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*<sup>5</sup>

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The melanocortin 1 receptor (MC1R), a G protein-coupled receptor (GPCR) positively coupled to adenyl cyclase, is a key regulator of melanocyte proliferation and differentiation and a determinant of pigmentation, skin phenotype, and skin cancer risk. MC1R activation stimulates melanogenesis and increases the ratio of black, strongly photoprotective eumelanins to yellowish and poorly photoprotective phaeomelanin pigments. Desensitization and internalization are key regulatory mechanisms of GPCR signaling. Agonist-induced desensitization usually depends on phosphorylation by a GPCR kinase (GRK) followed by receptor internalization in endocytic vesicles. We have shown that MC1R desensitization is mediated by two GRKs expressed in melanocytes and melanoma cells, GRK2 and GRK6. Here we show that in contrast with this dual specificity for desensitization, GRK6 but not GRK2 mediated MC1R internalization. Mutagenesis studies suggested that the targets of GRK6 are two residues located in the MC1R cytosolic C terminus, Thr-308 and Ser-316. A T308D/S316D mutant mimicking their phosphorylated state was constitutively desensitized and associated with endosomes, whereas a T308A/S316A mutant was resistant to desensitization and internalization. We studied the desensitization and internalization of three variant MC1R forms associated with red hair and increased skin cancer risk: R151C, R160W, and D294H. These variants showed a less efficient desensitization. Moreover, D294H was not GRK2 mediated MC1R internalization. Mutagenesis studies demonstrated that several variant alleles are associated with red hair and fair skin (the RHC phenotype) (8–11). Three frequent RHC alleles, R151C, R160W, and D294H, show odds ratios for red hair ranging from 50 to 120 (12, 13). These allelic variants are also associated with increased risk for melanoma and nonmelanoma skin cancers (14–17). Interestingly, this association seems at least partially independent on the effect on skin pigmentation and has been related with the ability of wild type (WT) MC1R, but not of the RHC variants, to activate DNA repair mechanisms after UV exposure (18, 19). Although it is widely agreed that the R151C, R160W, and D294H variant receptors correspond to diminished function forms, the degree of functional impairment and its molecular basis are not well understood. Therefore, the identification of the factors accounting for the genetic association between the RHC variants, the RHC phenotype, and skin cancer risk is a major area of research in skin biology.

MC1R is desensitized upon exposure to melancortin agonists (20), but no data are available on the desensitization of the penetrant RHC alleles R151C, R160W, and

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<sup>5</sup>The abbreviations used are: MC1R, melanocortin 1 receptor; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; IBMX, isobutylmethylxanthine; αMSH, α-melanocyte-stimulating hormone; NDP-MSH, norleucine 4-D-phenylalanine-7-melanocyte-stimulating hormone; PBS, phosphate-buffered saline; RHC, red hair color; WT, wild type; EGFP, enhanced green fluorescent protein; HEK cells, human embryonic kidney cells.
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FIGURE 1. Structure of the human MC1R. The boundaries of transmembrane fragments are drawn according to the two-dimensional model of Ringholm et al. (50). The amino acid sequence corresponds to the WT consensus (GenBank™ accession number AF326275 (31)). Positions of the mutations in the RHC alleles analyzed in this study are indicated by arrows, and the potential phosphorylation sites are shown on a gray background.

D294H. MC1R desensitization is likely mediated by GRK2 or GRK6, two GRKs expressed in melanocytes and melanoma cells. GRK6 seems more potent than GRK2 in that it is able to inhibit both constitutive and agonist-induced MC1R signaling, whereas GRK2 only inhibits agonist-dependent function. Phosphorylation of GPCRs by the GRKs is normally followed by recruitment of β-arrestins and endocytosis via clathrin-coated pits. This process may trigger new signaling events (21, 22). Internalization may target the receptor for degradation or be followed by recycling back to the plasma membrane. On the other hand, MC1R has a high agonist-independent constitutive activity (23), and it is, therefore, possible that the receptor may undergo constitutive endocytosis. Accordingly, it can be hypothesized that altered receptor desensitization, internalization, and/or recycling may cause changes in the level of MC1R cell surface expression. Interestingly, reduced cell surface expression has been shown for R151C and R160W expressed in heterologous systems, whereas D294H displayed a higher plasma membrane expression than WT (24, 25).

GRK-dependent desensitization of GPCRs is carried out by phosphorylation of specific serine (Ser) or threonine (Thr) residues, most often located in the cytosolic C-terminal extension of the receptor protein or in the second and third intracellular loops (26–28). Interestingly, many well-characterized RHC mutations impinge residues located in the second intracellular loop (7). MC1R displays eight potential phosphorylation targets for the loose specificity GRKs (Fig. 1) of which three are located in the second intracellular loop in the vicinity of residues mutated in RHC forms and four cluster in the short C-terminal tail. The phosphorylation sites responsible for MC1R desensitization have not yet been identified. Here we have studied MC1R internalization as related to homologous desensitization, and we have compared the desensitization and internalization of WT and RHC alleles. We report that internalization is partially mediated by GRK6. We show that two residues located in the cytosolic C terminus of MC1R, Thr-308 and Ser-316, are the most likely GRK targets. Finally, we also show significantly different desensitization and internalization rates for the variant RHC alleles as compared with WT.

EXPERIMENTAL PROCEDURES

Materials—A radioimmunoassay kit for cAMP and 125I-labeled NDP-MSH (2000 Ci/mmol) were from Amersham Biosciences. All restriction endonucleases were from Fermentas (Barcelona, Spain). The QuikChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). The transfection reagent Lipofectamine 2000, ligase, and compet DH5α cells were from Invitrogen. IGEPAL CA-630, bovine serum albumin, EDTA, phenylmethylsulfonyl fluoride, bicinchoninic acid, anti-FLAG M2 monoclonal antibody, and anti-FLAG M2-Peroxidase conjugate were from Sigma. G418 sulfate and the synthetic αMSH analogue NDP-MSH, were from Calbiochem. The anti-tyrosinase antiserum αPEP7h (29) was a kind gift from Dr. V. Hearing (NIH, Bethesda, MD). The anti-extracellular signal-regulated kinase 2 rabbit polyclonal IgG was from Santa Cruz Biotechnology (Santa Cruz, CA). Other reagents were from Merck or Prolabo (Barcelona, Spain).

Cell Lines and Transfection—Cell culture reagents were from Nunc (Roskilde, Denmark) or Invitrogen. HEK293 cells were grown in 12-well dishes using RPMI 1640 supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin sulfate. Cells grown to 75% confluence were transfected with 0.15 g of each plasmid and Lipofectamine. For co-transfections, 0.15 µg of each plasmid was employed unless specified otherwise. HBL and LND1 human melanoma cells (a gift from Prof. G. Ghanem, Free University of Brussels, Belgium) were grown in Dulbecco’s modified Eagle’s medium with antibiotics and 10% fetal calf serum. Stable transfectants derived from HBL cells have been already described (20, 25, 30) and were cultured in the presence of 800 µg/ml G418 sulfate.

Expression Constructs—All expression constructs were prepared in pcDNA3 (Invitrogen). The following constructs have been described: WT-MC1R (31), FLAG-MC1R (20), the FLAG-tagged RHC variants R151C, R160W, and D294H (25), and the GRK6 expression construct (20). cDNAs encoding bovine GRK2 and its dominant negative mutant GRK2-K220R cloned into pcDNA3 were gifts from Prof. F. Mayor Jr. (Madrid, Spain). The Rab5-EGFP and Rab7-EGFP constructs were a gift from
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Dr. Miguel Seabra (Imperial College, London, UK). The QuickChange mutagenesis kit was employed to generate one or two codon changes in the constructs next specified. A GRK6 dominant-negative mutant (32) was obtained by double mutagenesis using the human GRK6 plasmid as template and a pair of primers to convert Lys-215 and Lys-216 to Met. This mutant was designed GRK6-K215R/K216R. The 8 intracellular Ser and Thr residues present in MC1R (Ser-71, Ser-145, Ser-154, Thr-157, Thr-302, Thr-308, Thr-314, and Ser-316, Fig. 1) were independently mutated to either Ala or Asp to abolish or mimic (respectively) phosphorylation in these positions. Double mutants T308A/S316A and T308D/S316D were prepared using as template one single mutant and the pair of primers for the other residue. The sequences of mutagenic primers are provided as supplemental Table 1. After mutation, the coding strand of all mutants was excised from the plasmid using the restriction enzymes BglII and HindIII. The resulting plasmids were transfected into HEK293 cells to test for receptor expression and function. A dominant-negative mutant (32) was obtained by double mutagenesis using the human GRK6 plasmid to avoid undesirable mutations in the sequence. The plasmid was transfected into HEK293 cells to test for receptor expression and function. A dominant-negative mutant (32) was obtained by double mutagenesis using the human GRK6 plasmid to avoid undesirable mutations in the sequence.

Binding and Internalization Assays—Cells were serum-deprived at least 3 h before ligand addition. Radioligand binding assays were done with 10⁻¹⁰ M ¹²⁵I-labeled NDP-MSH and increasing concentrations of unlabeled NDP-MSH up to 10⁻⁷ M when required (33). For internalization assays, an acid wash protocol was employed (34). Briefly, cells were incubated with ¹²⁵I-labeled NDP-MSH and isotopically diluted to achieve a final concentration of 10⁻⁹ M corresponding to ~100 000 counts for 1.5 h (unless otherwise specified). Cells were then washed with cold serum-free RPMI followed by treatment with 0.5N NaOH for 10 min at room temperature and a final 10-min wash with PBS.

Confocal Microscopy—HEK293 cells grown on coverslips were transfected with the FLAG epitope-labeled WT or mutant MC1R constructs. In some experiments cells were co-transfected with MC1R variants and Rab5-EGFP or Rab7-EGFP (35), used as markers for endosomal vesicles. 24 h after transfection cells were serum deprived and incubated with 10⁻⁷ M NDP-MSH if needed. Cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.5% IGEPAI if required. Cells were then labeled with an anti-FLAG M2 monoclonal antibody (1:7000) followed by an Alexa 568-conjugated secondary antibody unless specified otherwise. For co-localization of FLAG-MC1R and tyrosinase, cells treated as above were incubated simultaneously with the anti-FLAG monoclonal (1:7000) and αPEP7h (1:1500) followed by Alexa 568-conjugated anti-mouse and Alexa 488-conjugated anti-rabbit secondary antibodies (both from Molecular Probes, Invitrogen). Samples were mounted by standard procedures using a mounting medium from DakoCytomation and examined with a Leica laser scanning confocal microscope.

Statistics—Unless otherwise specified, results are given as the mean ± S.E. for experiments performed at least 4 times, with independent duplicates or triplicates (n ≥ 8). Statistical significance was assessed with an unpaired Student’s t test performed with the GraphPad Prism package (GraphPad Software, San Diego, CA).

RESULTS

GRK6 Promotes MC1R Internalization—In HEK293 cells transfected with WT MC1R, agonist treatment (NDP-MSH, 10⁻⁷ M) mediated a rapid and strong increase in cAMP. Maximal cAMP levels were obtained ~30 min after addition of agonist and then decreased due to MC1R desensitization (20, 23). We used a functional receptor construct labeled with a FLAG epitope at the N terminus (25) to analyze MC1R cellular location during desensitization by confocal laser scanning microscopy (Fig. 2A). Staining of cells with an anti-FLAG monoclonal antibody showed a change of receptor distribution upon agonist treatment from a preferential location on the plasma membrane to a punctate intracellular staining indicative of receptor internalization in endocytic vesicles. Receptor sequestration away from the plasma membrane persisted for at least 90 min in the presence of agonist. The extent and rate of internalization were measured by following the appearance of acid-resistant binding (34). More than 36% of the bound ligand was internalized in the first hour of incubation (Fig. 2B).

We analyzed MC1R desensitization and internalization in a more physiological environment using two human melanoma cell lines, HBL and LND1, that express wild type MC1R (17). Cells were treated with 10⁻⁷ M NDP-MSH, and cAMP was measured using as template one single mutant and the pair of primers for the other residue. The sequences of mutagenic primers are provided as supplemental Table 1. After mutation, the coding strand of all mutants was excised from the plasmid using the restriction enzymes BglII and HindIII. The resulting plasmids were transfected into HEK293 cells to test for receptor expression and function. A dominant-negative mutant (32) was obtained by double mutagenesis using the human GRK6 plasmid to avoid undesirable mutations in the sequence.

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FIGURE 2. Agonist-promoted internalization of MC1R in HEK293 cells. A, HEK293 cells were transfected with FLAG epitope-labeled MC1R and stimulated with NDP-MSH (10^{-7} M) for the times shown. The distribution of MC1R in permeabilized cells was visualized by confocal microscopy. B, internalization assay performed on MC1R-transfected HEK293 cells. Transfected cells were incubated with 125I-labeled NDP-MSH and isotopically diluted to achieve a final concentration of 10^{-9} M and 10^{3} counts for the times shown. After the acid wash protocol (performed as described under “Experimental Procedures”), the radioactivity corresponding to radioligand bound on the cell surface (squares) or internalized (triangles) was measured. The internalization index (circles) represents the percentage of ligand internalized respect to total bound radioligand.

determined 30 min and 3 h after the challenge. The ratio of cAMP concentration at these time points is indicative of receptor desensitization (20, 36). MC1R desensitization was efficient in HBL and LND1 melanoma cells, as shown by decreased cAMP levels at longer incubation times (Fig. 3A), thus confirming previous results (20). To estimate the rate and extent of MC1R internalization, we used a clone of HBL human melanoma cells enriched in WT MC1R by stable transfection (clone HBL-20). This clone expresses a high density of plasma membrane melanocortin binding sites, thus facilitating accurate measurements of binding parameters (30). An internalization index of 40% was obtained (Fig. 3B) that decreased in the presence of known inhibitors of endocytosis such as 50 μM cytochalasin D (37), 0.25 mg/ml concanavalin A (37), 0.5 M sucrose (38), or low temperature (39). These data confirmed the specificity of the assay. Finally, internalization was visualized by confocal microscopy. For this purpose we used a clone of HBL cells expressing the FLAG epitope-tagged WT receptor (25). In resting cells, MC1R was present on the plasma membrane, in the perinuclear region, most likely corresponding to newly synthesized MC1R molecules located in the endoplasmic reticulum and in intracellular vesicles likely associated with receptor molecules in transit to or from the plasma membrane (Fig. 3C). The addition of the agonist caused a redistribution of receptor molecules from the plasma membrane to internal vesicles, evidenced by the fading of plasma membrane staining and the intensification of the labeling of intracellular vesicles. The kinetics of these changes were similar to those described in Fig. 2A, further supporting the use of HEK293 cells as a suitable heterologous model. Early work suggested that endogenous Mc1r expressed in mouse melanoma cells internalized to melanosomes after agonist binding (40). We extended these observations to human melanoma cells by using αPEP7h (29) for detection of the melanosomal enzyme tyrosinase (41), a suitable melanosomal marker. HBL cells expressing WT FLAG-MC1R were treated with NDP-MSH for 90 min, labeled with anti-FLAG and αPEP7h, and analyzed by confocal microscopy (Fig. 3D). The partial co-localization of MC1R and tyrosinase in resting cells increased markedly upon agonist treatment, further supporting that part of the MC1R is trafficked to the melanosome.

Homologous desensitization of MC1R was shown to be mediated by GRK2 and/or GRK6, two kinases expressed in human and mouse melanoma cells and normal melanocytes (20). Because desensitization and internalization are usually related processes, we analyzed the role of these kinases in MC1R sequestration. First, we excluded the involvement of the second messenger activated protein kinases C and A as well as p38 mitogen-activated protein kinase. No significant changes in internalization extent or kinetics were observed in the presence or absence of specific inhibitors of these kinases either in HBL-20 cells or HEK293 cells expressing WT MC1R (not shown). Next, HEK293 cells were transiently co-transfected with MC1R, GRK2, GRK6, or dominant negative mutants of the kinases (Fig. 4A). Neither GRK2 nor the dominant negative kinase-dead mutant GRK2-K220R (42) had any effect on internalization. Conversely, internalization was significantly stimulated by co-expression of GRK6 (p < 0.0001) and inhibited by a dominant negative form (GRK6-K215M/K216M, p = 0.038) (32). We also compared internalization in HBL cells and a clone obtained by stable transfection with the kinase gene (20). We found a slight but statistically significant increase in the internalization index in the GRK6-enriched clone (Fig. 4B). Therefore, internalization of the MC1R-agonist complex is likely mediated by GRK6-dependent phosphorylation.

Thr-308 and Ser-316 Are Involved in MC1R Desensitization and Internalization—To identify the phosphorylation sites targeted by GRK6, we obtained 8 individual Ser/Thr → Ala mutants, abolishing each one of the potential targets for the GRKs. The mutants were adequately expressed in HEK cells, as shown by Western blot (Fig. 5A). Binding studies indicated high densities of binding sites for all mutants, except T157A (Table 1). For this mutant the absence of significant binding apparently resulted from an inability to reach the plasma membrane as detected by fluorescence-activated cell sorter analysis (not shown). The behavior of T157A was not further analyzed. The other mutants showed moderate reductions of binding sites...
relative to WT without a parallel decrease in affinity, except T308A, which displayed a slightly higher Bmax. Internalization of the mutants was next analyzed (Fig. 5B). The T308A and S316A forms showed a statistically significant decrease in internalization (p < 0.0001), suggesting that residues Thr-308 and Ser-316 might provide targets for GRK6-dependent phosphorylation. Surprisingly, T314A, S71A, and S145A exhibited substantial increases in internalization. This was not further investigated.

Concerning functional coupling, no changes in coupling efficiency, desensitization, or sensitivity to GRK6 were seen for mutants in the first (S71A) or second (S145A and S154A) intracellular loops (not shown). The remaining potential phosphorylation targets are located in the cytosolic tail of the receptor. Of these, S302A, T314A, and S316A also behaved as WT (not shown). However, T308A was more resistant to desensitization, as demonstrated by higher cAMP levels than WT at long stimulation times in the presence or absence of GRK6 (Fig. 5C).

Based on these data, a series of new mutagenesis experiments was designed to further investigate the role of residues Thr-308 and Ser-316. We constructed the corresponding Ser/Thr→Asp mutants, expected to mimic the phosphorylated state of the residues, as well as double mutants to both Ala and Asp. The T308D, S316D, T308D/S316D, and T308A/S316A variants were expressed similarly to WT (Fig. 6A). However, the density of binding sites on the plasma membrane was lower for mutants T308D, S316D, and T308D/S316D and higher for T308A/S316A (Table 2). The mutants displayed reduced coupling efficiency and anomalous internalization behavior. T308D and S316D elicited agonist-mediated cAMP increases 2–3-fold lower than WT but were still able to undergo desensitization as shown by lower cAMP levels after 3 h of agonist treatment as compared with a 30-min challenge (Fig. 6B). Conversely, desensitization was strongly impaired for T308A/S316A MC1R, with cAMP levels increasing up to 3 h in the presence of agonist. Finally, T308D/S316D did not mediate significant increases in cAMP.

In the absence of exogenous GRK6, internalization was more efficient for T308D, S316D, and T308D/S316D than for WT (Fig. 6C). This further supported a role of Thr-308 and Ser-316 phosphorylation in promoting MC1R sequestration. When the
various forms were co-transfected with GRK6, internalization reached maximal levels for WT, T308D, S316D, and S316A but remained impaired for the T308A and T308A/S316A mutants. Finally, the high rate of internalization of T308D/S316D was only slightly increased upon co-transfection with GRK6.

The different densities of the T308D/S316D and T308A/S316A mutants on the plasma membrane suggested an altered subcellular distribution within transfected cells. This was analyzed by confocal laser microscopy. As shown in Fig. 7A, T308D/S316D exhibited a punctate pattern indicative of preferential association with intracellular vesicles. This pattern is very similar to WT MC1R in cells stimulated for 30 min with NDP-MSH, consistent with a constitutive internalization of T308D/S316D. On the other hand, T308A/S316A revealed a preferential association with the plasma membrane both in resting and stimulated cells, indicative of impaired ability to undergo agonist-induced internalization (Fig. 7B). Moreover co-localization with the early endosomal marker Rab5 was absent or very low for T308A/S316A, intermediate for WT, and extensive for T308D/S316D (Fig. 7C). Similar results were obtained with the late endosome/lysosome marker Rab7 (Fig. 7D), indicative of the location of MC1R molecules in different types of vesicles of the endocytic pathway. Overall, these data strongly suggest that Thr-308 phosphorylation triggers MC1R internalization in endosomal vesicles.

Altered Desensitization and Internalization Properties of the RHC Alleles—The highly penetrant RHC mutant alleles R151C, R160W, and D294H show reduced signaling via the cAMP pathway and abnormal cell surface expression (7, 24, 25). The possibility that they may also display altered desensitization

| Variant | $B_{\text{max}}$ (pmol/mg of protein) | $K_d$ (nM) |
|---------|-------------------------------------|------------|
| Wild type | 9.5 ± 1.4 | 2.2 ± 0.3 |
| S71A | 5.8 ± 0.6 | 1.8 ± 0.5 |
| S145A | 8.5 ± 0.3 | 3.2 ± 0.7 |
| S154A | 9.0 ± 1.1 | 2.3 ± 0.2 |
| T157A | Not detectable | Not determined |
| S302A | 3.4 ± 1.8 | 1.3 ± 0.3 |
| T308A | 13.4 ± 1.3 | 3.4 ± 0.9 |
| T314A | 7.7 ± 3.0 | 1.6 ± 0.4 |
| S316A | 8.0 ± 1.0 | 2.7 ± 0.4 |

FIGURE 4. Stimulation by GRK6 of MC1R internalization. A, HEK293 cells were cotransfected with MC1R and either GRK2 or GRK6 or the corresponding dominant negative (DN) expression constructs (0.15 μg each plasmid), and an internalization assay was performed. ***; p < 0.0001; *, p < 0.05. B, HBL melanoma cells and the stable clone 15 overexpressing GRK6 (20) were compared for internalization using the standard acid wash protocol. *, p < 0.05.

FIGURE 5. Functional analysis of Ser/Thr→Ala MC1R point mutants. A, comparable expression of WT MC1R and Ser/Thr→Ala mutants in HEK cells. 24 h after transfection with the FLAG epitope-tagged constructs, cells were harvested and solubilized for Western blot. Membranes were stripped and re-probed with an anti-extracellular signal-regulated kinase 2 antiserum for loading comparison. B, internalization of Ser/Thr→Ala mutants. HEK293 cells were independently transfected with each construct and incubated with 125I-labeled NDP-MSH for 1.5 h followed by acid wash and determination of externally bound and internalized ligand. Only the T308A and S316A mutants (hatched bars) showed a decreased internalization index relative to WT (solid bar). ***, p < 0.0001. C, impaired desensitization of T308D/S316A MC1R. HEK293 cells were transfected with WT MC1R and the T308A and S316A mutants alone or co-transfected with the receptor variants and GRK6. 24 h after transfection, cells were incubated with 10−7 M NDP-MSH for 30 min or 3 h, and intracellular cAMP was measured. **, p < 0.001.

TABLE 1

Equilibrium binding parameters of Ser/Thr→Ala MC1R mutants expressed in HEK293 cells

Results are the mean ± S.E. for three independent assays performed in duplicate dishes.
and/or internalization was next analyzed. HEK cells expressing the relevant forms were challenged with NDP-MSH for 30 min or 3 h, and their cAMP contents was measured (Fig. 8A). Consistent with published results, the mutant receptors mediated reduced but significant increases in cAMP, with similar residual activities for R151C and R160W and a higher degree of functional impairment for D294H. Interestingly, the three RHC forms exhibited reduced desensitization, as assessed from the ratio of cAMP levels after a 30-min or a 3-h treatment. This was confirmed by following the evolution of cAMP concentration over a 60-min period in cells pretreated with the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX). Phosphodiesterase inhibition quenches the contribution of enzymatic deg-

**TABLE 2**

Equilibrium binding parameters of the T308D, S316D, T308D/S316D, and T308A/T316A MC1R mutants expressed in HEK293 cells

Results are the mean ± S.E. for three independent assays performed in replicate dishes.

| Variant          | $B_{\text{max}}$ pmol/mg of protein | $K_d$ nM |
|------------------|-----------------------------------|----------|
| Wild type        | 9.5 ± 1.4                         | 2.2 ± 0.3 |
| T308D            | 1.5 ± 0.8                         | 1.6 ± 0.6 |
| S316D            | 3.6 ± 0.9                         | 1.3 ± 0.5 |
| T308A/S316A      | 20.0 ± 2.6                        | 8.6 ± 1.1 |
| T308D/S316D      | 0.5 ± 0.2                         | 10.2 ± 1.7 |

**FIGURE 6.** Functional analysis of mutants mimicking the phosphorylated state of Thr-308 and Ser-316 and double mutants. A, efficient expression of T308D and S316D MC1R and the double mutants T308A/S316A and T308D/S316D. The FLAG epitope-labeled mutants were expressed in HEK293 cells, and their levels were compared by Western blot analysis. An anti-extracellular signal-regulated kinase 2 antibody was used for loading control. B, functional coupling to cAMP production of T308D, S316D, and double mutants T308A/S316A and T308D/S316D. HEK cells expressing WT MC1R or the variants shown were challenged with $10^{-7}$ M NDP-MSH for 30 min or 3 h, and cAMP was measured. Note the lack of desensitization of the T308A/S316A form as compared with WT. **, $p < 0.001$. C, internalization of Thr-308 and Ser-316 single and double mutants. Co-transfection of each variant with GRK6 resulted in impairment of the internalization for the double Ala mutant. ***, $p < 0.0001$, calculated for cells expressing the receptor construct alone relative to control cells transfected with empty vector.

**FIGURE 7.** Opposite effects on MC1R internalization of mutation of residues Thr-308 and Ser-316 to Ala or Asp. A, laser scanning confocal micrographs of HEK cells transfected with WT (left) or the T308D/S316D forms (right). S308D-T316D staining pattern is very similar to the one corresponding to WT after a 30-min stimulation with $10^{-7}$ M NDP-MSH (middle). B, resistance of T308A/S316A to agonist induced internalization. Cells expressing S308A/T316A were challenged with $10^{-7}$ M NDP-MSH for the times shown and stained for MC1R with an anti-FLAG monoclonal antibody. C, association of T308D/S316D with Rab5-positive endocytic vesicles. HEK293 cells were transfected to express WT MC1R or the indicated mutants and the early endosome marker Rab5-EGFP. Cells were fixed, stained for MC1R, and examined in a confocal microscope. Left panels, MC1R is shown in red. Middle panels, Rab5-EGFP is shown in green. Right panels, overlays of MC1R and Rab5-EGFP. The boxed areas in the overlays are enlarged and depicted in the right column. Note the higher colocalization of T308D/S316D with the endosomal marker. D, association of T308D/S316D with Rab7-positive vesicles. Same as in panel C, except that a Rab7-EGFP construct was used.
radiation of cAMP. Therefore, in the presence of IBMX receptor desensitization should result in approximately stable cAMP levels, whereas lack of desensitization should yield linear kinetics. As shown in Fig. 8B, for WT cAMP increased during the first 30 min and then remained stable. Conversely cAMP levels increased linearly during the time course of the experiment in cells expressing the RHC forms, confirming impaired desensitization of the mutant receptors.

MC1R forms dimers in living cells and heterodimerization of WT, and mutant variants can modulate key pharmacological properties of the receptor (25). We looked for changes in receptor desensitization and internalization in cells co-expressing WT and RHC alleles. Cells expressing either WT alone or WT and R151C behaved similarly when cAMP levels were measured (Fig. 9A). Conversely, in cells co-expressing WT and either R160W or D294H, receptor desensitization was impaired, as shown by similar second messenger levels for the 30-min and 3-h treatment times. More conclusively, cAMP levels at 3-h treatment times were significantly higher in cells co-expressing WT and R160W or 294H as compared with cells expressing WT MC1R alone. We next looked at the internalization of the RHC alleles. In radioligand binding studies, internalization was slightly higher for the R151C and R160W variants as compared with WT and strongly impaired for D294H (p < 0.0001, Fig. 9B). Moreover, in cells co-expressing WT and D294H (but not R151C or R160W), the RHC allele exerted a dominant-negative effect on the internalization of WT (Fig. 9C). The altered internalization of D294H was confirmed by confocal microscopy (Fig. 10). The preferential association of this form with the plasma membrane of transfected cells was resistant to agonist, whereas the distribution of R151C and R160W was similar to WT, with formation of endocytic vesicles after agonist binding.

**DISCUSSION**

Desensitization and internalization are key regulatory mechanisms of GPCR signaling (21). Agonist-induced desensitization is most often carried out by receptor phosphorylation by GRKs. This is usually followed by binding of arrestin, which uncouples receptor and G protein and triggers internalization. The internalized receptor can be targeted for lysosomal degradation or dephosphorylated and recycled back to the cell surface for resensitization. MC1R mediates the multiple actions of the melanocortins on melanocytes and is a key regulator of human pigmentation and UV-dependent tanning (3, 7, 19, 43). Several natural MC1R alleles, designated RHC, are associated
with a preferentially pheomelanic phenotype and an increased risk of skin cancer (7, 8, 10, 13, 14, 16, 33). These alleles encode partial loss-of-function forms, with altered cell surface expression (7, 24, 25). In an attempt to further understand the functional basis of the genetic association of these variants with pigmentation phenotypes and skin cancer risk, we undertook a study of WT and variant MC1R desensitization and internalization. The key findings of this study were as follows. 1) MC1R expressed in an heterologous system or in human melanoma cells underwent similar desensitization and internalization in the continuous presence of the agonist, 2) in contrast with our previous findings that MC1R can be desensitized by GRK2 or GRK6 (20), receptor internalization was not promoted by GRK2 but was at least partially dependent on GRK6, 3) GRK6-mediated desensitization and internalization most likely depended on the phosphorylation of two residues located in the cytosolic C terminus of the receptor, Thr-308 and Ser-316, 4) desensitization of the RHMC1R variants was less efficient than WT, 5) internalization was strongly reduced for the D294H RH variant, and 6) heterodimerization of RH variants and WT receptors modulated desensitization and internalization.

The inability of GRK2 to promote MC1R internalization was established by the lack of effect of co-expression of MC1R and the kinase gene or a dominant negative form (GRK2-K220R). Conversely, internalization was stimulated by co-expression of GRK6 and inhibited by a dominant negative form of this kinase (GRK6 K215M/K216M). These data suggest that MC1R might be differentially regulated by GRK2 and GRK6. GRK6 would cause receptor desensitization and internalization. Because internalization of endogenous murine Mc1r likely targets the receptor for lysosomal degradation (44) and partially for delivery to the melanosomes (Ref. 40 and results presented in Fig. 3D), GRK6 would promote MC1R down-regulation and incorporation of internalized cargo to the melanosome. Conversely, GRK2 would desensitize MC1R without sequestration, thus avoiding protein degradation. Conceivably, MC1R could then be resensitized by internalization-independent dephosphorylation at the cell surface, a process that has been shown to occur for other GPCRs (45–47).

Given the different outcome of GRK2 and GRK6 action on MC1R, the specific Ser or Thr residues targeted by the two kinases should not be the same. Alternatively, GRK2 could phosphorylate only a subset of the residues targeted by GRK6, thus triggering only one of the effects associated with GRK6 phosphorylation. To identify the MC1R residues targeted by GRK6 we used a functional approach based on mutation to Ala of the eight intracellular Ser and Thr residues in MC1R and analysis of the effects of mutation on internalization and desensitization. These experiments were performed in cells transfected with the individual variants alone or in combination with GRK6. This mutational analysis clearly pointed to Thr-308 and Ser-316 as the key residues controlling the functional status of MC1R. This is strongly suggested by the resistance of the T308A/S316A double mutant to agonist-induced desensitization and internalization as well as by the lack of functional coupling and the constitutive association of the T308D/S316D mutant with vesicles positive for the small GTPases Rab5 or Rab7, two markers of endosomal compartments (48). Therefore, in MC1R the targets for GRK6 are located in the C-terminal cytosolic extension, as previously shown for various GPCRs (49). However, the relative importance of Thr-308 and Ser-316 seemed different. Mutation of Thr-308 to Ala was sufficient to impair desensitization, but a similar effect was not seen for S316A. On the other hand, both T308A and S316A showed impaired sequestration, and either S316D or T316D internalized more efficiently than WT. However, phosphorylation of Thr-308 was necessary for optimal internalization since the T308A mutant did not achieve maximal internalization even when co-expressed with GRK6, whereas S316A internalized as efficiently as WT in cells co-expressing the receptor and the GRK. Therefore, it would appear that the functional status of MC1R is mainly controlled by Thr-308, with Ser-316 playing an ancillary role.

The variant RHC alleles associated with red hair, poor tanning ability, and increased skin cancer risk are partial loss-of-function forms (7, 13), but their binding properties are similar to WT with dissociation constants for NDP-MSH in the low nanomolar range. Accordingly, the R151C, R160W, and D294H variants should be essentially saturated in the experimental conditions used for the measurements of cAMP production, namely stimulation with 10^{-7} M NDP-MSH. The lower cAMP production by the RH variants should, therefore, reflect the inability of these forms to undergo the agonist-induced conformational change leading to a fully active receptor state, particularly in the case of D294H, which is expressed at even higher levels.
Wild Type and Variant MC1R Desensitization and Sequestration

plasma membrane densities than WT. Thus, the impaired rate of desensitization of the RHC variants is not surprising and likely reflects a lower affinity of GRK6 for their agonist-bound form.

MC1R shows some degree of constitutive signaling (23), and the results presented here indicate that it underwent constitutive internalization in the absence of agonists, as suggested by detectable co-localization with the early endosome marker Rab5 and the late endosome/lysosome marker Rab7. This effect was exacerbated in T308D/S316D, with very low surface expression, constitutive association with endocytic vesicles, and extensive co-localization with Rab5 and Rab7. Conversely, the T308A/S316A mutant was resistant to internalization and co-localized poorly with Rab5 or Rab7, and its cell surface density was 2-fold higher than WT. These data point to the rate of internalization as a major determinant of MC1R surface expression. Previous studies suggested that an altered surface expression may contribute at least in some cases to reduced function of the RHC variant receptors (7, 24, 25). In our hands, the plasma membrane receptor numbers for R151C and R160W were 5–10-fold lower than WT but ~3-fold higher for D294H. The internalization index of D294H was similar to the T308A/S316A mutant, also expressed at high levels on the cell surface. Therefore, the primary defect underlying altered surface expression of D294H appeared to be an inability to undergo internalization, also demonstrated by confocal microscopy. For the R151C and R160W variants, an internalization index higher than WT might also contribute to the lower cell surface expression, but other factors are also very likely involved. Ongoing experiments suggest that these forms are aberrantly processed and retained in intracellular compartments, as we have detected extensive co-localization of R151C and R160W (but not D294H) with the endoplasmic reticulum marker calnexin.4

We have previously shown that WT MC1R exists as a constitutive dimer when expressed alone and as a constitutive heterodimer when co-expressed with variant alleles (25). This physical interaction of functionally non-equivalent receptor forms was found to modulate some properties of the receptor. For instance, co-expression of WT and R151C or R160W decreased the expression of cell surface binding sites. Here we investigated a possible effect of heterodimerization on desensitization and internalization. Receptor desensitization was easily evidenced by the decreased concentration of cAMP in cells continuously exposed to the agonist for 3 h as compared with cells challenged for 30 min (20, 36). Using this simple assay, we found that heterodimerization of WT and R160W or D294H strongly inhibited receptor desensitization. The effect was particularly clear for D294H. This form does not significantly contribute to cAMP production, yet the levels of cAMP in cells co-expressing WT and D294H were higher for the 3-h challenge than when WT was expressed alone. Moreover, co-expression of D294H and WT also caused a reduction of the internalization index to levels similar to those of D294H expressed alone. Taken together, these data show that the WT-D294H heterodimer retained a strong signaling activity but displayed desensitization and internalization parameters reminiscent of the RHC form. This suggests that, whereas monomers could be individually active within the dimer, efficient desensitization and internalization may require the contribution of the two units.

In summary, the results described above suggest novel and complex aspects of regulation of MC1R. These include 1) differential effects of the GRKs that may provide the basis for cell type-specific regulatory patterns, 2) different desensitization and internalization properties for the WT and natural variants, and 3) modification of key signaling properties by heterodimerization of the WT receptor with skin cancer-associated RHC alleles. It will be important to assess the actual impact of these regulatory mechanisms in normal human melanocytes of defined genotype naturally expressing different combinations of the frequent variant alleles. In any case, our results show that MC1R is an excellent model to study fundamental aspects of GPCR signaling such as the role of individual units within dimeric forms.

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