Palmitoylation of CCR5 Is Critical for Receptor Trafficking and Efficient Activation of Intracellular Signaling Pathways*

Received for publication, January 21, 2001, and in revised form, April 20, 2001
Published, JBC Papers in Press, April 25, 2001, DOI 10.1074/jbc.M100583200

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CCR5 is a CC chemokine receptor expressed on memory lymphocytes, macrophages, and dendritic cells and also constitutes the main coreceptor for macrophage-tropic (or R5) strains of human immunodeficiency viruses. In the present study, we investigated whether CCR5 was palmitoylated in its carboxyl-terminal domain by generating alanine substitution mutants for the three cysteine residues present in this region, individually or in combination. We found that wild-type CCR5 was palmitoylated, but a mutant lacking all three Cys residues was not. Through the use of green fluorescent fusion proteins and immunofluorescence studies, we found that the absence of receptor palmitoylation resulted in sequestration of CCR5 in intracellular biosynthetic compartments. By using the fluorescence recovery after photobleaching technique, we showed that the non-palmitoylated mutant had impaired diffusion properties within the endoplasmic reticulum. We next studied the ability of the mutants to bind and signal in response to chemokines. Chemokines binding and activation of Gi-mediated signaling pathways, such as calcium mobilization and inhibition of adenylate cyclase, were not affected. However, the duration of the functional response, as measured by a microphysiometer, and the ability to increase [35S]guanosine 5′-3-O-(thio)triphosphate binding to membranes were severely affected for the non-palmitoylated mutant. The ability of RANTES (regulated on activation normal T cell expressed and secreted) and aminooxyptenate-RANTES to promote CCR5 endocytosis was not altered by cysteine replacements. Finally, we found that the absence of receptor palmitoylation reduced the human immunodeficiency viruses coreceptor function of CCR5, but this effect was secondary to the reduction in surface expression. In conclusion, we found that palmitoylated cysteines play an important role in the intracellular trafficking of CCR5 and are likely necessary for efficient coupling of the receptor to part of its repertoire of signaling cascades.

CCR5 is a high affinity receptor for the CC chemokines MIP-1α, MIP-1β, RANTES1, MCP-2 (2), LD78β (3), and a proteolytically processed variant of HCC-1 (HCC-1-(9–78)) (4). It is also the principal coreceptor for macrophage-tropic strains of HIV (5, 6). HIV-1 infection is initiated by the interaction of the virion envelope glycoprotein with cellular CD4 and a coreceptor that belongs to the G protein-coupled receptor (GPCR) family. CCR5-using (R5) strains predominate during the early stages of the disease and are responsible for viral transmission. The major role of CCR5 in AIDS pathogenesis has been demonstrated by the almost complete resistance to HIV-1 infection of individuals homozygous for a 32-base pair deletion (32 allele) in the coding region of the receptor (7, 8). A prominent role of CCR5 has also been suggested in the recruitment of leukocyte populations in other human diseases, such as rheumatoid arthritis, multiple sclerosis, and asthma (9–13). CCR5 surface expression can be regulated at multiple levels. Repression of CCR5 gene transcription has been achieved in T cells by CD28 engagement (14). Sequestration of mutated receptor...
ceptors in the endoplasmic reticulum (ER) has been described for the Δ32 variant and for other CCR5 variants known to affect chemokine receptor or coreceptor functions (15, 16). The level of CCR5 at the cell surface is also regulated by endocytosis, and this mechanism contributes to the blocking of HIV entry by chemokines and chemokine analogs (17–19). Several post-translational modifications have also been shown to influence CCR5 expression. Activation of CCR5 by chemokines induces phosphorylation of serine residues within the carboxy-terminal domain, and this, in turn, plays an important role in receptor desensitization and internalization (20). Two disulfide bonds linking CCR5 extracellular domains are necessary for maintaining a structural conformation of the receptor compatible with efficient trafficking to the cell surface, ligand binding, and induction of intracellular signaling (21). Sulfation of CCR5 amino-terminal tyrosines has also been shown to play an important role in gp120 binding and HIV entry (22).

It is well established that for some GPCRs, such as the β2-adrenergic receptor, palmitoylation of cysteine residues in their cytoplasmic tails can modulate their biological activities (23). It has been suggested that this acylation provides a membrane anchor to the carboxyl-terminal domain and creates a fourth intracellular loop. Palmitate is bound to cysteine side chains through a reversible thioester bond and increases the overall hydrophobicity of the protein domain. Palmitoylation of GPCRs has been shown to affect different functions of the receptors, such as membrane targeting, signaling properties, endocytosis, and recycling. Because of the pleiotropic effects of palmitoylation that have been described on the function of various receptor families (23), it is not possible to predict how palmitoylation will affect a specific receptor. No information concerning palmitoylation and its role in chemokine receptors has been reported so far. Most, but not all, chemokine receptors display cysteines in their carboxy-terminal tails, at positions compatible with palmitoylation. CCR5 has a cluster of three cysteines in this region (Fig. 1A). In the present study, we investigated the role played by these three cysteine residues in various aspects of CCR5 function. We provide evidence that CCR5 is indeed palmitoylated on these residues. We also found that palmitoylation is necessary to allow efficient CCR5 trafficking to the cell surface and for efficient triggering of intracellular signal transduction pathways.

MATERIALS AND METHODS

Mutagenesis and CCR5 Constructs—The three cysteines located within the carboxy-terminal domain of CCR5 (Cy5-321, Cy5-323, and Cy5-324) were mutated to alanine, individually or in combination, by site-directed mutagenesis using the Quickchange method (Stratagene). The 3Cy5A mutant refers to a receptor in which all three cysteines have been mutated to alanine. Alamine substitutions were made instead of more conservative serine substitutions, in order to avoid additional phosphorylation of the receptor tail. Following sequencing of the constructs, the mutated reading frames were subcloned into the bicistronic expression vector pEFIN3 as described previously (24) for generation of stable cell lines and in pcDNA3 (Invitrogen) for the HIV-1 infection studies. Clones expressing similar amounts of wtCCR5 or 3Cy5A at the cell surface were selected (clones CCR5-c3 and 3Cy5A-c1 respectively) following establishment of clonal cell lines by limiting dilution and screening by FACS analysis. Clonal CHO-K1 cell lines stably expressing the fusion proteins wtCCR5-GFP and 3Cy5A-GFP were also established and selected by fluorescence microscopy.

HIV-1 Infection—Mixed cell populations expressing wtCCR5 or the various mutants, as well as the clonal lines CCR5-c3 and 3Cy5A-c1, were assayed for [3H]palmitic acid incorporation. Approximately 5 x 10⁶ cells, at 70% confluence in 10-cm dishes, were washed twice with cold PBS and incubated for 18 h in serum-free Ham's F-12 medium. Cells were washed once with cold phosphate-buffered saline (PBS, pH 7.4) and metabolically labeled with 0.2 μCi/ml [9,10-3H]palmitic acid (Amersham Pharmacia Biotech) for 4 h at 37°C. The incubation was stopped by washing the cells twice with cold PBS, and the cells were solubilized in 1 ml of solubilization buffer (10 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 2 mM EDTA, 1 tablet of Protease Inhibitor Mixture (Roche Molecular Biochemicals) per 10 ml, and 1% (w/v) Cymal TM5 (Antrace, Maumee, OH)) as described (26) for 1 h at 4°C under constant agitation, and cell debris was removed by ultracentrifugation for 30 min at 45,000 × g and 4°C. Solubilized receptors were incubated overnight at 4°C with 10 μl/mg 2D7, a mAb recognizing a conformation epitope in the second extracellular loop of CCR5, then immunoprecipitated by protein G-Sepharose (Amersham Pharmacia Biotech) for 2 h at 4°C, and washed three times in solubilization buffer. Samples were resuspended in SDS-PAGE sample buffer, heated for 5 min at 100°C, and resolved onto 12% polyacrylamide gels. One SDS-PAGE gel, performed with 20% of the immunoprecipitated samples, was transferred to nitrocellulose filters. The filters were incubated, for CCR5 immunodetection, with MC5 (0.5 μg/ml), a monoclonal antibody recognizing a linear epitope in CCR5 amino terminus, and then with a horseradish peroxidase-coupled goat anti-mouse antibody, and the immunoblot was revealed by enhanced chemiluminescence (Amersham Pharmacia Biotech). The other SDS-PAGE gel, performing staining samples was incubated in the Amplify fluorographic reagent (Amersham Pharmacia Biotech) and dried, and autoradiography was performed for 3 weeks at ~70°C with x-ray films (Fuji).

FACS Analysis—Cell surface expression of the CCR5 variants was measured by flow cytometry using phycoerythrin-conjugated 2D7 (Becton Dickinson), or MC-5. FACS analysis was performed on a FACScan flow cytometer using the CellQuest software (Becton Dickinson). The mean channel fluorescence (MCF) was used to compare the levels of receptor expression at the cell surface. Results were normalized for the MCF obtained for wtCCR5 with the 2D7 antibody (100%) after subtraction of the nonspecific fluorescence obtained for untransfected cells (0%).

Confocal Microscopy—For immunofluorescence studies, stable CHO-K1 cell lines expressing wild-type or mutant CCR5 were grown on uncoated glass coverslips for 24 h. Coverslips were rinsed with PBS, fixed for 10 min in 3% paraformaldehyde in PBS, and washed three times for 10 min with Tris-buffered saline (TBS). For intracellular staining, the cells were permeabilized by a 5-min incubation with 0.15% Triton X-100 in TBS and washed three time in TBS. Fixed or permeabilized cells were incubated for 1 h at room temperature with 5% normal sheep serum in TBS. Incubation with 5 μg/ml of the MC-5 mAb was performed overnight in the presence of 5% normal sheep serum. Cells were rinsed three times in TBS, incubated for 60 min in the dark with a fluorescein isothiocyanate-labeled sheep anti-mouse IgG antibody (1:30 dilution, Amersham Pharmacia Biotech), washed three times in TBS and once in water, and mounted with a drop of gelvatol solution.
containing 100 ng/ml 1,4-diazabicyclo(2.2.2)octane antifading agent (Sigma). Cells were observed on an MRC 1024 confocal microscope (Bio-Rad) fitted on an Axiovert 100 inverted microscope (Zeiss, Oberkochen, Germany) equipped with a Plan-Neofluar water immersion objective (Zeiss). The 488 nm excitation beam of an argon-krypton laser and a 522/32 nm band-pass emission filter were used for photobleaching (FRAP) using the MRC 1024 confocal microscope (see above). A defined region was photobleached at full laser power (100%) for 10 scans, and recovery was monitored by scanning the whole cell at low laser power (3 to 10%). The first image was recorded 30–40 s after photobleaching (the time required for adjusting the optics and resetting the instrument), and subsequent images were recorded every 20 s. Quantitative measurements of fluorescence, for generating recovery plots, and D and M values, were obtained by using macros written in the NIH Image freeware. The fluorescence signal of an unbleached cell area was measured before and at each time point after photobleaching. All FRAP values were normalized according to these control values, but even after 60 scans, the correction factor never exceeded 20% of the initial value. Diffusion parameters were obtained as described (27) and were calculated by nonlinear regression using the GraphPad PRISM software.

Binding Assays—Mixed populations of CHO-K1 cells expressing wild-type or mutant CCR5 were collected from plates with Ca++, and Mg²⁺-free PBS supplemented with 5 mM EDTA, gently pelleted for 2 min at 1000 × g, and resuspended in binding buffer (50 mM HEPES, pH 7.4, 1 mM CaCl₂, 5 mM MgCl₂, 0.5% BSA). Competition binding assays were performed in Minisorb tubes (Nunc, Roskilde, Denmark), using 0.08 mM 125I-MIP-1β (2200 Ci/mmol, PerkinElmer Life Sciences) as tracer for wtCCR5 and the single cysteine mutant or 0.12 mM 125I-MIP-1β for the double and triple cysteine mutants, variable concentrations of competitors, and 40,000 cells in a final volume of 0.1 ml. Total binding was measured in the absence of competitor, and nonspecific binding was measured in the presence of a 100-fold excess of unlabeled ligand. Samples were incubated for 90 min at 27 °C and then bound tracer was separated by filtration through GF/B filters presoaked in 1% BSA. Filters were counted in a β-scintillation counter. Binding parameters were determined with the PRISM software (Graphpad Software, San Diego, CA) using nonlinear regression applied to a one-site competition model.

Aequorin-based Functional Assay—The functional response to chemokines was analyzed by measuring the luminescence of aequorin as described (21, 28). Cells were collected from plates with Ca++, and Mg²⁺-free PBS supplemented with 5 mM EDTA, pelleted for 2 min at 1000 × g, resuspended in DMEM at a density of 5 × 10⁴ cells/ml, and incubated for 2 h in the dark in the presence of 5 µM coelenterazine H (Molecular Probes, Eugene, OR). Cells were diluted 7.5-fold before use. Agonists in a volume of 50 µl of DMEM were added to 50 µl of the cell suspension (33,000 cells), and luminescence was measured for 30 s in an EG & G Berthold (PerkinElmer Life Sciences). Functional parameters were determined with the PRISM software (Graphpad Software) using nonlinear regression applied to a sigmoidal dose-response model.

Immunoassays for CCR5 Accumulation—To determine CCR5 expression using an anti-3CysA-α antibody (Bio-Rad), cells were plated onto a 96-well microplate (Basic FlashPlates, PerkinElmer Life Sciences). [35S]GTPγS (0.1 nM, Amersham Pharmacia Biotech) was added, and microplates were shaken for 1 min and further incubated at 30 °C for 30 min. The incubation was stopped by centrifugation of the microplate for 10 min, at 800 × g and at 4 °C, and aspiration of the supernatant. Microplates were counted in a TopCount (Packard Instrument Co) for 1 min per well. Functional parameters were determined with the PRISM software (Graphpad Software) using nonlinear regression applied to a sigmoidal dose-response model.

Endocytosis Assay—Chemokine-induced CCR5 endocytosis was quantified as described (19). Briefly, CCR5-c3 or 3CysA-c1 cells were plated onto 96-well plates with 3.5 µl EDTA in PBS, washed with DMEM/F-12, and incubated for 45 min at 37 °C with RANTES or AOP-RANTES at different concentrations. Cells were washed twice with 3 ml of cold PBS supplemented with 0.1% sodium azide and 0.1% BSA, and incubated for 30 min at 4 °C with MC-5 (a monoclonal antibody that does not compete with RANTES binding), then stained with a phycoerythrin-conjugated anti-mouse Ig antibody (Sigma). Cells were further washed and cell fluorescence was analyzed by FACS, using a FACScan flow cytometer and the CellQuest software (Becton Dickinson, San Jose, CA). pcdNA3-transfected cells infected with the same amount of GFP reporter viruses were used as controls for background GFP expression. The MCF was used to compare the levels of receptor expression at the cell surface. HIV infection was determined by counting GFP-positive and dual-positive (GFP + DsRed) cells.

RESULTS

Palmitoylation is known to occur on cysteine residues located in the carboxyl-terminal domain of GPCRs. It has been associated with the modulation of various functional properties, depending on the specific receptor (23). CCR5 exhibits a cluster of 3 cysteines in its carboxyl-terminal domain (Cys-231, Cys-232, and Cys-234) (Fig. 1A, A, suggesting that it could be palmitoylated. In order to test the role of these intracellular cysteines on CCR5 function, we mutated these 3 residues into alanine, individually (mutants C321A, C323A, and C324A) or in all possible combinations (C321A/C323A, C321A/C324A, C323A/C324A, 3CysA = C321A/C323A/C324A). The various constructs were inserted into efficient bicistronic vectors and transfected in CHO-K1 cells stably expressing the reporter
protein apoaequorin. Mixed populations of transfected cells were used for testing surface expression, binding, and functional properties of the receptors. We also selected clonal cell lines expressing wtCCR5 and the 3CysA mutant at similar levels (respectively clones CCR5-c3 and 3CysA-c1) and performed the various assays on these two clones as well.

**CCR5 Is Palmitoylated and Mutation of the Cysteine Cluster in Its Carboxyl-terminal Domain Abrogates [3H]Palmitic Acid Incorporation**—Mixed CHO-K1 cells expressing wtCCR5 or the various mutants, as well as the CCR5-c3 and 3CysA-c1 clones, were metabolically labeled for 4 h with [3H]palmitic acid. The receptor was immunoprecipitated from cell lysates by the anti-CCR5 mAb 2D7. Immune complexes were separated by SDS-PAGE. CCR5 was either immunodetected following Western blotting of the samples using mAb MC-5 (A) or the gel was subjected to fluorography and autoradiography (B). Controls included immunoprecipitation using wtCCR5-expressing cells and an isotype (IgG1) of the 2D7 monoclonal (Ig control) and immunoprecipitation using untransfected CHO-K1 cells and the 2D7 monoclonal antibody (untransfected). The Western blot and fluorography shown are representative of two independent experiments.

Fig. 3, A and B, the mutation of any one of the three cysteines (mutants C321A, C323A, and C324A) reduced cell surface expression by 50%. No significant difference was seen between the three mutants. The double mutants (C321A/C323A, C321A/C324A, and C323A/C324A) and to a larger extent the triple mutant (3CysA) showed even greater decreases in surface expression. This gradual decrease in cell surface expression, according to the number of substituted cysteines, was also seen with MC-5, a mAb recognizing a linear epitope, suggesting a reduction in the number of receptors at the plasma membrane rather than an alteration of the receptor conformation. Surface expression of the selected clones CCR5-c3 and 3CysA-c1 is illustrated for comparison (Fig. 3C).

To investigate the relative role of decreased protein synthesis and export impairment, the subcellular distribution of receptor immunoreactivity was analyzed by confocal microscopy, using the MC-5 mAb. In the absence of permeabilization, strong staining was obtained for most cells of the mixed population expressing wtCCR5 but was markedly reduced for the 3CysA mutant and restricted to a small fraction of the cell population (Fig. 4A). When cells were permeabilized, strong intracellular staining could be seen in most cells of both wtCCR5 and the 3CysA mutant (Fig. 4B). The presence of strong perinuclear staining in 3CysA-expressing cells suggested that the mutant receptor is synthesized efficiently but sequestered in the endoplasmic reticulum and/or the Golgi complex where palmitoylation might take place (31).

**CCR5 Palmitoylation Increases Its Diffusion Rate in Intracellular Compartments**—The dynamics of fusion proteins between wtCCR5 or 3CysA and GFP were analyzed by confocal microscopy in clonal cell lines. wtCCR5-GFP was detected at the plasma membrane, although a fraction of the receptor was also seen intracellularly. The 3CysA-GFP mutant was mainly...
localized intracellularly (Fig. 4C). The ability of wtCCR5-GFP and the 3CysA-GFP mutant to diffuse within intracellular compartments was quantified using the FRAP technique (32), in which the fluorescent protein present in a small area is irreversibly photobleached by an intense laser flash. The fluorescence recovery through exchange of bleached and non-bleached protein was measured every 20 s using an attenuated laser beam (not shown). Quantitative FRAP analysis showed that fluorescence recovery was more rapid and more complete for wtCCR5-GFP, with a diffusion constant of $1.47 \times 10^{-9}$ cm$^2$/s and a mobile fraction of 100%, whereas the 3CysA-GFP mutant was characterized by a diffusion constant of $0.43 \times 10^{-9}$ cm$^2$/s with a mobile fraction of only 60% (Fig. 5), suggesting that palmitoylation of CCR5 increases its mobility in intracellular membrane compartments.

Role of CCR5 Palmitoylation in G Protein Coupling and Intracellular Signaling—The binding parameters ($B_{max}$ and $K_i$) of mixed cell populations expressing the various mutants were determined by a homologous competition binding assay, using $^{125}$I-MIP-1$\beta$ as tracer. A reduction in the number of binding sites (calculated $B_{max}$) was observed for all mutants, particularly when more than one cysteine was substituted, in agreement with the results of the FACs analysis. The affinity of the cysteine mutants for MIP-1$\beta$ did not change significantly, however, as compared with wtCCR5, with $K_i$ values ranging from 0.2 to 0.7 nM (Fig. 6A and Table I).

We next tested the ability of these non-clonal cell populations expressing the various cysteine mutants to trigger intracellular calcium release, using the aequorin assay. As shown in Fig. 6B, mutation of a single cysteine resulted in a moderate reduction of the efficacy of calcium signaling in response to MIP-1$\beta$ (50% of wtCCR5) but with a similar potency (Table I). The functional responses of the double and triple mutants were more severely affected, as no signal was obtained in response to the highest
CCR5 Palmitoylation and HIV Coreceptor Function—The coreceptor function of the 3CysA mutant for viruses pseudotyped with three R5 strains Envs (ADA, BaL, and JRFL) was reduced by about 50% as compared with wtCCR5 (data not shown). As for functional response to chemokines, HIV infection is also strongly influenced by the level of CCR5 expression (33). Therefore, to normalize for differences in receptor expression, we transfected cells with different amounts of the plasmid encoding wtCCR5 and then infected with GFP reporter viruses pseudotyped with the BaL Env. After 2 days post-infection, cells were stained with the PE-conjugated 2D7 mAb and assayed for both surface expression (PE fluorescence) and HIV infection (GFP fluorescence) by FACS analysis. As shown in Fig. 8A, dilution of the wtCCR5 plasmid resulted in a reduction of cell surface expression to a level similar to that obtained for the 3CysA mutant. For similar surface expression levels, there was no significant difference in the coreceptor function of wtCCR5 and the 3CysA mutant (Fig. 8B), suggesting that palmitoylation by itself was not important for HIV coreceptor function.

DISCUSSION

The mechanisms controlling intracellular trafficking of GPCRs play a fundamental role in the regulation of receptor functions. Trafficking of CCR5 to and from the plasma membrane has been shown to affect greatly its chemokine receptor and HIV coreceptor functions (7, 8, 16). The level of CCR5 at the cell surface has been correlated with HIV infectivity both in vitro and in vivo (33, 34), and ligand-mediated CCR5 internalization plays a prominent role in the inhibition of HIV infection by chemokines (17–19). Palmitoylation is a common post-translational modification of membrane and cytosolic proteins, consisting in the acylation of cysteine side chains by palmitic acid. A number of GPCRs have been shown to be palmitoylated on cysteines located within the intracellular carboxyl-terminal domain of the receptor. By anchoring this domain to the plasma membrane, this post-translational modification tends to create a fourth intracellular loop in these receptors (Fig. 1B). GPCRs belonging to different subfamilies are palmitoylated, and mutagenesis of the palmitoylated cysteines is associated with many, and sometimes opposite, effects on the functional properties of the receptors. These include modifications of receptor trafficking, signal transduction and desensitization processes, that might vary according to the receptor studied (23).

CCR5 exhibits a cluster of 3 cysteine residues in its carboxyl-terminal region, which we found represented the main site of CCR5 palmitoylation.

The integrity of the cysteine cluster was important for the transport of CCR5 to the cell surface. Since no significant difference was seen according to which cysteines were mutated in the single and double mutants, it is likely that all three cysteines can be palmitoylated. By using confocal microscopy, we found that the triple cysteine mutant was synthesized but sequestered largely in the ER or Golgi complex, suggesting that much of the protein was misfolded or retained by some other mechanism. Recently, it has been demonstrated that only a fraction of newly synthesized OP1 opioid receptor exits the ER and reach the cell surface (35), whereas the receptors retained in the ER are transported back to the cytosol, ubiquitinated, and degraded by the 26 S proteasome (36). The overall reduction of fluorescent material in cells stably expressing the 3CysA-GFP mutant, as compared with wtCCR5-GFP-expressing cells (data not shown), suggests that the retention of receptors in intracellular compartments probably leads to its early degradation as well. For several other GPCRs, including the thyrotropin, vasopressin V2, and adenosine A1 receptors (37–40), preventing receptor palmitoylation also leads to decreased receptor expression. The underlying mechanism was not dem-
The surface expression of wt and mutant CCR5 in CHO-K1 cells as determined by FACS analysis, using the 2D7 mAb, is represented as the MCF. The binding parameters (pEC<sub>50</sub> and calculated B<sub>max</sub>) were derived from competition binding assays using [125I]-MIP-1β as tracer. Functional parameters (pEC<sub>50</sub> and E<sub>max</sub>) were derived from dose-response curves in the aequorin assay. The data represent the mean ± S.E. calculated from three separate experiments. NM, not measurable.

| Expression, MCF (2D7) | Binding | Aequorin assay |
|-----------------------|---------|---------------|
|                       | pEC<sub>50</sub> | B<sub>max</sub> (sites/cell) | pEC<sub>50</sub> | E<sub>max</sub> (% of wt) |
| wtCCR5                | 497     | 9.21 ± 0.15   | 161,000 ± 80,000 | 7.71 ± 0.24 | 100 |
| C321A                 | 206     | 9.25 ± 0.11   | 108,000 ± 31,000 | 7.53 ± 0.26 | 51 ± 10.7 |
| C323A                 | 264     | 9.49 ± 0.22   | 53,500 ± 14,000  | 7.71 ± 0.22 | 43 ± 9.8  |
| C324A                 | 292     | 9.64 ± 0.42   | 94,000 ± 9,800   | 7.62 ± 0.38 | 53 ± 6.9  |
| C321A/C323A           | 43      | 9.57 ± 0.01   | 16,200 ± 1,100   | NM          | NM        |
| C321A/C324A           | 32      | 9.55 ± 0.01   | 11,600 ± 1,100   | NM          | NM        |
| C323A/C324A           | 31      | 9.63 ± 0.33   | 11,600 ± 7,600   | NM          | NM        |
| 3CysA                 | 24      | 9.70 ± 0.15   | 6,900 ± 3,300    | NM          | NM        |

The surface expression of wtCCR5 and the 3CysA mutant in CHO-K1 cell clones expressing similar level of wtCCR5 or the 3CysA mutant is demonstrated, however, except for the A1 receptor, for which enhanced degradation was indeed shown (40).

We used FRAP to investigate, in living cells, the diffusion of wtCCR5-GFP or the 3CysA-GFP mutant into intracellular compartments. The diffusion parameters for wtCCR5-GFP were similar to those derived previously for the α1-adrenergic and the gonadotropin-releasing hormone receptor (27, 41). The diffusion of the 3CysA-GFP mutant was severely affected, suggesting that the mutant is associated with slowly diffusing ER proteins and/or forms large aggregates (42). The fourth intracellular loop or the carboxyl-terminal tail of the receptor might interact with other proteins necessary for efficient CCR5 targeting to the plasma membrane, and the proper folding of these domains might require cysteine palmitoylation. For several GPCRs, such as olfactory receptors in Caenorhabditis elegans, opsins, and adrenomedullin receptor, specific chaperones or cargo receptors, necessary for their efficient transport to the cell surface, have been identified (43–48). Alternatively, unpalmitoylated receptors might leave accessible patches of hydrophobic residues, favoring interaction with chaperones or aggregation of the receptor, leading to its degradation (49).

Once CCR5 has reached the cell surface, whether it is palmitoylated or not, extracellular domains of the receptor appeared to be correctly folded, since the different cysteine mutants were recognized by mAbs binding conformational or linear epitopes with the same efficiency, and they bound MIP-1β with the same affinity. For some GPCRs, such as the β2-adrenergic or the endothelin ET<sub>A</sub> and ET<sub>B</sub> receptors, palmitoylation has been found to affect the signaling properties of the receptor (50–52). Low expression of the double and triple cysteine mutants reduced signaling efficiency in transfected cells. For similar expression levels, however, the 3CysA mutant promoted intracellular calcium release and inhibition of cAMP accumulation as efficiently as wtCCR5. In contrast, the kinetics of the cellular response measured by a microphysiometer was much shorter. Repeated stimulation of the mutant receptor did not uncover a more rapid desensitization of the cellular response. The different kinetics could result from differential coupling between palmitoylated and unpalmitoylated receptors. Indeed, the microphysiometer integrates the metabolic consequences of many different signaling cascades induced by receptor activation, and different signaling cascades are known to result in different profiles of metabolic activity (53). This modified kinetics, and the lower [35S]GTPγS binding increment to membranes expressing unpalmitoylated receptor, suggest that the 3CysA mutant does not couple as efficiently to some cascades activated by the wild-type receptor. Mutations in other GPCRs have been shown to affect selectively specific signal transduction pathways without affecting others (23). For example, the absence of palmitoylation in the endothelin A receptor prevents its coupling to Go<sub>i</sub> without affecting its ability to stimulate Go<sub>q</sub> (51). The carboxyl-terminal tail of CCR5 has been shown to be important for G protein coupling (54). A specific structural organization of the carboxyl-terminal domain of CCR5, dependent on its palmitoylation, might therefore be necessary for the efficient coupling to some G protein subtypes and signaling cascades. For example, it has been shown recently that the fourth intracellular loop of activated rhodopsin interacts directly with Go<sub>i</sub> and plays a role in the regulation of the βγ subunit binding (55, 56).

Many effectors of GPCR signaling are acylated as well. The α-subunits of G proteins are myristoylated and/or palmitoylated and the γ-subunits are prenylated (57, 58), and these posttranslational modifications control the association of functional G protein complexes to the plasma membrane (59, 60). Palmitoylation is required for the localization of some signaling proteins in detergent-resistant subdomains of the membrane (61–64). The presence of CCR5 in these raft microdomains has been described and was proposed to play a role in the organization of front-rear polarity in migrating cells (65). It is thus possible that palmitoylation targets CCR5 to particular membrane microdomains, favoring its interaction with specific sig-
naling proteins, enriched in these membrane subdomains. Further studies will be required to elucidate the role played by rafts in CCR5 (and other GPCRs) signaling.

Chemokine-induced activation of CCR5 promotes the recruitment of G protein-coupled receptor kinases to the plasma membrane, and G protein-coupled receptor kinases mediate the phosphorylation of serine residues in the carboxyl-terminal tail of the receptor, which is followed by receptor endocytosis (20). This mechanism was shown to contribute greatly to the inhibitory effect of chemokines on HIV infection (18). We have shown here that palmitoylation of CCR5 does not seem to affect receptor internalization (Fig. 8). The role of palmitoylation on GPCR endocytosis varies according to the specific receptor studied. Preventing receptor palmitoylation has been reported to increase (50, 66, 67), decrease (38, 39, 68–70), or not affect (40, 51, 52) internalization of specific GPCRs.

Finally, we have investigated the influence of palmitoylation onto the HIV coreceptor function of CCR5. We found that viral entry was reduced by 50% when the triple cysteine mutant was used as coreceptor. CD4 is also palmitoylated (71), and a constitutive association between CCR5 and CD4 has been demonstrated (72). It has been proposed that membrane raft microdo-

Fig. 6. Binding and functional properties of the cysteine mutants. A, competition binding assays were performed on pools of CHO-K1 cells expressing wtCCR5 or the mutants C321A, C321A/C323A and 3CysA, using 0.08 or 0.12 nmol/liter $^{125}$I-MIP-1$\beta$ as tracer. Results were analyzed by the Graphpad Prism software, using a single-site model, and the data were normalized for nonspecific (0%) and specific binding in the absence of competitor (100%). All points were run in triplicate (error bars, S.E.). Data are representative of three independent experiments. B, intracellular calcium release following addition of MIP-1$\beta$ was recorded in mixed cell populations coexpressing apoaequorin and the various cysteine mutants. The luminescent signal was recorded for 30 s in a luminometer. Results were analyzed by nonlinear regression. All points were run in triplicate (error bars, S.E.). The displayed curves represent a typical experiment out of three performed independently. C, aequorin-based calcium-mobilization assays in response to MIP-1$\beta$ were performed on clones expressing similar levels of wtCCR5 (CCR5-c3) or the 3CysA mutant (3CysA-c1), with or without preincubation with PTX (100 ng/ml) for 18 h. All points were run in triplicate (error bars, S.E.). Results were analyzed by non-linear regression, and the data were normalized for basal luminescence (0%) and maximal luminescence obtained by activation of endogenous P$_{2}$ receptors by 10 $\mu$M ATP (100%). The displayed curves represent a typical experiment out of three performed independently. D, inhibition of forskolin-stimulated cAMP accumulation. CCR5-c3 or 3CysA-c1 clones were incubated with 10 $\mu$M forskolin and/or MIP-1$\beta$ for 15 min, and cAMP was measured by radioimmunoassay. The results were analyzed by nonlinear regression and normalized for basal (0%) and maximal cAMP levels (100%, in the absence of forskolin) and maximal cAMP levels (100%, in the absence of chemokines). The experiments were performed in duplicate (error bars, S.E.), and the displayed curves represent a typical experiment out of three performed independently. E, the metabolic activity of the clones CCR5-c3 (filled squares) and the 3CysA-c1 (open squares) was monitored using a microphysiometer, and the functional response to MIP-1$\beta$ (100 nM) was recorded. The results were normalized relative to the basal acidification rates (100%) before addition of the chemokine. Initiation of the stimulation period (2 min) by MIP-1$\beta$ is indicated by the arrow. The displayed curves represent a typical experiment out of four performed independently. F, effect of RANTES on [35S]GTP$\gamma$S binding to membranes from clones CCR5-c3 and 3CysA-c1. Results were analyzed by non-linear regression. The experiments were performed in triplicate (error bars, S.E.), and the displayed curves represent a typical experiment out of two performed independently.
mains play an important role in HIV-1 infection (73), suggesting that absence of palmitoylation might affect coreceptor function (74, 75). However, at comparable levels of binding sites, the coreceptor activity was similar between wtCCR5 and the 3CysA mutant, demonstrating that, when the CD4 concentration is not limiting, palmitoylation of CCR5 is not by itself important for HIV entry.

In conclusion, palmitoylation represents an important post-translational modification influencing greatly the global function of CCR5. The relative conservation of cysteines in the carboxyl-terminal domain of about 80% of GPCRs suggests that palmitoylation likely plays similar structural roles in many receptors. Anchorage of the carboxyl-terminal domain of the receptor to the plasma membrane may secure the correct presentation of the receptor tail. Although the recently described crystal structure of bovine rhodopsin does not include lipids, the position of the side chains of Cys-322 and Cys-323 are consistent with this model (76). As not all GPCRs display cysteines in their carboxyl-terminal domain, palmitoylation is obviously dispensable in some receptors. In this study, we found that for CCR5, disruption of palmitoylation results in a strong reduction of its surface expression in CHO-K1 cells, by alteration of its intracellular trafficking, but also of its ability to interact with so far uncharacterized signaling and/or regulatory proteins. Whether these functions can be generalized to leukocytes in which CCR5 is naturally expressed and to the other members of the chemokine receptor subfamily that are potentially palmitoylated remains to be determined.

Acknowledgments—We thank M. J. Simons, M. E. Decobecq, K. Gillard, and H. Nguyen Tran for expert technical assistance. We thank A. Proudfoot for kindly providing AOP-RANTES, M. Mack for mAbs, D. Communi for running mass spectrometry, and the AIDS Research and Reference Reagent Program for sCD4.

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