Mutating the Four Extracellular Cysteines in the Chemokine Receptor CCR6 Reveals Their Differing Roles in Receptor Trafficking, Ligand Binding, and Signaling†

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ABSTRACT: CCR6 is the receptor for the chemokine MIP-3α/CCL20. Almost all chemokine receptors contain cysteine residues in the N-terminal domain and in the first, second, and third extracellular loops. In this report, we have studied the importance of all cysteine residues in the CCR6 sequence using site-directed mutagenesis and biochemical techniques. Like all G protein-coupled receptors, mutating disulfide bond-forming cysteines in the first (Cys118) and second (Cys197) extracellular loops in CCR6 led to complete elimination of receptor activity, which for CCR6 was also associated with the accumulation of the receptor intracellularly. Although two additional cysteines in the N-terminal region and the third extracellular loop, which are present in almost all chemokine receptors, are presumed to form a disulfide bond, this has not been demonstrated experimentally for any of these receptors. We found that mutating the cysteines in the N-terminal domain (Cys36) and the third extracellular loop (Cys288) neither significantly affected receptor surface expression nor completely abolished receptor function. Importantly, contrary to several previous reports, we demonstrated directly that instead of forming a disulfide bond, the N-terminal cysteine (Cys36) and the third extracellular loop cysteine (Cys288) contain free SH groups. The cysteine residues (Cys36 and Cys288), rather than forming a disulfide bond, may be important per se. We propose that CCR6 forms only a disulfide bond between the first (Cys118) and second (Cys197) extracellular loops, which confines a helical bundle together with the N-terminus adjacent to the third extracellular loop, creating the structural organization critical for ligand binding and therefore for receptor signaling.

The chemokines are a family of small, secreted, and inducible proteins that regulate leukocyte trafficking and are therefore important in lymphoid organogenesis, inflammation, and host defense (*1*). On the basis of the invariant cysteines near the N-terminus of chemokines, chemokines can be grouped into CXC, CC, CX3C, and C subfamilies (*2*). Functionally, chemokines can be divided into those that serve homeostatic and those that serve inflammatory functions (*3*). The majority of chemokines are proinflammatory and are important for recruitment of leukocytes to peripheral sites.

The effects of chemokines on target cells are mediated by a family of seven transmembrane domain G protein-coupled receptors (GPCRs)[1] (*4*−*6*). To date, 19 chemokine receptors have been identified, including 11 CC chemokine receptors (CCR1−11), six CXC chemokine receptors (CXCR1−6), and one each for CX3C and C chemokine receptors (*5*). The only chemokine ligand for CCR6 is MIP-3α/LARC/Exodus, renamed CCL20 according to the latest nomenclature (*2*). A recent report demonstrated that defensins are also active as ligands for CCR6 (*7*). The expression of CCR6 is restricted to lymphoid tissues, with the receptor found on memory T cells, B cells, and immature dendritic cells (*8*−*12*). Much biological data have suggested that CCR6 and MIP-3α/CCL20 may play an important role in inflammatory responses in skin and gut (*10, 13, 14*).

Despite the interest in the biology of CCR6, relationships between CCR6 structure and function have not yet been addressed. Studying the structure and function of CCR6 may provide information that is helpful in the development of receptor antagonists for treating inflammatory diseases. We have begun such studies of CCR6 using site-directed mutagenesis and domain swapping.

Extracellular cysteines in G protein-coupled receptors are thought to be important for forming disulfide linkages in maintaining the structure needed for ligand binding and receptor signaling (*15*). Two cysteine residues, in the first and second extracellular loops, are conserved in all GPCRs and are known to be linked by a disulfide bond. For all chemokine receptors except CXCR6, there are two additional cysteines, located in the N-terminal region and the third extracellular loop. To address the importance of these extracellular cysteines in CCR6, we substituted serine as well

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[1] Abbreviations: CCR, CC chemokine receptor; CCL, CC chemokine ligand; CXCR, CXC chemokine receptor; CXCL, CXC chemokine ligand; GPCR, G protein-coupled receptor; MIP-3α, macrophage inflammatory protein-3α; LARC, liver and activation-regulated chemokine; 5-IAF, 5-iodoacetamidofluorescein; FBS, fetal bovine serum; BSA, bovine serum albumin; DTT, dithiothreitol; HA epitope, Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala; HBSS, Hank's balanced salt solution; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

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as other amino acid residues for the four extracellular cysteines, singly or in combination, and examined the mutant receptors' expression, ligand binding, and functional activities. Our results indicate that the four extracellular cysteines have differing roles in receptor trafficking, ligand binding, and signaling.

MATERIALS AND METHODS

Site-Directed Mutagenesis of CCR6. Mutagenesis of specific amino acid residues was conducted using the pALTER site-directed mutagenesis system (Promega, Madison, WI). A plasmid containing the coding region of the human CCR6 gene in the pBK-CMV vector (Stratagene, La Jolla, CA) was used as the template together with the primer set (forward, 5′-GCGGTGGCGGCCGCGTGAGTAGGTAATTCTACAACCAGCTTGCATT-3′; reverse, 5′-GCGGTGGCGGCCGCGATGCCTTAGGGAGACTCAGC-3′) to generate a CCR6 fragment by PCR. This PCR fragment was cloned into pALTER-MAX and used as a template for performing site-directed mutagenesis as described by the manufacturer. The specific oligomer designed for each mutation contained ~10−12 matched nucleotides flanking the mismatched nucleotide. The resulting receptor mutants in pALTER-MAX were screened, and the entire region of the plasmid encoding each CCR6 mutant was sequenced by automated DNA sequencing to verify that no undesired mutations had been introduced during the mutagenesis process.

Construction of Epitope-Tagged CCR6. The primer containing the sequence encoding the HA epitope (5′-CCGCTCGAGCTAGCTAACTGAGCCACCATGTACCCATACGACGTCCCAGACTACGCTATGAGCGGGGAATCAATGAAT-3′) was used as the forward primer, and 5′-GCGGTGGCGGCCGCGATGCCTTAGGGAGACTCAGC-3′ was used as the reverse primer. This primer set was used for amplification of N-terminal HA-tagged wild-type and mutant receptors. The PCR products were purified and inserted into either pCIneo or pALTER-MAX at the *Xho*I and *Not*I sites, and the sequences were confirmed.

Cell Culture and Transfection. Jurkat-TAg cells, which had been transfected with the large T-antigen gene of SV40, were kindly provided by L. Samelson (National Institutes of Health, Bethesda, MD). Jurkat-TAg cells were grown in RPMI 1640 supplemented with 10% FBS and 2 mM glutamine. For all transfections of Jurkat-TAg cells, 400 μL of the cell suspension at a density of 2.5×10^7 cells/mL in RPMI 1640 medium containing 10 mM Hepes and 4 mM glutamine was transferred to a cuvette with a 0.4 cm gap, and electroporation was conducted in a BTX Pulse Generator, ECM 630 (Genetronics, Inc., San Diego, CA), at room temperature using 260 V, 1050 μF, and 725 Ω. The transfectants were cultured for 36−40 h before being harvested for the analysis of receptor expression, calcium flux, and chemotaxis.

COS-1 Cell Tansfection and Cell Surface and Intracellular Staining. COS-1 cells were grown in Dulbecco's modified Eagle's medium containing 10% FBS. COS-1 cells were transfected with wild-type or various mutant receptor-encoding DNAs using a DEAE-dextran protocol as described previously (*16*). Thirty-six to forty-eight hours after transfection, cells were washed and stained with antibody to CCR6 (BD PharMingen, San Diego, CA) followed by goat anti-mouse IgG conjugated with FITC. The cells were then fixed with a 4% paraformaldehyde/FBS mixture (1/1, v/v), washed, and permeabilized with 0.5% Triton X-100 in PBS. Intracellular staining were performed by blocking cells with 5% BSA in PBS, followed by staining cells with phycoerythrin (PE)-conjugated antibody to CCR6. The stained cells were examined using a laser scanning confocal microscope (MRC 1000; Bio-Rad Laboratories).

Flow Cytometry. Transfected cells were suspended at a density of 10^6 cells/mL in HBSS buffer containing 10 mM Hepes, 1% FBS, and 0.1% NaN_3, and were incubated with PE-conjugated monoclonal antibody to CCR6 (clone 11A9 from PharmMingen; clone 56811 from R&D System, Inc., Minneapolis, MN). After being washed, cells were analyzed for immunofluorescence using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA). In some experiments, cells were stained with anti-HA (HA.11, COVANCE, Richmond, CA) followed by PE-conjugated anti-mouse IgG, washed, and analyzed for immunofluorescence.

Measurements of Calcium Flux. For calcium measurements, transfected cells were resuspended in HBSS containing Ca/Mg, 10 mM Hepes (pH 7), and 1% FBS and were loaded with 2 μM Fura-2 AM (Molecular Probes Inc., Eugene, OR) for 45 min at 30 °C with occasional shaking. The loaded cells were stimulated, and the calcium flux was measured using a ratio fluorescence spectrometer (Photon Technology International, South Brunswick, NJ). Excitation was alternately at 340 and 380 nm with emission measured at 510 nm. The ratios of the signals obtained at the two excitation wavelengths were plotted as a function of time.

Assaying Chemotaxis. In vitro chemotaxis assays were performed as described previously (*17*), with modifications, using 6.5 mm Transwell tissue culture inserts with 5 μm pores (Corning Inc., Corning, NY). Transfected cells were suspended at a density of 10^7 cells/mL in RPMI 1640 with 10 mM Hepes and 0.5% BSA and prewarmed at 37 °C for 30 min in a CO_2 incubator. One hundred microliters of cell suspension was added to an insert above a well containing 600 μL of medium with or without MIP-3α/CCL20 (PeroTech Inc., Rocky Hill, NJ) and incubated for 90 min at 37 °C in a CO_2 incubator. After incubation, migrated cells in the wells were collected, pelleted, and counted. Duplicate wells were used for each condition.

Receptor Binding Assay. The binding of MIP-3α/CCL20 to transfected cells was analyzed with a competition binding assay. Transfected cells were incubated with 0.1 nM ^{125}I-labeled MIP-3α/CCL20 (2200 Ci/mmol, New England Nuclear Life Science Products, Boston, MA) and varying concentrations of unlabeled MIP-3α/CCL20 in a total volume of 100 μL of binding buffer (RPMI containing 1% BSA, 25 mM Hepes, and 0.02% NaN_3) for 1 h at room temperature with rotation. The cells were then overlaid on 10% sucrose in PBS and pelleted by centrifugation. The supernatants were aspirated; the bottoms of the microfuge tubes were cut, and the amount of cell-bound radioactivity was measured using a gamma counter. Each sample was done in duplicate.

Metabolic [^{35}S]Methionine/Cysteine Labeling and Immunoprecipitations. Jurkat-TAg cells transfected with HA-tagged wild-type or mutant receptors were washed once and incubated in methionine- and cysteine-free RPMI medium supplemented with 10% dialyzed FBS for 1 h in a CO_2

incubator. For metabolically labeling cells, 1 mCi of [^{35}S]-methionine/cysteine [Express (1175 Ci/mmol); New England Nuclear Life Science Products] was used per 10^8 cells in 1 mL of methionine- and cysteine-free RPMI medium with 10% dialyzed FBS for 20 min in a CO_2 incubator. After being labeled, cells were washed with PBS and lysed in lysis buffer [150 mM Tris-HCl (pH 7.5), 300 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 1 mM PMSF, 10 μg/mL leupeptin, and 10 μg/mL aprotinin]. After nuclei and cell debris had been pelleted by centrifugation, the lysates were subjected to immunoprecipitation with anti-HA antibody, HA.11 (COVANCE), followed by incubation with protein G−agarose (Pierce, Rockford, IL). The immunoprecipitates were washed with lysis buffer three times followed by heating in nonreducing sample buffer [200 mM Tris-HCl (pH 6.8), 3% SDS, 10% glycerol, 1 mM EDTA, and 0.05% bromophenol blue]. For reducing samples, 50 mM DTT was added to nonreducing sample buffer before heating. After being heated, samples were transferred into fresh tubes; 100 mM *N*-ethylmaleimide (Merck, Darmstadt, Germany) was added to each tube, and the proteins were resolved on 8% SDS−polyacrylamide gels. After electrophoresis, gels were dried and exposed to Kodak BioMax MR film.

For reacting the free SH group of extracellular cysteines of HA-tagged wild-type or mutant receptors (C36/288S, C118/197S, and C36/118/197/288S) with 5-iodoacetamidofluorescein (5-IAF) (Pierce), the cells were metabolically labeled with [^{35}S]methionine (1175 Ci/mmol), washed, resuspended, and incubated in RPMI and 10 mM Hepes containing 1 mM 5-IAF at room temperature for 2 h with rotation in the dark. The cells were washed with PBS four times, lysed, and immunoprecipitated as described above. The immunoprecipitates were washed, heated in reducing sample buffer, and resolved on 8% SDS−polyacrylamide gels. In some experiments, metabolically labeled cells were lysed, and the receptors were immunoprecipitated. The immunoprecipitates were then resuspended in RPMI and 10 mM Hepes containing 1 mM 5-IAF for reaction as described above. After reaction, the immunoprecipitates were washed, heated in reducing sample buffer, and resolved on 10% SDS−polyacrylamide gels.

RESULTS

Extracelluar Cysteines 118 and 197, but Not Cysteines 36 and 288, Are Critical for Receptor Expression. The four extracellular cysteines in CCR6 are located in the N-terminal domain (Cys36) and in the first (Cys118), second (Cys197), and third (Cys288) extracellular loops. To study the effects of these extracellular cysteines on receptor expression and function, we mutated them to serines (S), singly and in combination, and transfected Jurkat-TAg cells with these mutants. The expression of surface receptors was evaluated by staining transfectants with an antibody to the N-terminal peptide of human CCR6 (clone 11A9). C118S and C197S mutant receptors as well as the double-mutant receptor C118/197S exhibited dramatically diminished levels of surface expression (Figure 1). In contrast to the C118S and C197S mutant receptors, the surface expression of C36S, C288S, and C36/288S mutant receptors was comparable to that of the wild type. Mutation of all four extracellular cysteines (C36/118/197/288S) reduced the level of surface expression, making it similar to those of the C118S, C197S, and C118/197S mutant receptors (Figure 1). Mutant receptors where Cys36 and Cys288 were replaced with amino acid residues other than serine (C36A, C36G, C36D, C288G, C288A, and C288R) exhibited levels of expression similar to those seen for the C36S, C288S, and C36/288S mutants (Table 1). To demonstrate that the antibody used is not conformationally sensitive, we treated CCR6 transfectants with DTT to reduce the disulfide bonds, and examined the surface receptor with various antibodies, including two different clones of CCR6 antibodies (clone 11A9 and clone 56811), and an antibody to the HA epitope tag, which was placed at the N-terminus

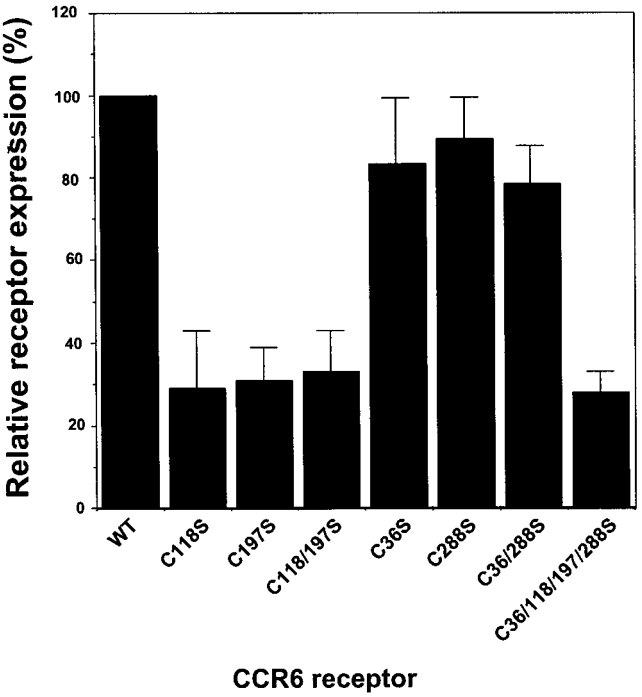

FIGURE 1: Mutation of first (C118) and second (C197) extracellular loop cysteines, but not the N-terminal (C36) and third (C288) extracellular loop cysteines, significantly reduces the level of receptor surface expression. Jurkat-TAg cells were transfected with the wild type or various cysteine mutants. Approximately 40 h after transfection, the cells were stained with anti-CCR6-PE and the level of expression of surface receptors was measured by flow cytometry. Multiple experiments were done, and the error bars (SE) are indicated.

Table 1: Surface Expression and Activities of Various Cys36 and Cys288 Mutant Receptors[a]

mutant receptor	surface expression (% of wild type)	calcium flux (% of wild type)	chemotaxis (% of wild type)
C36S	83.2 ± 18.4	5.3 ± 3.6	<1
C36A	75.1 ± 16.2	18.6 ± 3.6	<1
C36G	78.1 ± 15.6	0	<1
C36D	90.0 ± 8.8	0	<1
C288S	86.9 ± 16.2	31.6 ± 19.1	<1
C288A	78.6 ± 13.1	17.6 ± 9.4	<1
C288G	78.1 ± 22.9	15.4 ± 12.8	<1
C288R	90.6 ± 7.2	25.5 ± 5.6	<1

[a] DNAs encoding CCR6 mutants were transfected into Jurkat-TAg cells, and the cells were analyzed for surface receptor expression, calcium flux, and chemotaxis. Surface receptor expression was assessed by flow cytometry, calcium flux by fluorometry in response to 100 ng/mL MIP-3α/CCL20, and chemotaxis by assessing migration from chambers without chemokine to chambers containing 50 ng/mL MIP-3α/CCL20. Values using cells transfected with wild-type CCR6 were taken to be 100%. Means ± SE were calculated from four to six separate experiments.

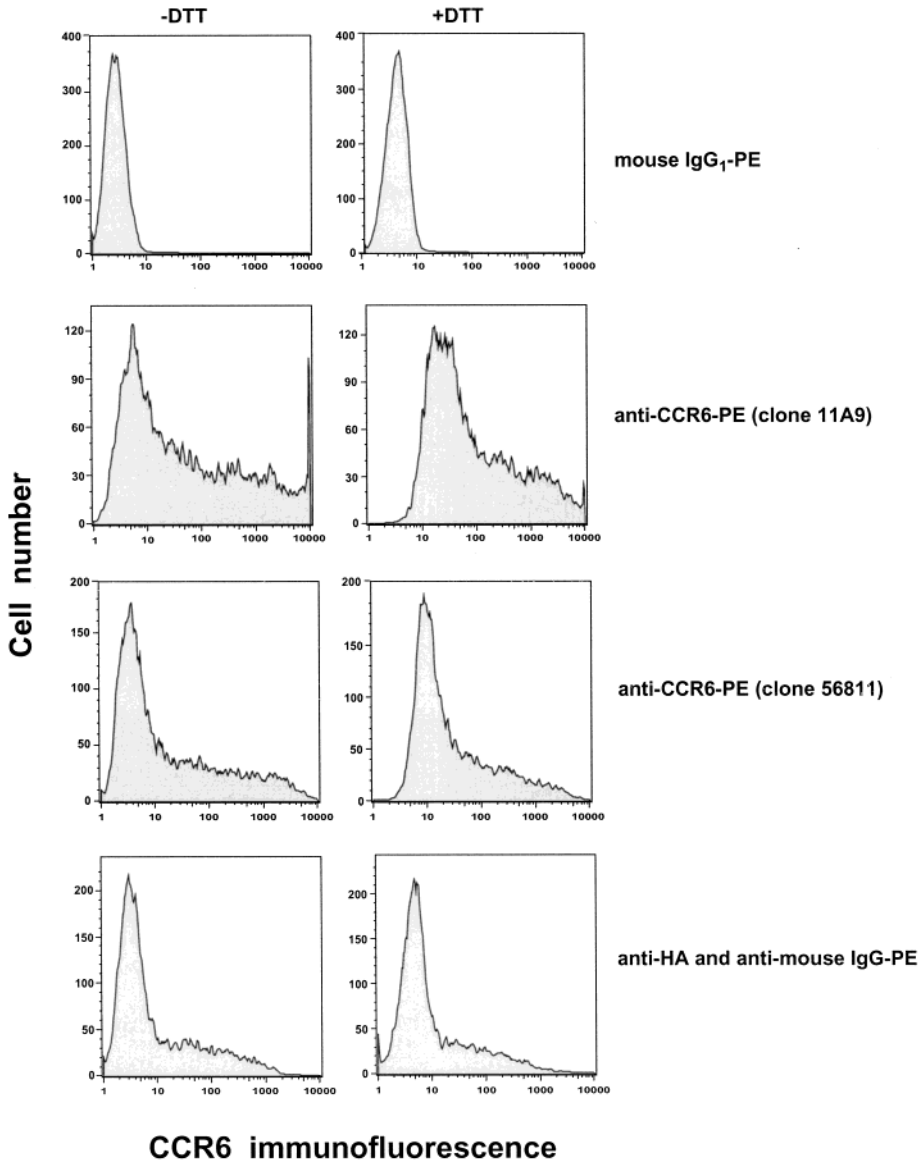

FIGURE 2: Antibodies can recognize CCR6 in DTT-treated transfectants. CCR6-transfected Jukat-TAg cells were incubated for 30 min at room temperature in the presence or absence of 100 mM DTT and then stained with various anti-CCR6 antibodies conjugated with PE (clone 11A9 from PharMingen and clone 56811 from R&D) or anti-HA antibody (clone HA.11) followed by anti-mouse IgG-PE. The cells were washed and analyzed by flow cytometry.

of CCR6. As shown in Figure 2, all the antibodies that weretested recognized both nonreduced and reduced forms of CCR6, although the antibody (clone 11A9) that we used routinely exhibited somewhat better staining. Additionally, we stained wild-type and mutant receptors with these antibodies, and all three antibodies gave similar staining profiles (data not shown). These results demonstrated that a diminished level of staining for CCR6 in C118S, C197S, C118/197S, and C36/118/197/288S was due to a reduced level of surface expression and not to conformational changes in the receptors that affected antibody binding.

Although the level of surface expression of the C118/197S and C36/118/197/288S mutant receptors in Jurkat-TAg transfectants was significantly reduced, levels of receptor expression in wild-type and cysteine mutants were comparable when detected by Western blot analysis (data not shown), indicating that the mutant receptors were being synthesized, but could not be properly expressed on the cell surface. To demonstrate the fact that receptors accumulated intracellularly, we performed surface and intracellular receptor staining simultaneously in transfected COS-1 cells. We chose COS-1 cells because their intracellular staining was clearer and more easily demonstrated than that of Jurkat-TAg cells. As shown in Figure 3, COS-1 cells transfected with C36S, C288S, and C36/288S mutant receptors showed levels of surface expression comparable to that of cells expressing the wild-type receptor. Although we occasionally observed intracellular staining in cells transfected with the wild type, C36S, C288S, and C36/288S, we always observed significant surface receptor expression in these cells. Differences in intracellular accumulation of these receptors probably reflect the difference in DNA taken up by cells. On the contrary, we consistently observed that C118S, C197S, and C118/197S as well as C36/118/197/288S mutant receptors exhibited dramatically reduced levels of surface receptor expression when compared with that of the wild type, and an increased level of accumulation of receptors intracellularly (Figure 3). We hypothesized that the reduced

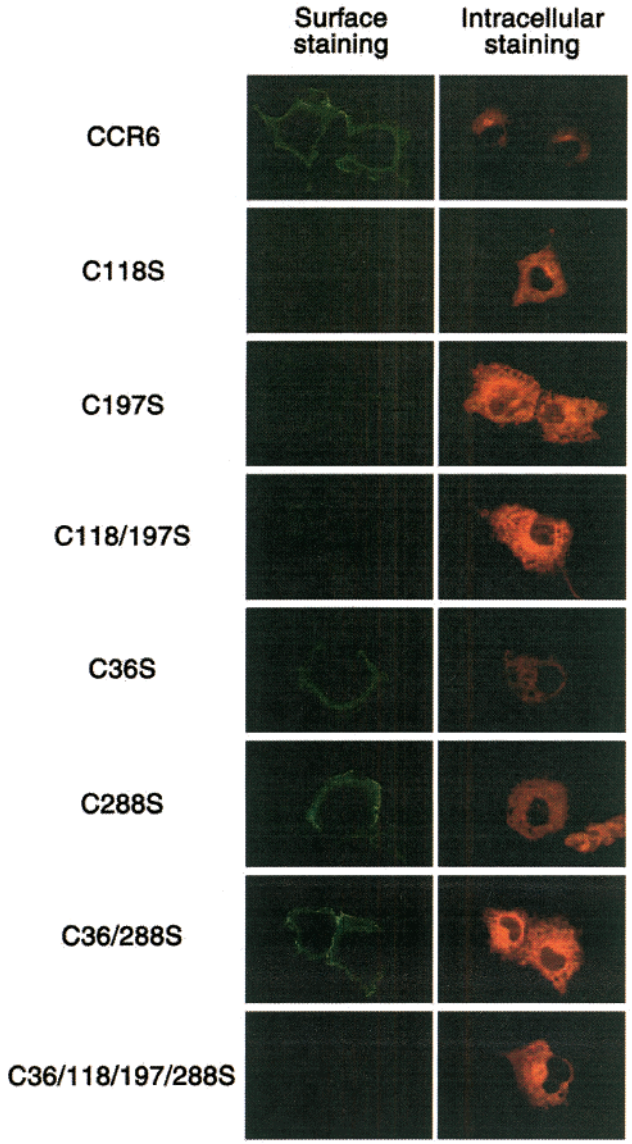

FIGURE 3: Mutation of first (C118) and second (C197) extracelluar loop cysteines singly or in combination dramatically reduces the level of surface receptor expression, but increases the level of receptor accumulation intracellularly. COS-1 cells transfected with the wild type or various cysteine mutants were first stained for surface receptor with anti-CCR6 followed by anti-mouse IgG-FITC. Cells were washed, fixed, permeablized, stained with anti-CCR6-PE, and washed again. Cells were mounted and visualized using confocal microscopy.

level of surface expression of cysteine mutant receptors (C118S, C197S, C118/197S, and C36/118/197/288S) existed because the loss of a disulfide bond resulted in the proteins' improper folding, leading to an impairment of receptor trafficking.

Mutation of Extracellular Cysteines of CCR6 Affects Calcium Flux and Chemotaxis Induced by MIP-3α/CCL20. Not surprisingly given the data presented above, mutation of extracellular cysteines (C118S and C197S) individually or in combination totally abolished the calcium flux induced by MIP-3α/CCL20 in Jurkat-TAg transfectants. However, mutating cysteines (C36S and C288S) singly or together reduced but did not eliminate the calcium flux induced by MIP-3α/CCL20. Calcium flux was less affected in the C288S mutant receptor than in the C36S and C36/288S receptors (Figure 4A). When Cys36 and Cys288 were mutated to

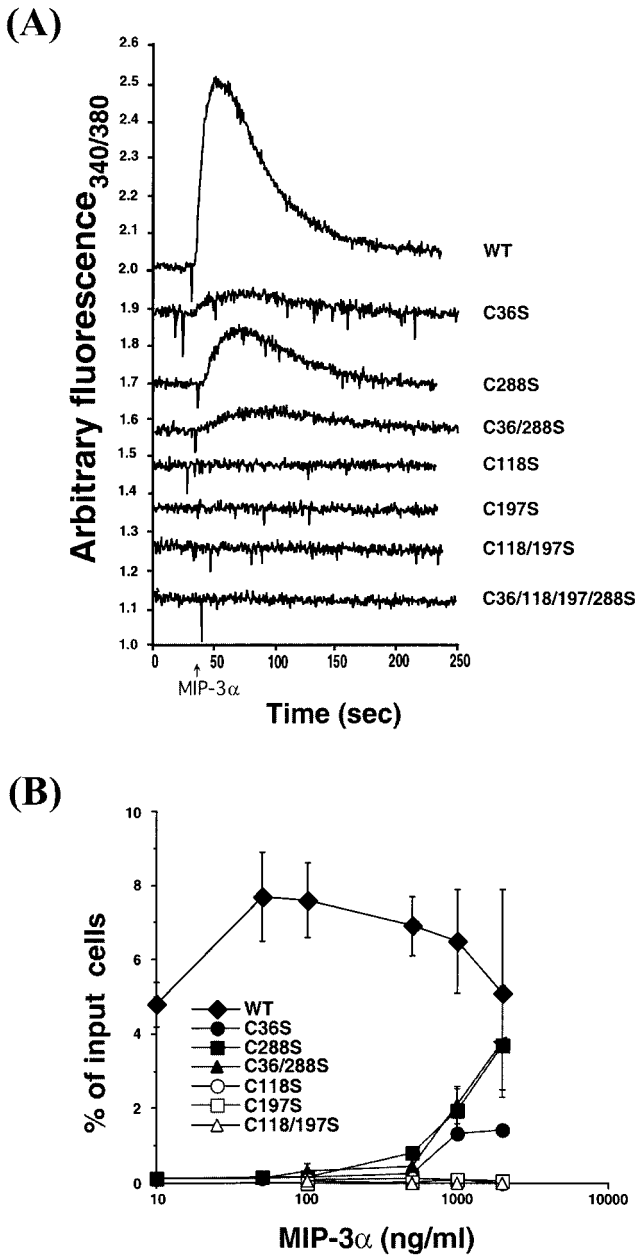

FIGURE 4: Mutation of extracellular cysteines in the first (C118) and second (C197) extracellular loops abolishes both calcium flux and chemotaxis, while mutation of extracellular cysteines in the N-terminus (C36) and the third loop (C288) reduces calcium flux and chemotaxis in response to MIP-3α/CCL20 stimulation. (A) Calcium flux. Jurkat-TAg cells transfected with the wild type or various cysteine mutants were loaded with Fura-2 AM and stimulated with 100 ng/mL MIP-3α/CCL20. Calcium flux was recorded by the fluorescence ratio. MIP-3α/CCL20 was added at the times indicated by the arrow. (B) Chemotaxis. Jurkat-TAg cells that had been transfected with the wild type or various cysteine mutants were added to inserts in wells containing medium with or without MIP-3α/CCL20 at various concentrations, and were incubated for 90 min at 37 °C in a CO_2 incubator. After incubation, migrated cells in the wells were collected, centrifuged, and counted. The results are expressed as the percentage of input cells, corrected for percentages of cells migrating in the absence of chemokines. Each point represents the average of three experiments, and the error bars (standard error of the mean) are indicated.

amino acid residues other than serine, some of the resulting mutant proteins were able to respond to MIP-3α/CCL20 at the concentration that was optimal for the wild-type receptor, and to produce a calcium flux (C36A, C288G, C288A, and

C288R), but some failed to do so (C36G and C36D) (Table 1). Interestingly, when assessed at the concentration of MIP-3α/CCL20 that was optimal for chemotaxis mediated by the wild-type receptor, the mutant proteins could not support chemotaxis (Table 1).

Because our data suggested that the C36S, C288S, and C36/288S mutant receptors were not completely dysfunctional, we tried to quantify the functional effects of the mutations by analyzing calcium flux and chemotaxis activities at various concentrations of ligand up to 1 μg/mL for calcium flux and 2 μg/mL for chemotaxis. In the calcium flux assay, the half-maximally effective concentration (EC_{50}) of MIP-3α/CCL20 was ~1 ng/mL with cells expressing the wild type and 500, 50, and 500 ng/mL for those expressing C36S, C288S, and C36/288S receptors, respectively (data not shown). The EC_{50} values for C118S, C197S, and C118/197S mutant receptors could not be calculated since they failed to respond to MIP-3α/CCL20 at concentrations up to 2 μg/mL. For chemotaxis, the wild-type receptor exhibited a typical bell-shaped curve in response to chemokines, and the optimal concentration of MIP-3α/CCL20 was ~50 ng/mL. However, even at 2 μg/mL, the C36S, C288S, and C36/288S mutant receptors only exhibited activity comparable to what was seen for the wild-type receptor at a concentration of 10 ng/mL (Figure 4B). The optimal concentration of MIP-3α/CCL20 for these mutants was not determined since concentrations higher than 2 μg/mL were not tested. As expected, C118S, C197S, and C118/197S mutant receptors failed to respond in the chemotaxis assay.

Mutation of Extracellular Cysteines Impairs MIP-3α/CCL20 Binding to Receptors. CCR6 receptor mutants were also tested for their ability to bind MIP-3α/CCL20. Using displacement of a constant concentration of [^{125}I]MIP-3α/CCL20 (0.1 nM) with various concentrations of unlabeled MIP-3α/CCL20, we were able to detect MIP-3α/CCL20 binding to wild-type transfectants with an equilibrium inhibitory constant (K_i value) of ~6 nM. Mutations in each of the four extracellular cysteines led to severely impaired ligand binding (Figure 5). Although the level of specific binding of MIP-3α/CCL20 to C36S and C288S mutant receptors was too low to be measured by this displacement binding assay, these mutations had not resulted in the complete loss of ligand binding, since stimulation of those receptors resulted in signaling as described (Figure 4A,B).

Extracellular Cysteines 118 and 197, but Not Cysteines 36 and 288, Form a Disulfide Bond. As described above, our data demonstrated that mutation of extracellular cysteines 118 and 197 completely abolished both receptor expression and function, consistent with the fact that a disulfide bond formed between the cysteines in the first and second extracellular loops of GPCRs is critical for receptor expression and biological activities. However, mutation of extracellular cysteines 36 and 288 neither affected receptor expression nor completely abolished receptor activity. Because elimination of a disulfide bond would be expected to abolish receptor activity, these findings suggested to us that Cys36 and Cys288 might not form a disulfide bond. We have taken two biochemical approaches to address this question. The first approach depended on the higher mobility of polypeptides on SDS−PAGE when disulfide bonds are intact due to the polypeptides' compact structure before as compared with after reduction. Therefore, we examined the

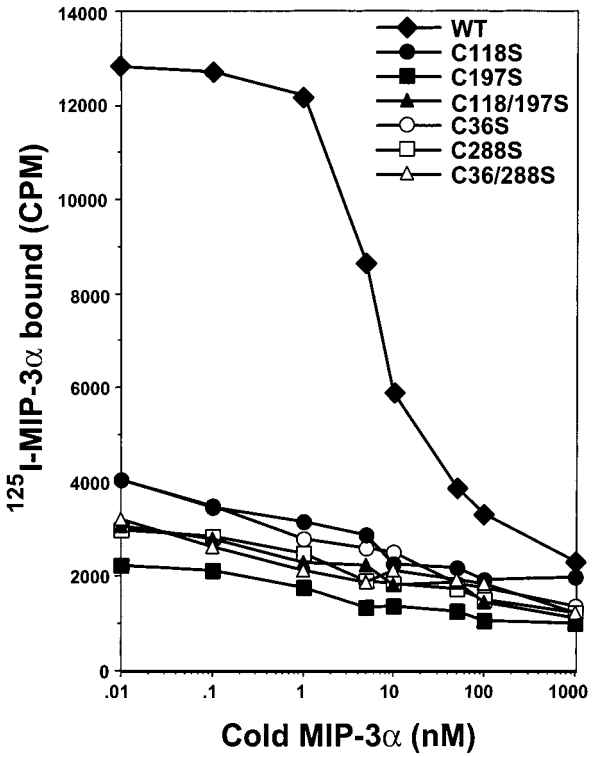

FIGURE 5: Mutation of extracellular cysteines impairs MIP-3α/CCL20 binding. Jurkat-TAg cells (2.5×10^7) transfected with the wild type or various cysteine mutants were incubated with 0.1 nM [^{125}I]MIP-3α/CCL20 together with various concentrations of unlabeled MIP-3α/CCL20 for 1 h at room temperature. The cells were then overlaid on 10% sucrose in PBS, pelleted by centrifugation, and counted in a gamma counter.

mobility of HA-tagged wild-type and mutant receptors (C36/288S, C118/197S, and C36/118/197/288S) on a SDS−polyacryalmide gel under reducing and nonreducing conditions. As shown in Figure 6, the wild type and C36/288S mutant showed higher mobility under nonreducing conditions (−DTT) than under reducing conditions (+DTT) (lanes 2 and 4 vs lanes 1 and 3), suggesting that a disulfide bond is present in both wild-type and C36/288S mutant receptors, presumably the disulfide bond between Cys118 and Cys197. In contrast, the C118/197S and C36/118/197/288S mutant receptors showed no difference in mobility under reducing and nonreducing conditions (lanes 5−8), suggesting that there is no disulfide bond in these two mutants.

If there were a disulfide bond between Cys36 and Cys288 in CCR6, in addition to the disulfide linkage between Cys118 and Cys197, we would expect that wild-type CCR6 should run ahead of C36/288S under nonreducing conditions. However, when the wild type is compared with C36/288S under nonreducing conditions, the wild type and C36/288S show no difference in electrophoretic mobilities (Figure 6, lane 2 vs lane 4), suggesting that Cys36 and Cys288 do not in fact form a disulfide linkage. Additionally, if Cys36 and Cys288 form a disulfide bond, the wild-type receptor should show a greater shift in electrophoretic mobility between nonreducing and reducing conditions than the C36/288S mutant. However, as shown in Figure 6, the wild type and C36/288S show the same difference in electrophoretic mobility between nonreducing and reducing conditions (lane 2 vs lane 4 and lane 1 vs lane 3), arguing against a disulfide bond between Cys36 and Cys288.

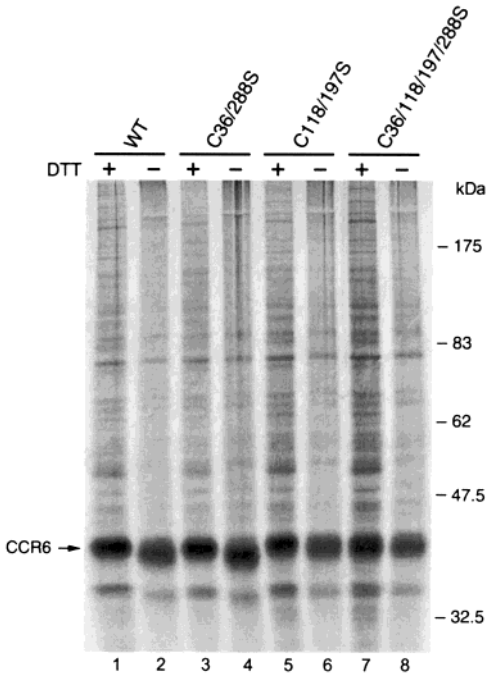

FIGURE 6: Both wild-type and C36/288S mutant receptors, but not C118/197S and C36/118/197/288S mutant receptors, show different electrophoretic mobilities under reducing and nonreducing conditions. Jurkat-TAg cells were transfected with HA-tagged wild-type or various HA-tagged mutant receptors, labeled with [^{35}S]methionine/cysteine, washed, and lysed. The receptors were immunoprecipitated with anti-HA antibody, incubated with or without 50 mM DTT, boiled, and resolved via 8% SDS−PAGE. The gel was dried and exposed to Kodak BioMax MR film for 3 days. The positions of prestained molecular mass markers are indicated on the right, and CCR6 is indicated by the arrow. Multiple experiments were done, and results from one representative experiment are shown.

To provide additional evidence for free SH groups on Cys36 and Cys288, we performed alkylation experiments to assay for free cysteines in these receptors. To detect any differences in electrophoretic mobilities among the wild type, C36/288S, C118/197S, and C36/118/197/288S mutant receptors when the extracellular free SH groups were alkylated, we chose a high-molecular mass alkylating agent, 5-IAF (515.26 Da). Transfected cells expressing HA-tagged wild-type, C36/288S, C118/197S, and C36/118/197/288S receptors were metabolically labeled and incubated with 5-IAF to react with the extracellular free SH groups. If Cys36 and Cys288 were present as free SH groups, then these cysteines in wild-type and C118/197S receptors would react with 5-IAF and lead to an increase in molecular mass of ~1 kDa compared with C36/288S and C36/118/197/288S receptors in which no extracellular free SH groups should be available. As expected, the wild-type and C118/197S receptors ran with a mobility corresponding to a molecular mass of ~43 kDa, while the C36/288S and C36/118/197/288S receptors ran at ~42 kDa (Figure 7A). Additionally, we also performed the reactions of 5-IAF with immunoprecipitates from wild-type, C36/288S, C118/197S, and C36/118/197/288S mutant receptors (Figure 7B). Receptors with higher molecular masses were obtained from the reaction of 5-IAF with immunoprecipitates, suggesting that other than surface cysteines, some intracellular and/or transmembrane cysteines are also able to react with 5-IAF (panel A vs panel B of Figure 7). In each case, we consistently observed an increase in molecular mass of ~1 kDa in the wild type and the C118/197S mutant

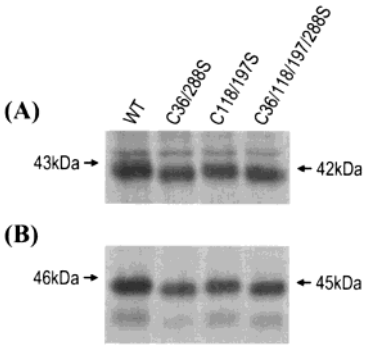

FIGURE 7: Wild-type CCR6, but not the C36/288S mutant, receptor reacts with the alkylating agent, 5-IAF. (A) Jukat-TAg cells were transfected with HA-tagged wild-type, C36/288S, C118/197S, or C36/118/197/288S mutant receptors, labeled with [^{35}S]methionine, washed, and incubated with 5-IAF for 2 h in the dark. After incubation, cells were lysed and the receptors were immunoprecipitated and resolved via SDS−PAGE. The apparent molecular mass is ~43 kDa for the wild-type and C118/197S mutant receptors and ~42 kDa for the C36/288S and C36/118/197/288S mutant receptors. Multiple experiments were done, and results from one representative experiment are shown. (B) The metabolically labeled cells were lysed, and the receptors were immunoprecipitated. The immunoprecipitates were then reacted with 5-IAF. After reaction, the immunoprecipitates were washed, heated in reducing buffer, and resolved on SDS−polyacrylamide gels. The apparent molecular mass is ~46 kDa for the wild-type and C118/197S mutant receptors and ~45 kDa for the C36/288S and C36/118/197/288S mutant receptors.

compared with the masses of the C36/288S and C36/118/197/288S mutants. All these results further support the contention that Cys36 and Cys288 do not form a disulfide bond in CCR6.

Cys336 in the Carboxyl-Terminal Region Is Critical for Receptor Expression and Functional Activity. CCR6 contains two cysteine residues in the carboxyl-terminal tail, Cys336 and Cys348. Mutation of Cys348 affected neither expression nor activity (Figure 8). Therefore, Cys348 is unlikely to participate in a disulfide bond. In contrast to Cys348, mutation of Cys336 to serine reduced the levels of surface expression, ligand binding, and function (Figure 8). The reduced level of ligand binding and function in the Cys336 mutant receptor is likely due to the reduced level of surface expression of the mutant receptor.

We also examined the importance of transmembrane cysteines (Cys57, Cys131, Cys138, Cys168, Cys233, Cys266, Cys309, and Cys310) in CCR6 by mutating them individually to serine. Transmembrane cysteine mutants exhibited wild-type levels of surface expression, calcium flux, and chemotaxis activity (data not shown). Thus, transmembrane cysteines in CCR6 probably do not directly interact with ligand, nor do they seem likely to be involved in disulfide bond formation.

DISCUSSION

Four highly conserved cysteines are present in the extracellular domains of all chemokine receptors found to date with the exception of CXCR6 (*18*−*20*). Two of these cysteines, located in the first and second extracellular loops, are common to most seven-transmembrane domain G protein-coupled receptors and are known to be linked by a disulfide bond. Mutation of either of these cysteines results in little or no expression of receptors at the cell surface, and

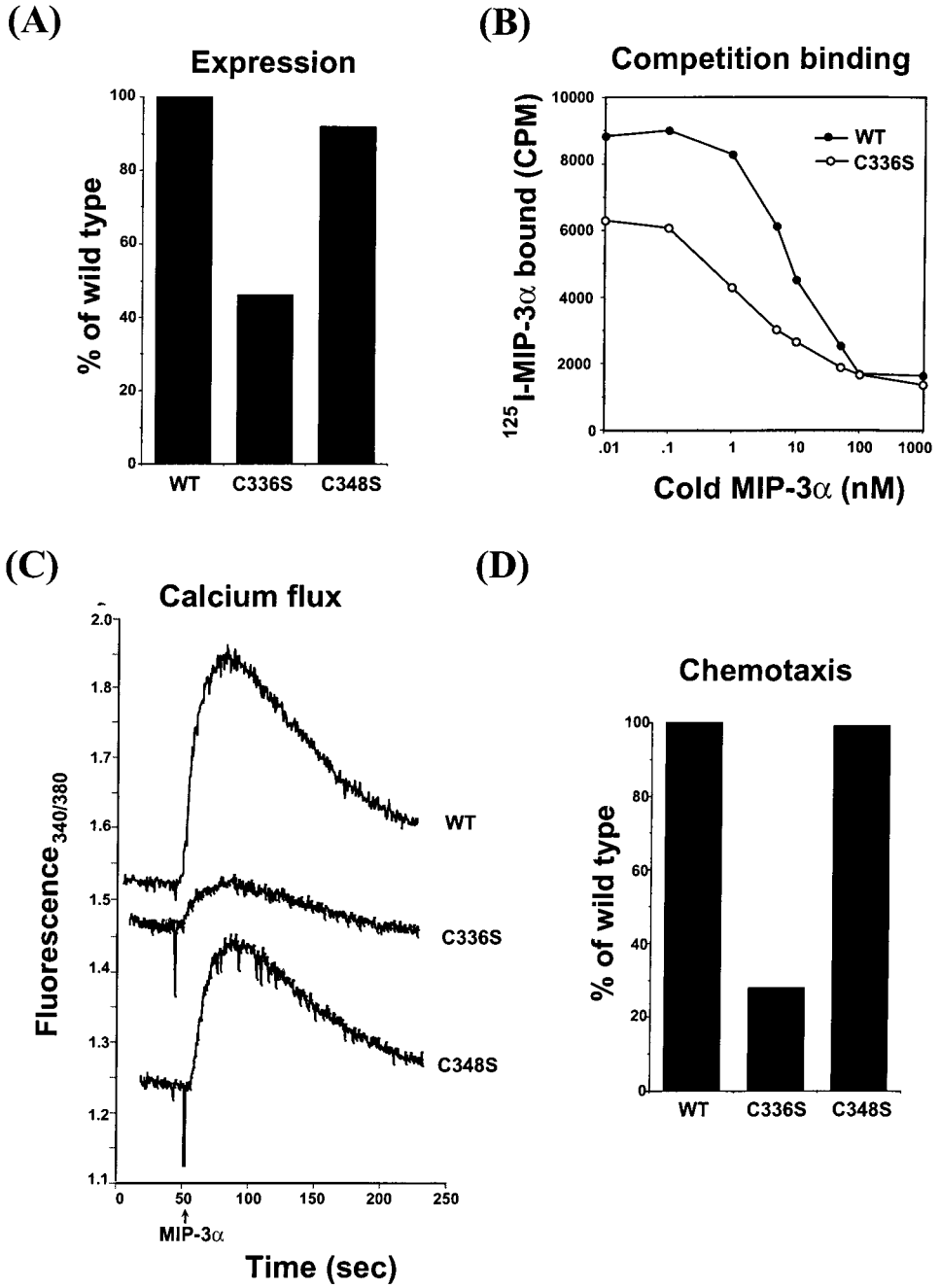

FIGURE 8: Intracellular Cys336, but not Cys348, is important for CCR6 expression, function, and ligand binding. (A) Jurkat-TAg cells transfected with the wild type, C336S, or C348S were harvested and stained with anti-CCR6-PE. The data were normalized against the wild type as 100%. (B) Ligand binding was carried out as described in the legend of Figure 5. (C) Calcium fluxes in response to MIP-3α/CCL20 (100 ng/mL) were measured as described in the legend of Figure 4A. (D) Chemotaxis in response to MIP-3α/CCL20 (50 ng/mL) was measured as described in the legend of Figure 4B, and the data were also normalized against the wild type as 100%. Multiple experiments were done, and results from a representative experiment are shown.

decreased levels of ligand binding and activation, presumably due to perturbations in the structure and stability of the proteins (*21*−*27*). Consistent with these observations, we have demonstrated that mutation of the analogous cysteines Cys118 and Cys197 in CCR6 dramatically reduced the level of receptor surface expression. We further demonstrated that the decrease in the level of expression of cell surface receptors was accompanied by an increase in the level of accumulation of intracellular receptors. Our observation suggests that the cysteines in the first (Cys118) and second (Cys197) extracellular loops form a disulfide bond that is critical for proper folding of CCR6 that is in turn necessary for efficient trafficking to the plasma membrane. The loss of functional activities in these mutant receptors could be explained primarily by the dramatic reduction in the level of expression of surface receptors.

There are two other cysteines found in the amino-terminal region and in the third extracellular loop in all chemokine receptors other than CXCR6, but not in all G protein-coupled receptors (*28*), suggesting that these cysteines may play a crucial role in ligand binding and biological functions for chemokine receptors but are not required for GPCR structure generally. For several chemokine receptors, although cysteines in the N-terminus and the third extracellular loop are

proposed to form an additional disulfide bond, this hypothesis has not yet been demonstrated experimentally (*29*−*33*). In this report, we used both genetic and biochemical approaches to examine this hypothesis, and we demonstrated that cysteines in the N-terminal region and in the third extracellular loop in CCR6 do not form a disulfide linkage. To our knowledge, this is the first study to provide biochemical evidence addressing the roles of these cysteines and showing that there is no formation of disulfide bonds between cysteines in the amino terminus and the third extracellular loop of a chemokine receptor. Our conclusions are supported by the observation that the N-terminal and the third loop cysteines are found singly in some GPCRs. Only the N-terminal cysteine, but not the third loop cysteine, is found in the human platelet thromboxane A_2 receptor and human EP1 prostaglandin E receptor (*34*); conversely, only the third loop cysteine, but not the N-terminal cysteine, is found in many GPCRs, e.g., human muscarinic acetylcholine receptors (*35*) and some human odorant receptors (*36*).

Although mutation of these cysteines (C36S and C288S) in CCR6 did not affect receptor expression, these mutations impaired ligand binding and reduced function as measured by calcium flux and chemotaxis, suggesting that these cysteines are important for ligand binding and biological function. We further demonstrated that these cysteine (C36S and C288S) mutant receptors could produce calcium signals as well if high concentrations of ligand were used, demonstrating that these receptors were not completely dysfunctional. We also demonstrated that although at the concentration of MIP-3α/CCL20 that was optimal for the wild-type receptor Cys36 and Cys288 mutant receptors showed detectable calcium flux, no chemotactic activity could be detected (Figure 4 and Table 1). The lack of a correlation between calcium flux activity and chemotaxis in Cys36 and Cys288 mutant receptors may reflect the different signaling pathways responsible for calcium flux and chemotaxis. Calcium flux induced by GPCRs is mediated through PLC-β isotypes that are activated by G proteins, including members of the Gi and Gq subfamilies. Gi proteins activate PLC-β through G$\beta\gamma$, while Gq proteins activate PLCβ through Gαq, although different PLC-β isotypes have different affinities for different types of G proteins (*37*). The activation of PLC-β leads to the generation of 1,4,5-trisphosphate [Ins(1,4,5)P_3], which is the key component for mobilizing calcium from intracellular stores. Jurkat T cells express both Gi and Gq subfamilies ($G\alpha_{11}$ and $G\alpha_{16}$) (*38*), and therefore, it is likely that Gi as well as Gq participated in the generation of calcium flux after CCR6 activation in these cells. In contrast, several reports have demonstrated that Gαi is the only G protein that can mediate chemotactic function through the release of G$\beta\gamma$ dimers that activate downstream signal molecules, such as Rac1/cdc42 (*39*−*41*). Calcium flux induced by activation of GPCRs is an early, rapid, and transient event, while chemotaxis is a more complicated and sustained response. Therefore, the threshold for activation of calcium flux is likely lower than that for activation of chemotaxis in Jurkat-TAg cells transfected with CCR6 receptors.

We also observed that mutation of the cysteine in the third loop (C288S) had a less deleterious effect than mutation of the N-terminal cysteine (C36S) in the calcium flux assay as reflected in the EC_{50} for the N-terminal cysteine mutant receptor (C36S), which was much higher than for the third loop cysteine mutant receptor (C288S). In addition, some of the Cys36 replacement mutants gave no activity. In contrast, replacement of the third loop cysteine (Cys288) with various amino acids gave activity that was attenuated but detectable. Taken together, these results suggest that cysteine residues Cys36 and Cys288, although not participating in a disulfide bond, may be important per se. The differences between mutating Cys36 and Cys288 may be due to other conformational constraints on a functional N-terminus such that Cys36 cannot tolerate most amino acid substitutions that have a lesser effect at Cys288.

For G protein-coupled receptors, some C-terminal cysteines near the seventh transmembrane domain have been shown to be palmitoylated, presumably to anchor the C-terminal domain in the membrane and create a fourth intracellular loop. Many lines of evidence have shown that these palmitoylated cysteines are critical for receptor activation and internalization (*42*−*46*). Inspection of the amino acid sequence upstream of Cys336 reveals a consensus sequence for palmitoylation, Phe-X-X-Leu/Ile-Leu/Ile-$(X)_n$-Cys^P (where X is any amino acid, $n = 0-4$, and P is palmitoylation) (*42*). Reduction of the levels of receptor expression and biological function in the C336S mutant receptor suggests that palmitoylation of this cysteine may be important for proper receptor expression. Consistent with our observation, impairing the palmitoylation of CCR5 reduced its level of surface expression (*44*, *45*). The reduced levels of receptor expression and ligand binding without an effect on the K_i in the C336S mutant suggest that the reduced level of biological function in the C336S mutant receptor probably reflects the reduction in the level of receptor surface expression.

In summary, we have presented studies demonstrating that the disulfide bond between the first and second extracellular loops and the cysteines in the N-terminus and the third extracellular loop are important for ligand binding and activation of human CCR6. It is possible that the disulfide bond between the first and second extracellular loops constrains a helical bundle together with the N-terminus, which might be adjacent to the third extracellular loop as proposed for the bradykinin receptor (*47*) or rhodopsin (*48*), creating the structural organization that is critical for ligand binding and therefore for receptor signaling. For the cysteines in the N-terminal domain and the third extracellular loop, their contribution to CCR6 structure and function is of a different sort, since these cysteines, while important, do not exert their effects by means of a disulfide bond.

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