the darkest induced skin color, with mean skin reflectances of 60.5% for the inner arm and 49.8% for the back of hand. To address the quantitative relationship of variant MC1R alleles with skin reflectance, we calculated the increase in the mean skin reflectance measurement per variant allele relative to the consensus genotype. In general, alleles acted in an additive manner to increase skin reflectance, *R* alleles showing a greater effect (+1.9%) than *r* alleles (+0.9%) on inner arm reflectance.[4] Red-haired subjects had the greatest number of freckles and least number of moles, and this phenotypic association was further tested for association with MC1R genotype. No consensus homozygote (+/+) had a severe freckling score, and there was little effect of the *r* allele upon this correlation, but the *R/R* genotype had the lowest number of moles and greatest number of freckles, consistent with the phenotypic association found in redheads. In the heterozygous state, the *R/+* genotype displays an initial positive association with moliness until severe freckling is reached, after which there is a significant decrease in the number of moles. The attributable fraction due to carrying at least one variant MC1R allele increased linearly with the degree of freckling from 23.4% for mild to 100% for severely freckled subjects.

APPROACHES TO FUNCTIONAL ASSAY OF MC1R VARIANT ALLELES

Several approaches have been used for functional analysis of variant MC1R receptors including expression in MC1R-null transgenic mice to determine their effects on coat color and in the MC1R-deficient mouse melanoma cell line, B16G4F, to monitor cellular and physiological response to ligand. The resultant coat color pigmentation phenotypes suggest that these alleles do not result in a complete loss of activity nor are they functionally equivalent.[5] However, only the wild-type receptor was responsive in B16G4F cell line growth inhibition experiments.[6]

Variant MC1R receptors have been tested through heterologous expression in nonmelanocytic cells for ligand binding and cAMP dose-response coupling. Amino acid substitutions can result in altered receptor activity either by reduced affinity for hormone binding[7] or through deficient coupling to intracellular cAMP activation without significantly affecting ligand binding.[8–10] Heterologous expression in non-

melanocytic cells overcomes the problem of endogenous genomic MC1R-allele expression and allows for testing in a range of cell lines with potentially greater sensitivity through higher levels of receptor expression. However, this must be offset against potential artifacts seen with levels of receptor produced in a nonphysiological cellular environment. The advantages of functional testing in homologous cell systems such as melanoma lines are that melanocytic pigmentary functions, cell growth, and adhesion can be assayed in a cellular system partially equating to a normal cellular milieu. Considering these limitations, primary human melanocyte strains may represent the best system for MC1R functional analysis because they are a normal cell, and in them the pigmentation pathway may give a true response to physiological melanogenic stimulators. Unfortunately, there are several impediments to their use because they are slow-growing cells with limited growth span that must be freshly established and genotyped. With these factors in mind, we have used both homologous and heterologous test systems for RHC variant MC1R receptors.

FUNCTIONAL TESTING OF MC1R VARIANT ALLELES IN HEK293 CELL LINES

Functional testing of the wild-type and R151C and R160W receptors was first approached by stable expression in HEK293 cells by using the pcDNA3.1 plasmid vector. Total membrane extracts[11] from several initial cell isolates were tested for binding specificity and affinity of radiolabeled MC1R superagonist ligand ^{125}I-NDP-MSH and demonstrated specific binding of each receptor-expressing cell line compared with the parental HEK293 cells, with relative assay levels 10- to 20-fold above background.

The MC1R receptor is known to couple to second messenger systems that result in the increase of intracellular cAMP levels[12]; therefore, the PathDetect in Vivo Signal Transduction Pathway *cis*-Reporting System for cyclic-AMP responsiveness (Stratagene) was used to measure agonist response in MC1R-expressing HEK cells transfected with the pCRE-Luc reporter plasmid vector as a surrogate measurement of cAMP activation.[13,14] The HEK-pcDNA3.1 parental control vector–transfected cells did not respond to ligand administration but demonstrated several hundredfold activation after cotransfection with pFC-PKA encoding the protein kinase A catalytic subunit (FIG. 1A). This level of fold-activation over background in response to PKA was also seen in each of the MC1R-transfected HEK cells, demonstrating the utility of this approach to normalize activation relative to this positive control. In the presence of 10 nM NDP-MSH, cells expressing the wild-type receptor had an equivalent level of activation to that seen with pFC-PKA, but with the R151C and R160W variant receptors, activation was reduced to approximately half that level. In an attempt to competitively inhibit agonist activation, we also added a synthetic agouti peptide derived from the agouti protein, known to be an antagonist at the MC1R receptor.[15] No inhibition was seen. This system though may be dependent on agonist/antagonist ratios which would need to be determined using a range of NDP-MSH and agouti peptide concentrations.

To further test the function of wild-type and variant receptors, we assayed for phosphorylation of the CREB using Western blots on MC1R-expressing HEK293 cell line extracts to measure the level of the 43-kDa activated form of CREB using a phosphoserine-133–specific antibody (Cell Signaling Technology). Assay of CREB

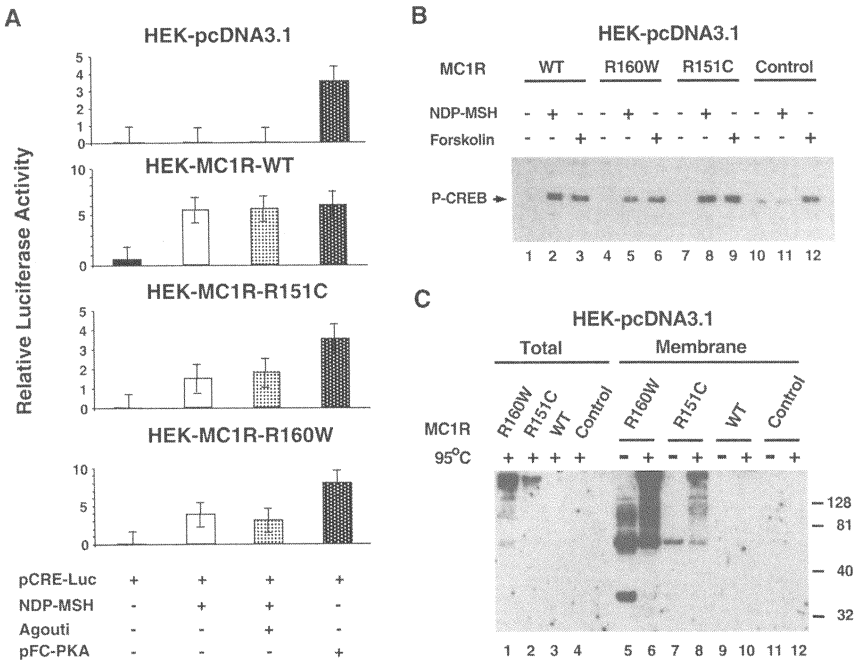

FIGURE 1. (**A**) Relative luciferase activities detected in HEK293 cells in response to NDP-MSH. HEK293 cells permanently transfected with the pcDNA3.1 vector (Invitrogen) containing MC1R receptors as indicated were harvested and plated into 12-well tissue culture plates at 80% confluence and allowed to attach for 24 h. The next day cells were transfected with 100 ng of pCRE-Luc reporter plasmid per well using Lipofectomine reagent (LifeTechnologies, Gaithersburg, MD), with control activation seen through cotransfection of 50 ng of pFC-PKA encoding the protein kinase A catalytic subunit. At 5 h after transfection, the cells were stimulated with 10 nM NDP-MSH ligand included in the DMEM media plus 10% fetal calf serum and left for another 6 h. The assay medium was aspirated and cells were washed twice with PBS and frozen dry at -20°C overnight. These cells were harvested in 100 mL of DMEM media without phenol red indicator and used to assay relative luciferase levels (Roche Luciferase Reporter Gene Assay, Constant Light Signal). (**B**) CREB phosphorylation in MC1R-expressing HEK293 cell lines. HEK293 cells stably transfected with cDNAs for MC1R receptor variants (**lanes 1**–**9**) or vector alone (**lanes 10**–**12**) were stimulated for 30 min with either NDP-MSH (10 nM) or forskolin (10 μM). Whole-cell lysates were analyzed by Western immunoblot using a specific anti–phospho-CREB antibody (Cell Signaling Technology). (**C**) MC1R levels in HEK293 cell lines. Whole-cell lysates or membrane preparations from stably transfected HEK293 cell lines were analyzed by Western immunoblot using an antibody raised against the amino terminus of the human MC1R (N19 Santa Cruz Biotechnology, Santa Cruz, CA). Whole-cell lysates were denatured in SDS-PAGE sample buffer (0.025M Tris-HCl, pH 6.8; 10% glycerol; 2% SDS; 5% 2-mercaptoethanol) for 2 min at 95°C prior to separation using 10% SDS-PAGE gels. Membrane preparations either were heat denatured in a manner identical to that for the lysates or were simply resuspended in sample buffer and incubated at room temperature for 10 min. Similar levels of protein were loaded for each cell line.

phosphorylation after 30-min stimulation with either NDP-MSH or the adenylate-cyclase activator forskolin induced similar levels of CREB phosphorylation in HEK293-expressing wild-type, R151C, and R160W receptors, with the HEK-pcDNA3.1 parental control again nonresponsive to ligand administration (FIG. 1B). The similar pattern of CREB-phosphorylation activity levels seen with each of these receptors contrasts with the lower activity of reporter gene transcriptional activity directed by the RHC variant alleles, suggesting that there may be differences in the temporal activation of adenylate-cyclase during extended periods of NDP-MSH administration, which were 30 min for kinase activity but 6 h for the luciferase reporter assay.

In the process of selecting MC1R-expressing HEK293 cell lines, we initially screened for receptor activation by assaying for luciferase reporter activity. Although this approach confirmed that RHC variant MC1R receptors do indeed have the ability to respond to ligand activation, indicating that these receptors are still partially functional, this may have biased the selection process toward cell lines with higher than normal physiological levels of the receptor. To investigate this possibility, we assayed total protein and membrane fractions from cell extracts for MC1R levels by immunoblotting using an N-terminal MC1R antibody (FIG. 1C). Only high molecular weight MC1R cross-reactive aggregates were detected in heat-denatured samples, as has been reported previously.[16] However, using non–heat denaturing conditions,[17,18] it was possible to resolve this aggregate into several bands, an expected monomeric species slightly above the 32-kDa marker, together with several other distinct higher molecular weight complexes with a predominant 66-kDa band. Considerably greater levels of receptor expression were detectable in the R151C and R160W variant cell lines than was seen with wild-type MC1R-expressing cells. No cross-reactivity was seen in pcDNA3.1 control–transfected cells. Therefore, although the wild-type and variant receptors have some functional activity, the excess levels of the variant receptors suggest that qualitative differences in receptor coupling are apparent. We have now established additional HEK293 cell lines expressing comparable levels of the MC1R receptor to address this issue. In addition, cell lines expressing V60L, V92M, and D294H MC1R receptor variants have been established for functional testing.

FUNCTIONAL TESTING OF MC1R VARIANT ALLELES IN MELANOCYTIC CELLS

Human melanoma cells are known to express from 400 to 1,600 specific receptors for MSH ligands per cell.[19,20] These have been utilized to study effects on hormone induction of melanogenesis and proliferation/growth inhibition and have suggested new modalities for melanoma treatment using toxic analogues. There have been conflicting reports on the actions of hormones on melanoma cell lines which reflect differences in the cell lines and culture conditions used. Moreover, the MC1R genotype of each cell line must be considered in assessment of any such response to ligand. To begin to address the functional properties of MC1R receptors in melanoma cells, we genotyped 56 melanoma cell lines. To attribute function solely to an individual variant receptor, we sought melanoma lines that were homozygote for each allele, thus restricting the capacity of these cells to expression of a single MC1R receptor

isoform. Heterozygous and compound heterozygous cell lines having the potential to produce two forms of receptor cannot be used to assign function to a specific allele. We found several consensus, compound heterozygote and homozygote MC1R variant–carrying lines.

Selected homozygote consensus and variant genotype lines were assessed for their ability to respond to either NDP-MSH or forskolin via coupling to CREB phosphorylation. These cell lines included two wild-type, homozygote V60L, R151C, R160W, and one D294H compound heterozygote (FIG. 2A) and were compared with the response observed in HEK293 wild-type MC1R-expressing cells. Direct stimulation of adenylate-cyclase by forskolin showed a variable pattern with apparent stimulation in some melanoma cells such as MM200, MM96L, ME10538, and A06MLC with nonresponsiveness in MM418 and CJM; however, no cell line showed an increase equivalent to that obtained in the HEK293 wild-type MC1R control. In contrast and irrespective of genotype, none of the cell lines increased the phosphorylation of CREB after NDP-MSH treatment. Variability in response to MSH stimulation of melanoma cell lines has been reported, but increases in cAMP above the basal level also have been described,[21–23] and this lack of CREB responsiveness may reflect innate diversity in MC1R signal transduction pathways between homologous and heterologous cell lines (see below). Receptor levels on each of the melanoma cell lines used in this assay must also be determined before definitive conclusions can be reached.

Given the variability and genetic instability that are characteristic of melanoma cell lines, possibly reflecting disrupted signaling pathways, we next turned to primary melanocytic cell strains as a suitable system to assess MC1R receptor function. Cultured human melanocytes have been found to express high-affinity receptors for the α-MSH ligand on their surface with approximately 700 binding sites per cell.[24–26] We have performed a systematic genotype screen for several of the common MC1R polymorphisms by using melanocytes cultured from individual foreskins to ascertain clonal cell strains. Over 1,000 foreskin samples were processed. The haplotype frequencies for the three R151C, R160W, and D294H RHC alleles were 8.3%, 7.3%, and 1.6%, respectively, which is comparable to the haplotype frequency range seen in the South East Queensland population (TABLE 1). It was our intention to isolate homozygote strains for each of these alleles to allow the specific functional characterization of individual forms of the receptor.

More than 300 clonal genotyped melanocyte cell strains were successfully established in culture from this initial screen with many wild-type +/+ and heterozygote +/– strains, including five R151C–/– and one R160W–/– homozygotes. However, no D294H–/– homozygotes were identified. When MC1R genotype was considered, all consensus +/+ allele strains had very dark pellets, being scored as black or dark brown by eye. When strains with a variant genotype were grouped, homozygous R151C–/– and R160W–/– cell pellets were considerably less pigmented, whereas heterozygous R151C+/– and R160W+/– cell pellets varied from black to white but were generally of intermediate pigmentation between the consensus and homozygotes. Upon culture of these cells with known second messenger inducers of pigmentation including IBMX and dbcAMP, cell growth was stimulated as seen by larger cell pellets, but there was no effect on color of the R151C–/– and R160W–/– cell pellets. However, when these cells were incubated with the melanogenic precursor substrate DOPA, we were able to demonstrate melanin production in each of

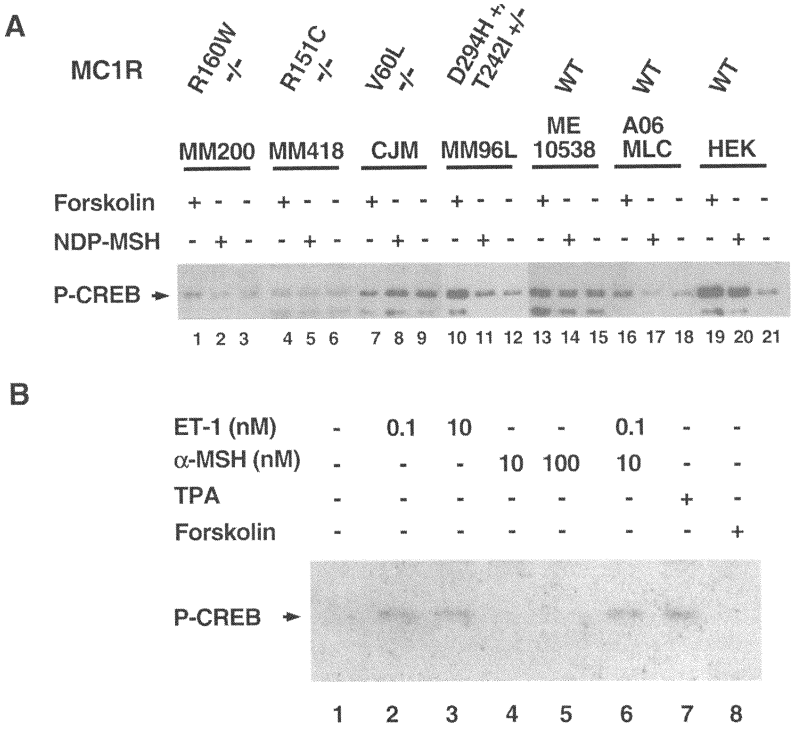

FIGURE 2. (A) CREB phosphorylation in melanoma cell lines. Cells were stimulated for 30 min with either NDP-MSH (10 nM) or forskolin (10 μM). Whole-cell lysates were analyzed by Western immunoblot using a specific anti–phospho-CREB antibody. Analysis of HEK293 cells expressing wild-type MC1R (**lanes 19–21**) is included for comparison. (**B**) CREB phosphorylation in primary human melanocytes. Cells were stimulated for 15 min with ET-1, α-MSH, TPA (16 nM), forskolin (10 μM), or combinations of each as indicated above the panel before whole-cell lysates were prepared for immunoblot.

these cell strains as compared with the nonmelanocytic HeLa cell line. Under the growth conditions used, a wild-type MC1R receptor appeared to be required for full melanization, and the cAMP second messenger stimulators could not mimic the pigmentary effects in variant MC1R homozygote melanocytes.

Melanocytes respond to a variety of growth factors, hormones, and ultraviolet light to increase pigmentation,[27–29] and attention increasingly is being focused on the interplay between the various signaling pathways. Recently, a synergistic action between endothelin, α-MSH, and bFGF was demonstrated for the phosphorylation of CREB.[30] Our initial studies have utilized a consensus melanocyte strain to investigate CREB phosphorylation in response to various mitogens. Both endothelin and TPA were able to couple to CREB phosphorylation. However, as previously seen for melanoma cells, α-MSH was not able to elicit such phosphorylation (FIG. 2B). Co-stimulation of melanocytes with endothelin and α-MSH did not reveal any obvious

latent synergy between these factors. Lack of α-MSH–induced CREB phosphorylation has also been described previously.[30]

Note that α-MSH–mediated cAMP induction does occur in melanocytes,[31] but these measurements are yet to be performed in our melanocyte strains. A direct loss of receptor-activated cAMP intracellular signaling recently has been reported in other studies of primary human melanocytes of defined MC1R genotype,[32] although kinase signaling pathways were not examined. In these studies, R160W homozygote and R151C/D294H, R160W/D294H compound heterozygote melanocyte strains demonstrated an impairment of tyrosinase activation in response to α-MSH stimulation and pronounced sensitivity to ultraviolet radiation treatment. These results confirm the utility of using primary melanocytes for MC1R functional analysis, but pose new questions about the signaling pathways that function in this cell which form a new tissue-specific paradigm.

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