

## Characterization of Sequence Determinants within the Carboxyl-terminal Domain of Chemokine Receptor CCR5 That Regulate Signaling and Receptor Internalization\*

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The CC chemokine receptor CCR5 mediates chemotaxis of leukocytes and serves as a principal co-receptor for macrophage-tropic human immunodeficiency virus type 1. To identify determinants on the CCR5 carboxyl-terminal domain that regulate receptor signaling and internalization, we generated several CCR5 mutants, which were progressively shortened from the COOH terminus or had carboxyl-terminal serine, cysteine, or leucine residues substituted by alanine and expressed them in RBL-2H3 cells. Using fluorescence resonance energy transfer between  $\beta$ -arrestin and CCR5 tagged with cyan and yellow variants of green fluorescent protein, we show that high affinity association of the two molecules in living cells requires intact carboxyl-terminal serine phosphorylation sites. Phosphorylation-deficient truncation or Ser/Ala replacement mutants of CCR5 mediated a sustained calcium response and enhanced granular enzyme release in RANTES-stimulated cells. Carboxyl-terminal serine residues are critically involved in CCR5 endocytosis and a dileucine motif, similar to that implicated in the regulation of CXCR2 and CXCR4, contributes to the internalization of CCR5 in a phosphorylation-independent manner. Despite their prominent role in receptor desensitization and internalization,  $\beta$ -arrestins are dispensable for the CCR5-mediated stimulation of mitogen-activated protein kinase pathways in RBL-2H3 cells. We also show that CCR5 is palmitoylated on carboxyl-terminal cysteine residues. Inhibition of CCR5 palmitoylation by alanine mutagenesis of cysteines or treatment with a palmitate analogue inhibitor profoundly reduces phorbol 12-myristate 13-acetate- and RANTES-induced receptor phosphorylation, homologous desensitization, and internalization. Alanine mutagenesis of serine, cysteine, or leucine residues or the limited carboxyl-terminal truncation of CCR5 did not impair chemokine-stimulated migration of RBL-2H3 cells. Together these results indicate that post-translational modifications of carboxyl-terminal serine and cysteine residues have a significant impact on receptor deactivation and internalization.

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Chemokines comprise a family of more than 40 structurally related molecules which play important roles in cell migration, proliferation and host inflammatory responses (1). They mediate their biological effects by binding to heptahelical receptors which interact with and signal through heterotrimeric G proteins. Several chemokine receptors, most notably CXCR4 and CCR5, also serve as coreceptors for the human immunodeficiency virus (HIV),<sup>1</sup> which together with CD4 facilitate viral entry into target cells (2). Natural ligands of the CCR5 receptor include the CC chemokines RANTES, MIP-1 $\alpha$ , MIP-1 $\beta$ , and MCP-2. Ligand binding to CCR5 induces the activation and dissociation of pertussis toxin-sensitive heterotrimeric G proteins, which, in turn, activate phospholipase C $\beta$  isoforms (3). This results in a transient increase in intracellular inositol 1,4,5-trisphosphate and cytosolic calcium. RANTES binding to chemokine receptors on T-lymphocytes activates both classical G protein signaling pathways as well as pertussis toxin-resistant protein tyrosine kinase-dependent pathways (4). Ligand binding to CCR5 was also shown to initiate mitogen-activated protein kinase (MAPK) cascades, which result in the activation of extracellular signal-regulated kinases (ERK1/2), p38 MAPK, and c-Jun amino-terminal kinase/stress-activated protein kinase (JNK/SAPK) (5).

Despite good evidence for a significant role for phosphatidylinositol 3-kinase  $\gamma$  in these processes (6, 7), the signaling pathways that are utilized to initiate chemotaxis, the prototypic function of chemokines, are largely unknown. Rapid modulation of signaling at the level of the receptor may be critical for the ability of chemokine receptors to sense small changes in chemokine concentrations as cells move along chemoattractant gradients. Several studies have tested the concept that receptor deactivation and internalization are essential for chemotaxis, although with contrasting results (8–11). Previously, we and others have shown that CC chemokines induce the rapid phosphorylation of CCR5 on carboxyl-terminal serine residues by G

<sup>1</sup> The abbreviations used are: HIV, human immunodeficiency virus; AP-2, adaptin 2; CCR5, CC chemokine receptor 5; ECFP, enhanced cyan fluorescent protein; ERK, extracellular-regulated kinase; EYFP, enhanced yellow fluorescent protein; FRET, fluorescence resonance energy transfer; GPCR, heterotrimeric GTP-binding protein-coupled receptor; GRK, G protein-coupled receptor kinase; HEK-293, human embryonic kidney 293; JNK, c-Jun NH<sub>2</sub>-terminal kinase; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; PAR-2, proteinase-activated receptor 2; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RANTES, released on activation normal T cell expressed and secreted (also known as CCL5); RBL-2H3, rat basophilic leukemia; BM, binding medium; BSA, bovine serum albumin; MES, 4-morpholineethanesulfonic acid; PBS, phosphate-buffered saline; PKC, protein kinase C; SAPK, stress-activated protein kinase.

protein-coupled receptor kinases (GRK) and protein kinase C (12–14). By analogy with other G protein-coupled receptors (GPCR),  $\beta$ -arrestins are thought to bind to the phosphorylated receptor and thereby sterically interfere with further G protein coupling. In addition to attenuating receptor signaling,  $\beta$ -arrestins also act as adapter proteins that facilitate clathrin-mediated endocytosis of certain G protein-coupled receptors (15). A role for  $\beta$ -arrestins in CCR5 internalization has been suggested previously by the ability of functionally active or dominant-negative  $\beta$ -arrestins to modulate receptor internalization in a differential way (12). Recently it was shown that  $\beta$ -arrestins fulfill additional functions in GPCR signaling as scaffolding proteins, which recruit components of MAPK cascades into multiprotein signaling complexes (16). Thereby,  $\beta$ -arrestins connect certain G protein-coupled receptors to the activation of ERK1/2 or JNK/SAPK. Whether this is a mechanism that also applies to CCR5-mediated mitogenic signaling is an open question.

Many GPCRs have been shown to be post-translationally modified by palmitoylation at cysteine residues in their carboxyl-terminal domains. Palmitoylation modifies GPCR signaling in ways that appear to vary among different receptors (for a review, see Ref. 17). CCR5 contains three carboxyl-terminal cysteines, which conform to a predicted palmitoylation site. Whether this lipid modification of the receptor is relevant for normal CCR5 function has not been studied in detail. In order to analyze the role of the carboxyl-terminal domain of the CCR5 receptor for ligand binding, signal transduction, and receptor internalization, we generated several receptor mutants with progressive carboxyl-terminal truncations of the receptor or with selective alanine mutation of amino acid residues at defined positions. These receptor mutants allowed us to investigate the functional significance of post-translational modifications and of specific conserved sequence motifs that are located in the CCR5 carboxyl-terminal domain.

#### EXPERIMENTAL PROCEDURES

**Materials**—Most reagents have been reported (13). [ $^3$ H]Palmitic acid and [ $^{25}$ I]-RANTES were from PerkinElmer Life Sciences; Amplify and Hyperfilm- $^3$ H were from Amersham Pharmacia Biotech; protease and kinase inhibitors were from Calbiochem; 2-bromopalmitate was from Fluka; all other reagents, unless otherwise indicated, were from Sigma.

**Plasmid Construction and Generation of Cell Lines**—The cDNA of either wild type CCR5 or phosphorylation-deficient receptor mutant CCR5-SSSS/AAAA (13) was amplified by polymerase chain reaction with a mutagenic 5' primer containing a *Hind*III site and a 3' primer, which replaces the CCR5 stop codon with a *Bam*HI restriction site. The restricted fragments were inserted in frame into the polylinker of pECFP-N1 (clontech). Rat  $\beta$ -arrestin with EYFP conjugated to its carboxyl terminus ( $\beta$ -arrestin-EYFP) was constructed by replacing the terminal stop codon of  $\beta$ -arrestin (18) with an *Acc*65I restriction site and inserting the modified cDNA in frame into the *Xho*I/*Kpn*I sites of pEYFP-N1 (clontech). Oligonucleotide-directed mutagenesis was performed on pEF-BOS-CCR5 (13) using the Gene Editor kit (Promega) according to instructions by the manufacturer to generate cysteine to alanine replacement mutants. The CCR5 truncation mutants were generated by inserting stop codons at various positions within the CCR5 carboxyl-terminal domain using site-directed mutagenesis. The CCR5-LL/AA, CCR5-LL/AA-SSSS/AAAA, and CCR5-Y/A replacement mutants were generated by polymerase chain reaction-mediated overlap extension technique. Coding sequences of all mutated cDNAs were verified by automated DNA sequencing. All CCR5 truncation and replacement mutants were stably expressed in RBL-2H3 cells using the pEF-BOS expression vector together with the ph $\beta$ APr1-neo plasmid as described previously (13). By the same technique, a RBL-CCR5, C5aR cell line was generated which stably expressed wild type CCR5 together with the C5a anaphylatoxin receptor (C5aR).

**Palmitoylation and Phosphorylation of CCR5**—RBL cells expressing either wild type or mutant CCR5 were incubated in serum-free RPMI 1640 medium for 16 h. Cells were then labeled for 4 h with 4 ml of RPMI 1640, 0.1% BSA and [ $^3$ H]palmitic acid (400  $\mu$ Ci/ml) at 37 °C for 4 h. Prior to use, [ $^3$ H]palmitic acid was dried under nitrogen and redissolved

in a minimal volume of dimethyl sulfoxide. After treatment with 50 nM RANTES or medium alone for 15 min at 37 °C, cells were washed once with cold PBS and solubilized in detergent buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.05% SDS with protease inhibitors as described (Ref. 19)) on ice. Receptors were immunoprecipitated by the incubation of precleared cellular lysates with 20  $\mu$ g of anti-FLAG M2 antibody and protein G-Sepharose. Receptors were eluted by incubation at 37 °C for 30 min in SDS sample buffer and subjected to 10% SDS-PAGE under non-reducing conditions. After fluorography with Amplify<sup>®</sup>, gels were dried and exposed to Hyperfilm- $^3$ H at –80 °C for 8 weeks. To ensure loading of equivalent amounts of receptors per lane of the gel, aliquots from each sample were analyzed by immunoblotting using an anti-CCR5 monoclonal antibody (T21/8),<sup>2</sup> which reacts with a CCR5 amino-terminal epitope and an enhanced chemiluminescence-based detection system. The effect of RANTES stimulation on [ $^3$ H]palmitate incorporation into the receptor was quantitated by densitometric analysis (ImageMaster TotalLab software; Amersham Pharmacia Biotech) of digitized images of autoradiograms and enhanced chemiluminescence films.

The phosphorylation of CCR5 in intact cells was determined as described (13). Briefly, RBL cell lines expressing wild type or mutant CCR5 were metabolically labeled with  $^{32}$ P<sub>i</sub>, treated with either PMA or RANTES, and phosphorylated CCR5 were routinely immunoprecipitated with FLAG M2 antibodies. For the purification of CCR5 and C5aR from cells that co-express both receptors, we employed antibodies (T21/8, S5/1; Serotec) with specificity for amino-terminal epitopes on the respective receptors. To be able to compare phosphorylation of the different receptor mutants, the same amount of surface expressed receptors, determined by flow cytometry, was applied to each lane of 10% SDS-PAGE gels. Quantitation of receptor phosphorylation was done by PhosphorImager analysis (Molecular Dynamics).

**Fluorescence Resonance Energy Transfer (FRET)**—HEK-293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were transiently transfected with either CCR5-ECFP or CCR5-SSSS/AAAA-ECFP alone or in combination with  $\beta$ -arrestin-EYFP (10  $\mu$ g/100-mm dish) by the calcium phosphate precipitation technique. Assays were performed 36–48 h after transfection; transfection efficiencies as judged by flow cytometry ranged from 45 to 85%. Cells were detached by mild trypsinization or incubation (10 min) with PBS plus 10 mM EGTA on ice, washed in fetal calf serum-containing medium, resuspended in PBS, and immediately measured for FRET using a Spex Fluoromax-2 spectrofluorometer. Exposure of cells to trypsin for brief periods of time (10–15 s) was found not to affect CCR5 signaling or FRET measurements. At various time points after addition of a saturating concentration of RANTES, the samples were excited at 424 nm, which represents the excitation maximum of ECFP. Emission spectra were collected from 450 to 600 nm and corrected for background fluorescence. Data are represented in a modification of the method used by Angers *et al.* (20) as FRET ratios, which are defined as [(emission at 526 nm) – (emission at 475 nm)  $\times$  cf]/(emission at 475 nm), where cf is the ratio (emission at 526 nm)/(emission at 475 nm) of CCR5-ECFP expressed in HEK-293 cells alone and in the absence of stimulus.

**Binding and Internalization of Radiolabeled Chemokines**—Equilibrium competition binding assays were carried out by analyzing the displacement of [ $^{125}$ I]-MIP-1 $\alpha$  from CCR5-expressing RBL cells by unlabeled ligand, and IC<sub>50</sub> values were calculated as described (21).

The internalization of [ $^{125}$ I]-RANTES by RBL-2H3 cells that express wild type or mutant CCR5 was determined by following a protocol by Signoret *et al.* (22). Briefly, RBL-CCR5 cells were grown to 70% confluence in 24-well plates and were incubated with binding medium (BM: RPMI 1640 without bicarbonate, 0.2% BSA, 10 mM HEPES, pH 7.4) containing 125 pM [ $^{125}$ I]-RANTES for 90 min at 4 °C. Unbound chemokines were removed by washing at 4 °C. Cells were incubated with 37 °C BM for different times (0, 3, 10, or 30 min) to initiate internalization. In half of the wells for each time point, surface-bound radioligand was removed by two 3-min acid washes with RPMI 1640, BSA, 10 mM MES, pH 2.5. All cells were washed again with 4 °C BM. The specific radioligand uptake was calculated as acid-resistant counts in 0.1 N NaOH extracts of acid-washed cells divided by the total cell-associated activity in cells washed at pH 7.4 after subtraction of non-specific binding at time 0.

**Functional Assays**—The assay for RANTES-induced *N*-acetyl- $\beta$ -D-glucosaminidase release from CCR5-expressing RBL-2H3 cells has been described before (23). Values (mean  $\pm$  S.E.) were expressed as a

<sup>2</sup> M. Oppermann, unpublished data.

percentage of total enzyme present in cells after lysis with 0.1% Triton X-100, and data were analyzed using nonlinear regression applied to a sigmoidal dose-response model by Sigma-Plot software (SPSS).

Agonist-dependent intracellular calcium mobilization was measured in transfected RBL-2H3 cells as described (13). To calculate the decrease in intracellular calcium concentrations after RANTES stimulation, we applied a mathematical analysis previously used by Christophe *et al.* (24). Calcium decays from 80% of the peak height to basal levels were fitted to an exponential ( $a + be^{-t/\tau}$ ), where the time constant  $\tau$  reflects the ability of CCR5 variants to evoke a more or less sustained calcium response.

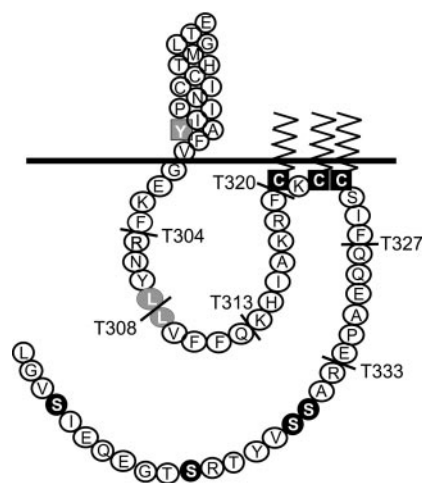
CCR5-mediated activation of MAPK pathways was determined in serum-starved cells grown in six-well dishes, which were stimulated for 5 or 15 min with 50 nM RANTES. Cellular lysates in Laemmli buffer were separated by 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with a 1:1000 dilution of monoclonal anti-phospho-ERK1/2, rabbit polyclonal anti-phospho-p38, or rabbit polyclonal anti-phospho-JNK/SAPK antibodies (New England Biolabs). After visualization of immune complexes by enhanced chemiluminescence, the membranes were stripped and reprobed for total cellular MAPKs with polyclonal rabbit anti-ERK1/2 (Calbiochem), anti-p38, or anti-JNK/SAPK (New England Biolabs) antibodies. Subsequent quantitation of phospho-MAPK immunoblots was normalized to the total amount of MAPK present using scanning laser densitometry and ImageMaster TotalLab software (Amersham Pharmacia Biotech).

Chemotaxis assays were performed on stably transfected RBL-2H3 cells using 48-well chemotaxis chambers (Neuroprobe) and polycarbonate filters with a 12- $\mu$ m pore size. Briefly,  $5 \times 10^4$  cells in 50  $\mu$ l of RPMI 1640, 1% BSA were seeded in the upper compartment and the lower compartment was filled with 27  $\mu$ l of RANTES diluted in the same medium. The chamber was incubated for 90 min at 37 °C in a 5% CO<sub>2</sub> moist atmosphere, membranes were then removed, and the number of cells that passed through the membrane were quantified by staining with a Diff-Quik kit and counting the number of cells present in three microscope fields (20 $\times$  objective). All experiments were performed in quadruplicate and repeated at least three times.

## RESULTS

**Characterization of CCR5 Carboxyl-terminal Mutants**—To study the functional role of the CCR5 carboxyl-terminal domain, which encompasses a total of 51 amino acid residues, we generated a series of truncation variants with progressive deletions of the COOH-terminal portion of the receptor (Fig. 1). These receptor constructs lack the carboxyl-terminal 18, 24, 31, 38, 43, or 47 residues of the native receptor and were designated by "T" followed by the position of their carboxyl-terminal amino acid. All truncation mutants lack the four COOH-terminal serine residues, which we previously identified as the exclusive phosphorylation sites on CCR5 for GRKs and PKC (13). To directly address the potential significance of post-translational receptor modifications, site-specific CCR5 mutants were generated with alanine exchange of the four carboxyl-terminal serine phosphorylation sites at positions 336, 337, 342, and 349 (CCR5-SSSS/AAAA) and of three adjacent and potentially palmitoylated cysteine residues at positions 321, 323, and 324 (CCR5-CCC/AAA). Additionally, the two adjacent leucine residues at positions 308 and 309, which form a potential AP-2 binding motif, were alanine-mutated either alone (CCR5-LL/AA) or together with the four serine phosphorylation sites (CCR5-LL/AA-SSSS/AAAA). A receptor with alanine replacement of Tyr-297 in transmembrane domain VII (CCR5-Y/A) had previously been reported to be impaired in its ability to signal and to undergo agonist-induced sequestration (12) and was included in this study for control purposes.

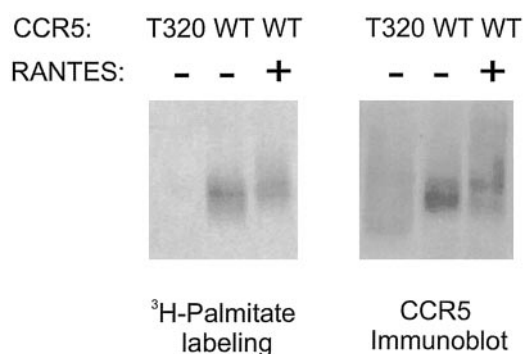
The wild type and mutant CCR5 cDNAs were transfected into RBL cells and stable clones derived by selection with G418. Receptor expression at the cell surface was tested by flow cytometry using a monoclonal antibody (Q10/19),<sup>2</sup> with specificity for a CCR5 epitope. The mutants were expressed at levels comparable to wild type receptor, except for the truncation mutant T304, which had significantly less receptor expression



**FIG. 1. Schematic representation of transmembrane helix VII with the CCR5 carboxyl-terminal domain and receptor mutants.** Truncated receptors with variable length C-tail were generated by introducing stop codons after residues 304, 308, 313, 320, 327, and 333. Serine phosphorylation sites (black circles) were mutated to alanine in the SSSS/AAAA mutant; three cysteines (black squares) which are potentially palmitoylated were changed to alanine in the CCC/AAA mutant; a potential dileucine sequence motif (shaded circles) was replaced by two alanines in the LL/AA mutant; in LL/AA-SSSS/AAAA, the dileucine and the serines were eliminated in combination; Tyr-297 (shaded square), which is part of a conserved NPXXY motif was alanine-mutated in CCR5-Y/A.

at the cell surface. This defect in cell surface expression was reproducibly observed in several independent RBL cell clones as well as in transfected HEK-293 cells. The CCR5-T304 mutant was, however, strongly expressed within cells at levels comparable to wild type CCR5 when receptors were detected by immunofluorescence in saponin-permeabilized cells (data not shown).

**Palmitoylation of CCR5**—The ability of RBL cells to incorporate [<sup>3</sup>H]palmitic acid into CCR5 wild type or a COOH-terminal truncation mutant (CCR5-T320) of the same receptor was examined by metabolic labeling experiments. The receptors were purified by immunoaffinity chromatography and subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. As shown in Fig. 2, only cells that express wild type receptor incorporated [<sup>3</sup>H]palmitic acid into a 40-kDa protein, which corresponds to mature CCR5. In contrast, no radioactivity was associated with the CCR5 truncation mutant, which lacks three carboxyl-terminal cysteine residues. Immunoblotting with an antibody that reacts with a CCR5 amino-terminal epitope confirmed expression of both receptors at equivalent levels. Since palmitoylation of proteins occurs through covalent modification of cysteine residues, this result implies that palmitoylation of CCR5 occurs through formation of thioester bonds with up to three adjacent cysteine residues at positions 321, 323, and 324, which are located in the receptor's carboxyl terminus in close proximity to transmembrane domain VII. Since palmitoylation of other G protein-coupled receptors was shown to be modulated by external stimuli, we investigated the possible effect of short term RANTES stimulation on the extent of CCR5 palmitoylation. When RBL-CCR5 cell were exposed to RANTES for 15 min, the electrophoretic mobility of the receptor decreased, a phenomenon that previously was found to be correlated with ligand-induced CCR5 phosphorylation (13). Exposure of cells to RANTES did not modulate [<sup>3</sup>H]palmitate incorporation into the receptor ( $94 \pm 7\%$  of the signal obtained with unstimulated wild type cells;  $n = 3$ ) in a significant manner when radiolabel incorporation was normalized to receptor expression levels, as determined by immunoblotting and scanning laser densitometry.

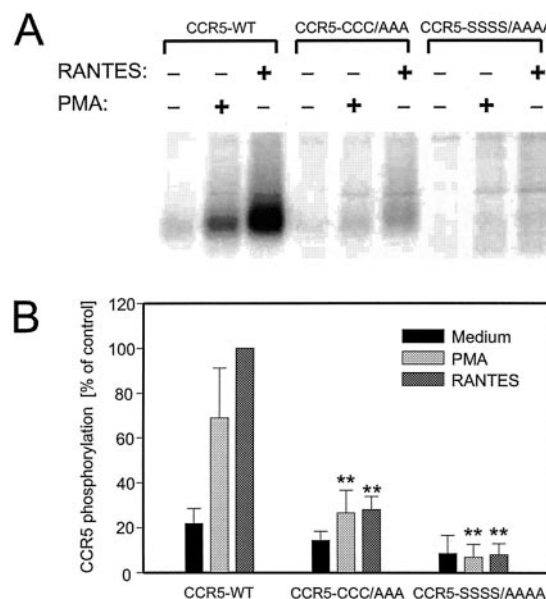


**FIG. 2. Palmitoylation of wild type CCR5, but not of CCR5-T320.** RBL-2H3 cells expressing wild type (WT) or truncated (T320) CCR5 were metabolically labeled with [ $^3\text{H}$ ]palmitate and incubated with or without 50 nM RANTES for the last 15 min of the labeling period. Solubilized receptors were immunoprecipitated and subjected to SDS-PAGE under non-reducing conditions followed by fluorography and autoradiography (left panel). Aliquots were analyzed in parallel by immunoblotting using a CCR5-specific monoclonal antibody (right panel).

**Phosphorylation of CCR5**—Using a series of receptor mutants that were transiently expressed in HEK-293 cells, we had shown previously that four distinct CCR5 carboxyl-terminal serine residues are phosphorylated upon RANTES stimulation by a GRK-mediated mechanism (13). As seen in Fig. 3, a CCR5-SSSS/AAAA mutant with alanine replacement of all four relevant carboxyl-terminal serine residues stably expressed in RBL cells was not phosphorylated following treatment with either RANTES or PMA. To investigate the functional significance of receptor palmitoylation, we generated cell lines that express a palmitoylation-deficient mutant with alanine substitution of all three COOH-terminal cysteine residues. Despite the presence of intact COOH-terminal phosphorylation sites, normal cell surface expression, and ligand binding affinity, no significant chemokine- or PMA-induced phosphorylation of the CCR5-CCC/AAA mutant with alanine mutation of the three potential palmitoylation sites was observed, and basal levels of receptor phosphorylation were also reduced as compared with wild type CCR5.

Although similar mutational approaches have been frequently used in other receptor systems to address the functional significance of protein palmitoylation, the possibility remains that the observed effects are due to loss of the cysteine residue *per se*, rather than loss of the palmitate. We therefore used 2-bromopalmitate as an inhibitor of receptor palmitoylation and investigated its effects on dually transfected RBL cells, which stably express wild type CCR5 together with C5aR. Human C5aR lack carboxyl-terminal cysteine residues and are, therefore, presumably not palmitoylated. After treatment with 2-bromopalmitate, RBL-CCR5,C5aR cells were incubated with PMA or the respective agonists and phosphorylation of the two receptors was monitored in parallel by immunoprecipitation with CCR5- or C5aR-specific monoclonal antibodies. Flow cytometry revealed that 2-bromopalmitate has no effect on cell surface expression levels of both receptors. Pre-treatment with the palmitate analogue profoundly reduced PMA- and RANTES-induced phosphorylation of CCR5 (Fig. 4). Inhibition of receptor phosphorylation by 2-bromopalmitate was unlikely to be due to effects on signaling molecules downstream of the receptor since phosphorylation of C5aR through PKC- or GRK-mediated mechanisms remained unaffected.

Ligand- and PMA-induced phosphorylation of CXCR4 was reported to depend on an intact carboxyl-terminal dileucine motif in this particular receptor (25). In contrast, the CCR5-LL/AA mutant and wild type receptors were phosphorylated to

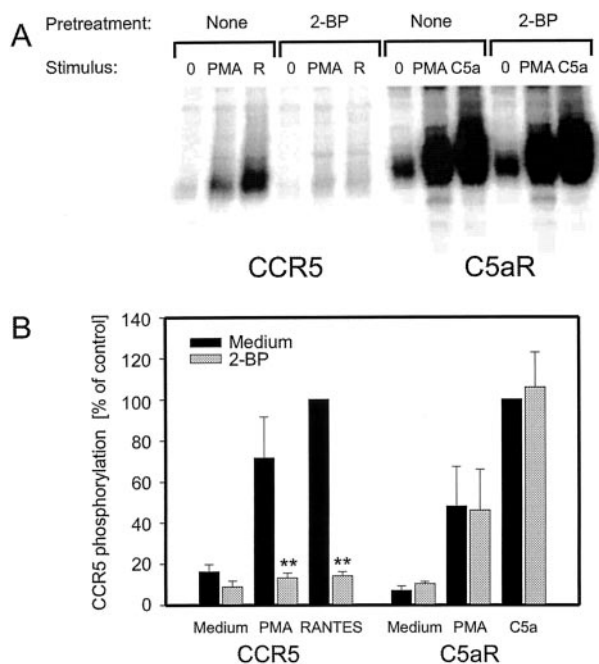


**FIG. 3. RANTES- and PMA-induced phosphorylation of wild type and mutant CCR5.** Cells expressing wild type, Cys/Ala-, or Ser/Ala-mutated receptors were metabolically labeled with  $^{32}\text{P}_i$  and exposed to 200 nM PMA or 50 nM RANTES for 5 min. Equal amounts of receptors were loaded into each lane of a 10% SDS-polyacrylamide gel and  $^{32}\text{P}$  incorporation into CCR5 was detected by autoradiography (A) and quantitative phosphoimager analysis (B). Data in the figure are expressed as a percentage of receptor phosphorylation measured in RANTES-stimulated wild type receptors and represent mean  $\pm$  S.E. from three experiments. \*\*,  $p < 0.001$  compared with wild type cells.

a similar extent upon cellular stimulation with either PMA or RANTES, and no difference was observed in phosphorylation of these receptor variants in GRK3-overexpressing COS-7 cells (data not shown).

**Detection of CCR5- $\beta$ -Arrestin Interaction by FRET**—Evidence for the chemokine-induced association of  $\beta$ -arrestin with CCR5 was obtained previously from functional studies (12) as well as from direct co-immunoprecipitation experiments (14). To determine the significance of receptor phosphorylation for agonist-induced association of  $\beta$ -arrestin with CCR5, we used FRET to monitor real time interaction between wild type or phosphorylation-deficient receptors and  $\beta$ -arrestin in living cells.

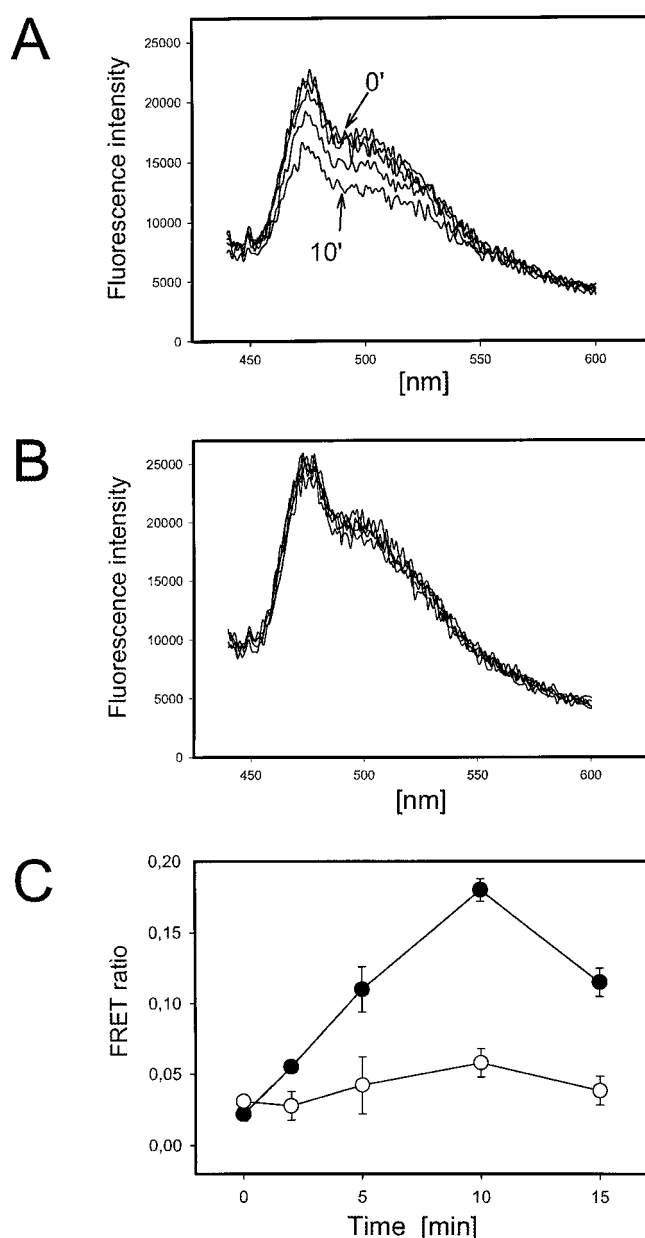
CCR5 fusion proteins with an additional ECFP moiety expressed on the carboxyl terminus (CCR5-ECFP) were validated to ensure that their functional characteristics resemble those of untagged wild type CCR5. In experiments not shown, we demonstrated that, upon RANTES stimulation, the fluorescent receptor induced intracellular calcium mobilization and underwent phosphorylation in a manner indistinguishable from wild type receptor. Fluorescence emission spectra were recorded in transfected HEK-293 cells, which expressed CCR5-ECFP and  $\beta$ -arrestin-EYFP together or individually at regular time intervals after stimulation with saturating concentrations of RANTES (Fig. 5). FRET data are represented as the ratio of fluorescence intensity recorded at 526 nm, which corresponds to the emission peak wavelength of EYFP, over that emitted at 475 nm using the equation given under "Experimental Procedures." Cells that co-expressed wild type CCR5-ECFP together with  $\beta$ -arrestin-EYFP showed an agonist-induced and time-dependent decrease of donor fluorescence with a maximal effect being observed after 10 min. This results in an increase of the FRET ratio of  $0.18 \pm 0.01$  relative to cells that express only CCR5-ECFP. The lack of a detectable increase in acceptor fluorescence is probably due to the relative inefficiency of this system using transiently transfected cells. In marked contrast,



**FIG. 4. Effect of 2-bromopalmitate on PMA- and ligand-induced phosphorylation of CCR5 and C5aR.** RBL-CCR5,C5aR, which co-express both receptors in the same cells, were incubated overnight with or without 100  $\mu$ M 2-bromopalmitate (2-BP) and labeled with  $^{32}$ P<sub>i</sub>. After stimulation with PMA (200 nM), RANTES (50 nM), or C5a (20 nM) for 5 min, receptors were immunoprecipitated by monoclonal antibody with specificity for CCR5 (T21/8) or C5aR (S5/1), respectively. *A*, the autoradiogram from one representative experiment is shown. *B*, quantitative phosphoimager analysis. Data were normalized to values obtained from cells which were stimulated with RANTES (*left panel*) or C5a (*right panel*) in the absence of the inhibitor and represent mean  $\pm$  S.E. from three experiments. \*\*,  $p < 0.001$  compared with cells without 2-bromopalmitate.

no significant changes in FRET ratios were observed upon RANTES stimulation in cells which co-expressed  $\beta$ -arrestin-EYFP together with a phosphorylation-deficient CCR5-SSSS/AAAA-ECFP mutant. The low basal FRET ratios observed in these cells probably derive from close proximity of EYFP and ECFP moieties upon overexpression of these proteins.

**Desensitization of the CCR5-mediated Calcium Response—**According to a classical model of GPCR signaling,  $\beta$ -arrestin binds to the agonist-occupied, GRK-phosphorylated receptor and thereby uncouples the receptor from its cognate heterotrimeric G protein. We tested the hypothesis that CCR5 mutants that were impaired in their abilities to interact with  $\beta$ -arrestin in an agonist-dependent manner also showed a defect in receptor desensitization. Upon RANTES stimulation a robust calcium response was observed in RBL-2H3 cells, which expressed truncation mutants T333, T327, and T320 (Fig. 6). No intracellular mobilization was observed following stimulation even with high concentrations (1  $\mu$ M) of RANTES in the T313, T308, and T304 as well as the Y/A mutants. Except for the cells that express the LL/AA mutant, which responded to RANTES stimulation in a manner indistinguishable from the wild type receptor, in all truncation and alanine replacement mutants a significant prolongation of the rise in intracellular calcium levels was observed as compared with the wild type receptor. This defect in receptor desensitization was analyzed by calculating calcium decay time constants, which were found to be 3–4 times higher in cells expressing the phosphorylation- and the palmitoylation-deficient mutants as compared with wild type receptors. With the CCR5-CCC/AAA mutant, we also reproducibly observed a retardation of the initial rise in intracellular calcium concentrations after RANTES addition. These

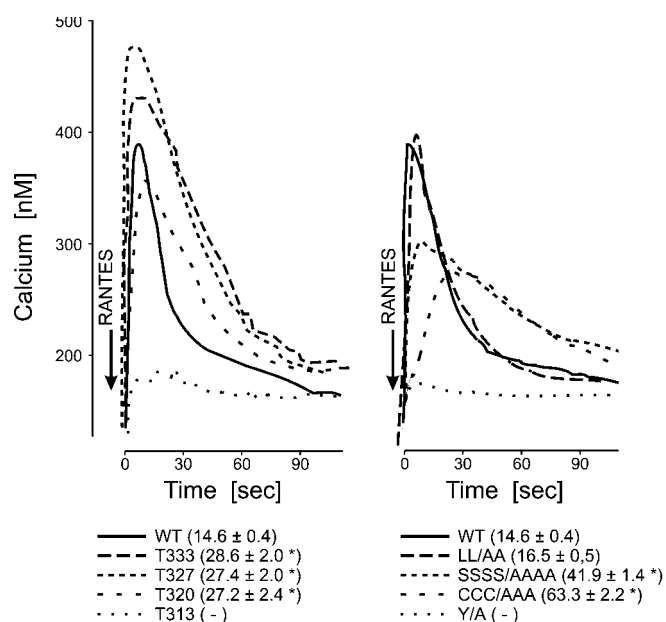


**FIG. 5. RANTES-induced association of  $\beta$ -arrestin with wild type, but not with phosphorylation-deficient CCR5-SSSS/AAAA as determined by FRET analysis.** Transfected HEK-293 cells, which co-express  $\beta$ -arrestin-EYFP together with either CCR5-ECFP (*A*) or CCR5-SSSS/AAAA-ECFP (*B*), were excited at 424 nm, and fluorescence emission spectra (450–600 nm) were recorded at various time points after RANTES addition. *C*, data are represented as FRET ratios, which were calculated as described under “Experimental Procedures” (●, CCR5-ECFP/ $\beta$ -arrestin-EYFP; ○, CCR5-SSSS/AAAA-ECFP/ $\beta$ -arrestin-EYFP).

results show an important role for post-translational modifications of carboxyl-terminal residues on CCR5 in the desensitization of this receptor.

**Internalization of CCR5—**In previous studies functional or dominant-negative  $\beta$ -arrestin proteins were shown to modulate CCR5 internalization in HEK-293 cells in an opposite manner (12), which suggests a role for  $\beta$ -arrestin in the regulation of CCR5 cell surface expression. To identify molecular determinants on the receptor that are involved in this process, we measured the capacity of RBL-2H3 cells to internalize  $^{125}$ I-RANTES.

In RBL-2H3 cells that express wild type CCR5, ~60% of the radiolabeled ligand was internalized within 3 min, and recep-



**FIG. 6. RANTES-induced calcium mobilization in RBL-2H3 cells expressing wild type (WT) and mutant CCR5.** RBL-2H3 cells, which stably express CCR5 wild type, truncation (*left*), or alanine replacement (*right*) mutants, were loaded with Fluo-3 AM, stimulated with 45 nM RANTES in the presence of extracellular calcium, and analyzed for their ability to induce intracellular calcium mobilization. Data shown are representative of at least three experiments. The numbers shown in parentheses represent time constants ( $\tau$ ) for calcium decay, which were calculated as described under "Experimental Procedures." \*,  $p < 0.001$  compared with cells expressing wild type CCR5.

tor endocytosis was maximal after 30 min incubation time when 80% of ligand initially present at the cell surface was detected intracellularly (Fig. 7). No significant RANTES internalization was observed with RBL-2H3 cells, indicating that ligand uptake was dependent on specific receptor binding rather than pinocytosis. Truncation of the carboxyl terminus to 333, 327, or 320 residues resulted in a significant decrease in the amount of internalized ligand after 3 min and 10 min but had little effect on receptor endocytosis at later time points. Thus, truncation of the CCR5 carboxyl terminus profoundly affects kinetics rather than overall efficiency of the endocytotic machinery. Truncation of the receptor to 308 or 304 residues almost completely eliminated specific uptake of radiolabeled RANTES, consistent with the defect in ligand binding and signaling of these receptor mutants. Analysis of the CCR5-SSSS/AAAA mutant revealed a similar defect in RANTES internalization as was observed in the T333, T327, or T320 mutants. This result is consistent with the notion that phosphorylation of carboxyl-terminal serine residues is largely responsible for chemokine-induced receptor internalization. The palmitoylation-deficient CCR5 mutant, which is also not phosphorylated, was internalized with even lower efficiency. Treatment of RBL-CCR5 cells with 2-bromopalmitate impaired ligand internalization, but this effect was less pronounced than in the cells that express the CCR5-CCC/AAA mutant. We also investigated the potential significance of the carboxyl-terminal dileucine element in CCR5 for receptor endocytosis. Internalization of the CCR5-LL/AA mutant was slightly, but significantly reduced at early time points compared with wild type CCR5. A mutant (CCR5-LL/AA-SSSS/AAAA) with combined alanine mutation of serine phosphorylation sites, and the dileucine motif had an even greater sequestration defect than a mutant (CCR5-SSSS/AAAA) deficient only in receptor phosphorylation.

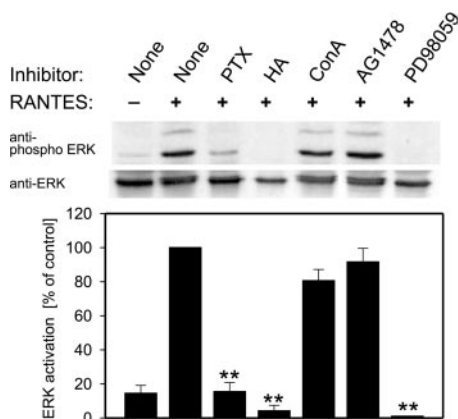
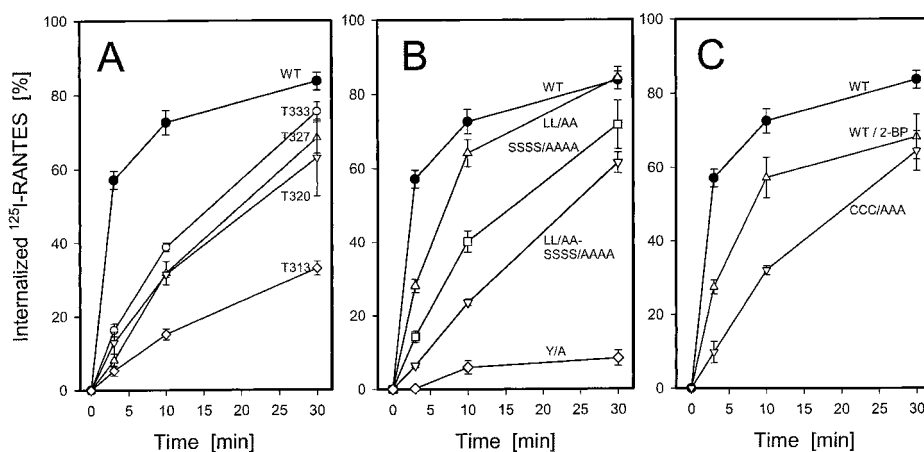
#### MAP Kinase Activation in Response to CCR5 Stimula-

tion—In  $\beta_2$ -adrenergic receptor as well as several other GPCR systems,  $\beta$ -arrestins were shown to act as adapters that recruit signaling molecules involved in tyrosine kinase signaling to ligand-activated GPCR. As shown in Fig. 8, stimulation of RBL-CCR5 cells with saturating concentrations of RANTES induces the activation/phosphorylation of ERK1/2 kinases by a mechanism that is sensitive to pertussis toxin, the tyrosine kinase inhibitor herbimycin A, and the MEK1 inhibitor PD98059. No effect on RANTES-stimulated ERK activation was observed for concanavalin A, an inhibitor of clathrin-mediated endocytosis, or the epidermal growth factor receptor tyrosine kinase inhibitor AG1478. Alanine replacement of carboxyl-terminal serine residues resulted in a receptor variant, which showed enhanced ( $p < 0.05$ ) ERK activation after RANTES stimulation compared with wild type CCR5 (Fig. 9B). The same tendency was observed with the T333 and T327 truncation mutants (Fig. 9A). Although T304 and T308 were completely inactive (not shown), a weak signal, which, however, was significantly higher than background values was observed in chemokine-treated cells that express the T313 mutant. Treatment of cells with 1  $\mu$ M PMA for 10 min resulted in maximal PKC-mediated ERK activation and showed that all cell lines were equally able to activate this MAPK pathway. In the PAR-2 system, a phosphorylation-deficient receptor retains the ability to activate ERK1/2, but wild type and phosphorylation-deficient receptors apparently utilize pathways that are differentially affected by PKC or tyrosine kinase inhibitors (26). We therefore investigated whether activation of ERK1/2 via wild type and phosphorylation-deficient CCR5 was affected by kinase inhibitors in a differential manner. However, pathways leading to ERK1/2 activation in cells expressing either receptor variant were affected by several inhibitors (bisindolylmaleimide, herbimycin A, AG1478, wortmannin) in the same way (data not shown). Phosphorylation-deficient CCR5 mutants were also not impaired in their capacities to induce activation of p38 ( $115 \pm 4\%$  compared with wild type CCR5; mean  $\pm$  S.E.,  $n = 5$ ) and SAPK/JNK ( $78 \pm 10\%$  compared with wild type CCR5, mean  $\pm$  S.E.,  $n = 3$ ) (Fig. 9C).

**CCR5-mediated Granule Release from Transfected RBL-2H3 Cells**— $\beta$ -Arrestins were reported to regulate the chemokine-induced granule exocytosis in RBL-2H3 cells (27). We therefore examined whether wild type and phosphorylation-deficient CCR5 variants differed in their capacities to mediate  $\beta$ -hexosaminidase release from these cells. Treatment of RBL-CCR5 cells with PP-2 (10  $\mu$ M/20 min) inhibited *N*-acetyl- $\beta$ -D-glucosaminidase release upon stimulation with saturating concentrations of RANTES by 66%. Thus, a role for Src family tyrosine kinases in the chemokine-induced release of glucosaminidase in transfected RBL-2H3 cells was confirmed. However, receptors that lacked carboxyl-terminal phosphorylation sites due to truncation or alanine replacement of serine residues mediated a robust enzyme release from these cells with even higher efficiency than wild type CCR5 (Table I).

**RANTES-induced Chemotaxis by RBL-CCR5 Cells**—Desensitization as well as internalization of chemotactic leukocyte receptors have been postulated to be important mechanisms in receptor-mediated chemotaxis. To determine whether mutations in the CCR5 carboxyl-terminal domain affect cellular migration, the chemotactic response of transfected RBL-2H3 cells toward different concentrations of RANTES was investigated. RBL-2H3 cells stably transfected with wild type CCR5 exhibited a classical biphasic response to RANTES with a maximal response at 0.6–1.7 nM (Fig. 10). Consistent with our results of RANTES-induced calcium mobilization and granule enzyme release, we observed that RBL-2H3 cells that expressed CCR5 truncation mutants T333, T327, or T320 mi-

**FIG. 7. Time-dependent internalization of  $^{125}\text{I}$ -RANTES by wild type (WT) or mutant CCR5 in RBL-2H3 cells.** Radioligand was bound to the cells at 4 °C, and the cells were warmed to 37 °C for the times indicated.  $^{125}\text{I}$ -RANTES that remained at the cell surface was removed by acid wash, and internalization was calculated as the percentage of acid-resistant counts in lysates of acid-washed cells to total cell-associated activity after correction for nonspecific binding (mean  $\pm$  S.E. of three to seven independent experiments performed in duplicate). Data are arranged to demonstrate the effects of carboxyl-terminal truncation (A), alanine substitution of the dileucine and phosphorylated serine residues (B), and inhibition of receptor palmitoylation (C) on CCR5 endocytosis. 2-BP, 2-bromopalmitate.



**FIG. 8. Effect of various inhibitors on the RANTES-induced ERK1/2 activation in wild type CCR5-expressing RBL-2H3 cells.** Cells were pre-incubated with medium, pertussis toxin (PTX; 100 ng/ml for 16 h), herbimycin A (HA; 1  $\mu\text{M}$ ), concanavalin A (ConA, 250  $\mu\text{g}/\text{ml}$ ), AG1478 (250 nM), or PD98059 (50  $\mu\text{M}$ ), and the activation of ERK1/2 upon exposure (5 min/37 °C) to 50 nM RANTES was detected by immunoblotting using antibodies specific for the phosphorylated/activated form of ERK1/2. Afterward the membranes were stripped and reprobed with an ERK-specific antibody to detect total cellular ERK levels. Visualization of horseradish peroxidase-labeled secondary antibodies was performed using enhanced chemiluminescence and quantified by scanning laser densitometry. Results are expressed as the percentage of the maximal signal obtained in the absence of inhibitors. Histogram represents the mean  $\pm$  S.E. from three experiments. \*\*,  $p < 0.001$  compared with RANTES-treated cells without inhibitor.

grated toward optimal concentrations of RANTES even better ( $p < 0.001$ ) than wild type CCR5-expressing cells. In cells that expressed the T313 mutant, the dose-response curve was shifted to the right by a factor of  $\sim 100$ , whereas T308, T304, or CCR5-Y/A cells did not migrate even toward micromolar concentrations of RANTES. The chemotactic response of RBL-2H3 cells that expressed alanine replacement mutants CCR5-SSSS/AAA, CCR5-LL/AA, and CCR5-CCC/AAA resembled those observed in wild type CCR5-expressing cells.

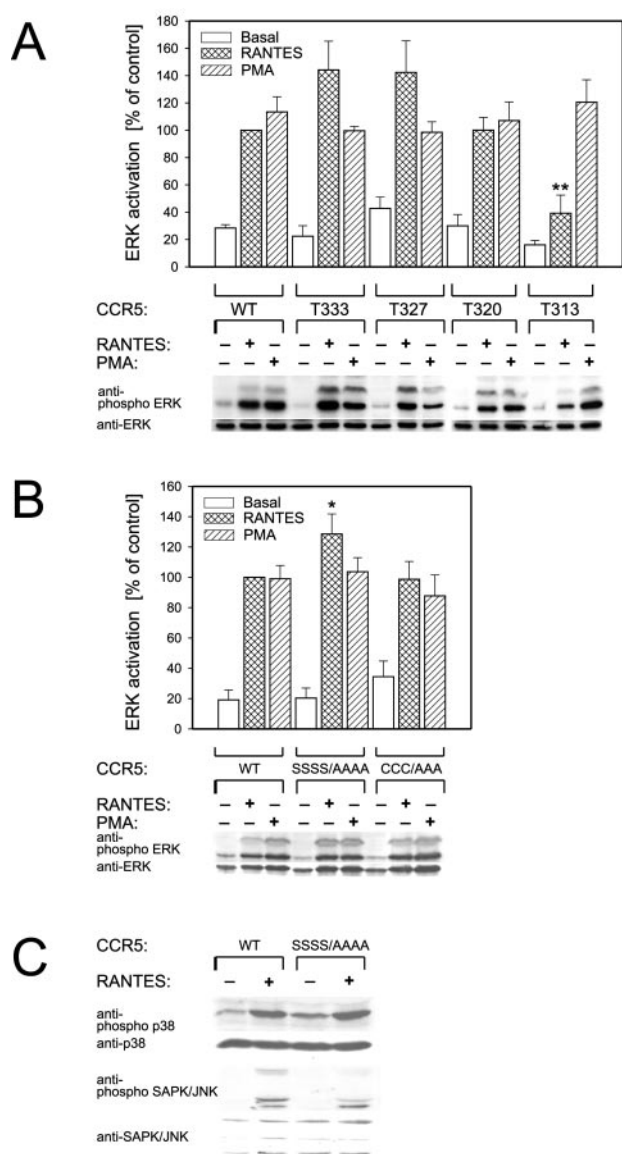
#### DISCUSSION

In this study we aimed at the identification of sequence elements within the CCR5 carboxyl terminus that regulate receptor signaling and internalization in transfected RBL-2H3 cells. There are two major results of this study. First, we demonstrate that CCR5 is palmitoylated on carboxyl-terminal cysteine residues and this lipid modification is required for efficient phosphorylation of the receptor by Ser/Thr protein kinases and for CCR5 endocytosis. Second, we show that interaction of  $\beta$ -arrestins with phosphorylated serine residues, which are located in the receptor carboxyl-terminal tail, fulfills

classical roles in CCR5 deactivation and internalization. However, this interaction appears dispensable for the chemokine-mediated activation of tyrosine kinases, which initiate the stimulation of MAP kinase pathways and granule enzyme release in these cells. Furthermore, we identify a conserved carboxyl-terminal dileucine motif that regulates CCR5 endocytosis by a mechanism independent of receptor phosphorylation.

Many GPCR and, specifically, 8 out of the 11 human CC chemokine receptors known to date contain one or several cysteine residues in their carboxyl-terminal domains that are potentially modified by thioesterification with palmitic acid. A palmitoylation-deficient mutant of the  $\beta_2$ -adrenergic receptor, a prototypic GPCR, is constitutively desensitized and hyperphosphorylated (28). In contrast, in the present study, we found that the palmitoylation-deficient CCR5-CCC/AAA mutant is not efficiently phosphorylated by either PKC or GRK kinases and shows a profound defect in agonist-induced receptor desensitization and internalization. This defect is not due to cysteine to alanine mutation *per se* since treatment of wild type CCR5-expressing cells with the palmitate analogue 2-bromopalmitate, which blocks palmitoylation (29), also results in a receptor phosphorylation defect.

Fatty acylation of GPCR may anchor the carboxyl-terminal segment of a receptor to the plasma membrane, thus creating an additional fourth intracellular loop. It was hypothesized that this conformational constraint imposed on the receptor modulates access of the carboxyl-terminal segment of the receptor to regulatory protein kinases (30). Indeed, with the  $\beta_2$ -adrenergic receptor, it was shown that a palmitoylation-deficient receptor mutant is a better substrate for the cyclic AMP-dependent protein kinase and elimination of the cyclic AMP-dependent protein kinase site from the depalmitoylated receptor restores normal G protein coupling (31). The CCR5 receptor is not a cyclic AMP-dependent protein kinase substrate but is phosphorylated by both PKC and GRKs on serine residues, which are located in close proximity to the palmitoylated carboxyl-terminal cysteines (13). It is conceivable that access of these kinases to CCR5 is also regulated by receptor palmitoylation, yet in a different manner. As shown with other receptors (32, 33), phosphorylation and palmitoylation appear to be closely interrelated mechanisms in the regulation of CCR5. Our finding that the palmitoylation-deficient CCR5-CCC/AAA mutant less efficiently mediated a RANTES-stimulated calcium response and granule release reaction from RBL-2H3 cells suggests that the palmitoylated carboxyl tail may also influence coupling of the receptor to heterotrimeric G proteins. Although this may have an effect on the ligand-induced phosphorylation of the receptor by GRKs, it clearly does



**FIG. 9. RANTES-induced activation of MAP kinases ERK1/2, p38, and SAPK/JNK in RBL-2H3 cells mediated by wild type (WT) or mutant CCR5.** Effect of progressive shortening of the carboxyl tail of CCR5 (A) or alanine replacement of carboxyl-terminal serine or cysteine residues (B) on the activation of ERK1/2 by RANTES in CCR5-expressing RBL-2H3 cells. ERK activation induced by RANTES (5 min, 50 nM) or PMA (10 min, 1  $\mu$ M) was detected by immunoblotting using phosphoERK- and total ERK-specific antibodies, and values were expressed as percentage of RANTES-induced ERK activation in wild type CCR5-expressing cells. Histograms represent mean  $\pm$  S.E. from three independent experiments. \*\*,  $p < 0.001$ ; \*,  $p < 0.05$  compared with CCR5-WT. C, RANTES-induced (15'/37  $^{\circ}$ C) activation of p38 and SAPK/JNK mitogen-activated protein kinases mediated by wild type and Ser/Ala CCR5 mutants as detected by immunoblotting using polyclonal rabbit antibodies, which specifically recognize only activated/phosphorylated forms of these MAP kinases. Membranes were reprobed with antibodies with specificity for total p38 or SAPK/JNK. Densitometric analysis of SAPK/JNK activation was performed by measuring ligand-induced phosphorylation of the p46 isoforms.

not explain the inability of PKC to phosphorylate CCR5-CCC/AAA upon PMA stimulation.

An alternative explanation for the phosphorylation defect in the non-palmitoylated CCR5 mutants relates to the property of palmitate to target proteins to specialized plasma membrane microdomains or rafts, which are enriched in sphingolipids and cholesterol (34). Membrane rafts act as specialized platforms within the lipid bilayer, which are characterized by spatial

concentration of membrane proteins with, among other cellular functions, important roles in signal transduction and endocytosis (35). Of note, CCR5 were reported to be constitutively associated with rafts (36). Whether CCR5 is present in membrane rafts in RBL-2H3 cells and what role, if any, palmitoylation has in targeting of receptors to these specialized microdomains remain to be determined in future studies.

Progressive truncation of the CCR5 carboxyl terminus revealed that this domain contains several sequence elements that regulate receptor expression at the cell surface and signal transduction. In keeping with previous studies (38, 39), a receptor mutant truncated after leucine 308 was normally expressed at the cell surface, but it lacked the ability to bind ligand with high affinity and to evoke cellular responses. The T304 mutant was largely retained intracellularly, indicating a critical role for amino acid residues 305–308 in proper targeting of CCR5 to the cell surface. Truncation of the receptor after Gln-313 resulted in a CCR5 mutant that did not induce calcium mobilization or granule release, even at high concentrations of ligand. However, the ability to activate MAPK pathways and mediate a chemotactic response toward micromolar concentrations of RANTES observed with this mutant indicates that this receptor may still possess residual G protein coupling capabilities. A receptor mutant T320, which lacked 31 carboxyl-terminal amino acid residues, was fully functional in all assay systems that were employed in this study. Altogether, our results suggest an important role for amino acid residues 308–320 of the CCR5 carboxyl terminus in coupling to heterotrimeric G proteins. Using a similar approach, Arai *et al.* (10) identified an analogous region within the carboxyl tail of the closely related CCR2 to be important for G protein coupling in this receptor.

The CCR5 carboxyl terminus contains sequence elements, which, by inference from other GPCR, can be assumed to present binding sites for intracellular adapters that facilitate receptor endocytosis, such as  $\beta$ -arrestins and AP-2. In this study we used FRET measurements to monitor the kinetics of the RANTES-induced association of CCR5 with  $\beta$ -arrestin. The interacting proteins were expressed in a functional form as carboxyl-terminal fusion proteins with the ECFP or EYFP variants of green fluorescent protein. Because the efficiency of energy transfer varies inversely with the sixth power of the donor to acceptor distance (40), FRET technology is an extremely sensitive method for determining the relative proximity of labeled protein partners. In our study we found that  $\beta$ -arrestin rapidly associated with the ligand-activated receptor and maximal FRET values were determined 10 min after addition of the stimulus. This time course differs from previously reported kinetics of RANTES-induced association of  $\beta$ -arrestin with this receptor as determined by co-immunoprecipitation, which was detected 30 min after RANTES stimulation (14). More importantly, we found that the formation of stable  $\beta$ -arrestin/CCR5 complexes depends on the presence of four carboxyl-terminal serine residues, which we showed in a previous study to represent the exclusive GRK phosphorylation sites on CCR5 (13). Our findings are consistent with the notion that GRK-mediated phosphorylation of carboxyl-terminal serine residues produces a localized concentration of negative charges, which facilitates ionic interactions with the positively charged phosphorylation recognition domain of  $\beta$ -arrestin. This may either directly enable a firm interaction between the two proteins or, alternatively, induce an intramolecular conformational change that exposes a secondary high affinity binding site on  $\beta$ -arrestin (41). Our data indicate that alanine mutation of four distinct carboxyl-terminal serine residues is sufficient to prevent such a high affinity interaction between  $\beta$ -arrestin and CCR5.



TABLE I

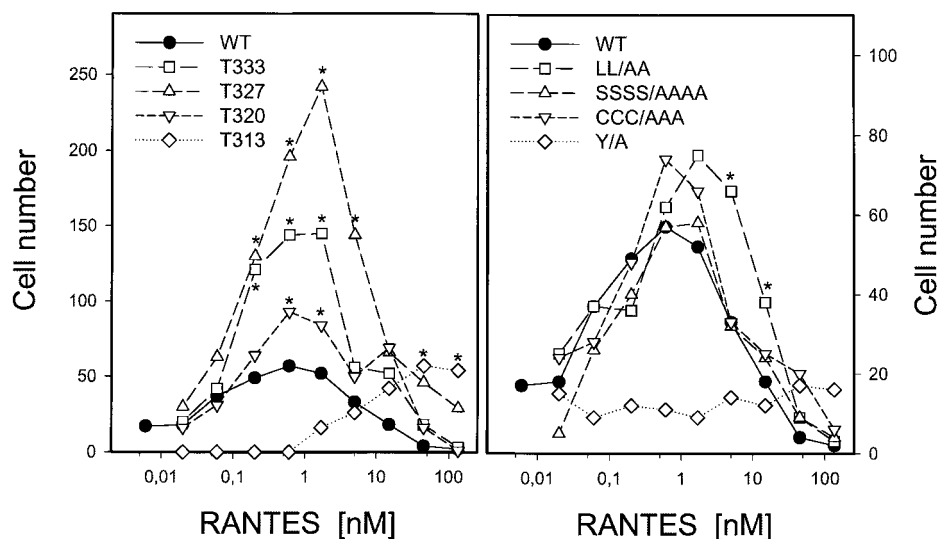
Cell surface expression, ligand binding affinity, and functional characteristics of CCR5 wild type and mutant receptors expressed in RBL cells

Data represent mean values from at least three independent experiments; ND denotes absence of detectable ligand binding or enzyme release even at high ligand concentrations; \*,  $p < 0.001$  versus CCR5-WT.

Receptor	Receptor expression <sup>a</sup>	MIP-1 $\alpha$ binding IC <sub>50</sub>	Glucosaminidase release	
			EC <sub>50</sub> $\pm$ S.E.	% $\pm$ S.E. of maximum
	<i>MCF</i>	<i>nM</i>	<i>nM</i>	
CCR5 wild type	7.7	19	9.5 $\pm$ 0.6	12.1 $\pm$ 2.7
CCR5-T333	13.2	30	2.8 $\pm$ 0.5	23.5 $\pm$ 3.0
CCR5-T327	7.8	41	7.8 $\pm$ 0.7	16.3 $\pm$ 3.3
CCR5-T320	9.7	48	3.8 $\pm$ 0.6	22.3 $\pm$ 0.9
CCR5-T313	3.6	293*	ND	ND
CCR5-T308	5.3	ND	ND	ND
CCR5-T304	1.5	ND	ND	ND
CCR5-SSSS/AAAA	6.8	15	2.7 $\pm$ 0.2	35.3 $\pm$ 0.9*
CCR5-CCC/AAA	7.9	27	16.9 $\pm$ 2.4	4.6 $\pm$ 0.4
CCR5-LL/AA	18.5	31	8.9 $\pm$ 0.6	18.1 $\pm$ 0.4
CCR5-LL/AA-SSSS/AAAA	16.1	15	3.7 $\pm$ 0.3	45.3 $\pm$ 0.8*
CCR5-Y/A	14.5	160*	ND	ND

<sup>a</sup> The mean channel of fluorescence (MCF) in non-transfected RBL-2H3 cells was 0.3.

FIG. 10. Chemotactic response of RBL cells expressing carboxyl-terminal truncation CCR5 mutants (left) or alanine mutated receptors (right). The experiments were performed as described under "Experimental Procedures," and the results represent mean values of one representative out of three to four experiments per receptor mutant. \*,  $p < 0.001$  compared with CCR5-WT cells. WT, wild type.



The CCR5-SSSS/AAAA mutant allowed us to specifically address the functional significance of the  $\beta$ -arrestin/CCR5 interaction without perturbing other receptor functions. Indeed, we observed that phosphorylation-deficient truncation mutants, but not the CCR5-Ser/Ala mutant, mediated a much higher chemotactic response to RANTES compared with wild type CCR5. This implies the existence of sequence elements encoded on the CCR5 carboxyl-terminal tail that negatively affect chemotaxis, but which are independent of receptor phosphorylation. Our study is the first to show a functional role for  $\beta$ -arrestin binding to phosphorylated receptors in the regulation of CCR5 in the context of a normal cellular complement of receptor kinases and of  $\beta$ -arrestin. Previously, Aramori *et al.* (12) showed that the inhibition of forskolin-stimulated cAMP formation by MIP-1 $\beta$  in HEK-293 cells that express CCR5 was not desensitized, unless the cells were co-transfected with GRKs. In this and another independent study (25), it was shown that the effect of  $\beta$ -arrestin on agonist-promoted receptor sequestration also required GRK overexpression. In our own experiments there was a clear difference between cells that express wild type or phosphorylation-deficient CCR5 mutants in their ability to desensitize the RANTES-induced calcium response and to sequester receptors from the cell surface in RBL-2H3 cells, even in the absence of GRK overexpression. The variations between these two studies probably relate to differences both in agonistic efficacy between RANTES and

MIP-1 $\beta$  and in expression levels of regulatory proteins in these two cell lines (13, 42).

Our study also identifies a conserved carboxyl-terminal dileucine sequence element that is involved in CCR5 endocytosis. Alanine mutation of the dileucine motif alone had a modest, yet significant effect on receptor internalization and a receptor variant (CCR5-LL/AA-SSSS/AAAA) in which both the serine phosphorylation sites and the dileucine sequence were replaced by alanines was more impaired in its ability to undergo ligand-induced sequestration than a Ser/Ala CCR5 mutant with an intact dileucine motif. The CCR5-LL/AA mutant induces cellular responses and is phosphorylated and desensitized in a manner indistinguishable from wild type CCR5. Thus, the dileucine motif and GRK-phosphorylated serine residues appear to regulate CCR5 endocytosis through independent mechanisms. The underlying mechanism that is responsible for the internalization defect of the CCR5-LL/AA mutant was not addressed in the present study. However, a LLKIL motif, which is located in the carboxyl tail of CXCR2 at a position analogous to the dileucine motif in CCR5, was shown to be largely responsible for interleukin-8-stimulated endocytosis of CXCR2, and alanine mutation of the first Leu-Leu pair was sufficient to produce the full internalization defect (11). Co-immunoprecipitation of adaptin 2 together with the ligand-stimulated CXCR2 required an intact LLKIL motif, but was independent of carboxyl-terminal phosphorylation sites. To-

gether, these results suggest a mechanism for agonist-induced CCR5 endocytosis via binding of two adapter proteins ( $\beta$ -arrestin, AP-2) to separate domains on the CCR5 carboxyl terminus in an independent manner that link the ligand-occupied receptor to clathrin-coated pits. Although the GRK/ $\beta$ -arrestin mechanism is clearly dominant in RBL-2H3 cells, other proteins may have a more prominent role in CCR5 endocytosis in cells that have a different cellular complement of receptor kinases or adapter proteins.

There is accumulating evidence to suggest that  $\beta$ -arrestins are involved not only in termination, but also in the initiation of different GPCR-mediated signaling events (16). The prominent role of  $\beta$ -arrestin in CCR5 deactivation and internalization that we identified in this study prompted us to investigate whether CCR5 mutants deficient in receptor phosphorylation can still activate tyrosine kinase pathways that are potentially regulated by  $\beta$ -arrestin. These studies are important for a better understanding on a molecular level of chemokine-regulated proliferation, migration, and granular enzyme release in inflammatory cells. By means of a specific inhibitor (PP-2), we confirm a critical role for Src-like tyrosine kinases in the chemokine-induced granular release reaction from RBL-2H3 cells. However, in striking contrast to the interleukin-8-induced glucosaminidase release from CXCR1-expressing RBL-2H3 cells, which requires intact carboxyl-terminal serine/threonine residues on CXCR1 (27), we find that cells that express phosphorylation-deficient CCR5 release significantly more enzyme upon RANTES stimulation as compared with wild type receptor-expressing RBL-2H3 cells. These divergent results provide an interesting example for the usage of different biochemical pathways by two closely related receptors expressed in an identical cellular background, which result in the activation of the same effector function. In RANTES-stimulated T cells, p56<sup>lck</sup> associates with CCR5 via activated Jak kinases (43) and thus provides an example of how Src kinases may be recruited to this receptor in a manner independent of  $\beta$ -arrestin.

We find that  $\beta$ -arrestin is also not essential for the RANTES-induced activation of the ERK1/2, p38, and JNK/SAPK mitogen-activated protein kinases in RBL-CCR5 cells. RANTES stimulation of phosphorylation-deficient CCR5 mutants leads to the activation of ERK1/2 kinases via pathways, which, as judged from kinase inhibitor studies, do not differ from pathways utilized in cells that express wild type CCR5. Particularly, we did not observe differential sensitivity of the two cell lines toward PKC or tyrosine kinase inhibitors, as was described in the PAR-2 system (26). The ability of various phosphorylation-deficient CCR5 mutants to evoke a larger and sustained calcium response correlated with their higher capacity to activate ERK1/2 in comparison to wild type receptors. This implies that a calcium-responsive tyrosine kinase such as Pyk2 may have a prominent role in CCR5-mediated MAP kinase activation (5).

The results of our chemotaxis experiments demonstrate that neither receptor desensitization nor internalization are required for directed cellular migration of RBL-CCR5 cells. On the contrary, several CCR5 truncation mutants produced an even higher chemotactic response than wild type cells. Although this result could be explained, in part, by greater levels of secondary messengers that are generated in desensitization-deficient cells, the failure of cells that express CCR5-SSSS/AAAA to show the same effect implies the existence of sequence elements within the CCR5 carboxyl terminus that potentially affect chemotaxis in a negative way. Our conclusion that chemotaxis does not require adaptation mechanisms that operate at the receptor level is supported by similar findings with CCR2b, CXCR2, and N-formyl peptide receptor mutants defi-

cient in receptor desensitization and internalization (9, 10, 44). Whereas a dileucine-containing motif in CXCR2 is critical for receptor-mediated chemotaxis in response to interleukin-8 (8, 11), mutation of an analogous region within the CCR5 carboxyl terminus does not impair cellular migration.

CCR5 is a principal HIV-1 co-receptor and has been implicated in the pathogenesis of various chronic inflammatory diseases, including multiple sclerosis and rheumatoid arthritis. Natural mutations in the CCR5 coding region are found in various human populations in surprisingly high frequencies, and several of these affect the cytoplasmic tail (45). The identification of discrete regions within the CCR5 carboxyl-terminal domain that influence transport of the receptor to the plasma membrane, effector coupling, deactivation, and internalization of this receptor may, therefore, have significant pathophysiological relevance.

*Addendum*—While this manuscript was under revision, we became aware of two electronic pre-publications (46, 47), which also demonstrate that CCR5 is palmitoylated on up to three carboxyl-terminal cysteine residues and, furthermore, imply an important role for CCR5 palmitoylation in proper targeting of the receptor to the cell surface. In keeping with the results of our own study, it was shown that palmitoylation-deficient receptors that reach the cell membrane bind ligand with normal affinity and are also functional, albeit with reduced potency. However, the report by Blanpain *et al.* (46) of normal chemokine-induced internalization of a palmitoylation-deficient CCR5 Cys/Ala mutant contrasts with our finding of a significant endocytosis defect of an identical receptor mutant. These divergent results might possibly be due to differences in the methods used to assess receptor internalization. In the study by Blanpain *et al.* (46), CCR5 endocytosis was determined by antibody staining of receptors that are detectable at the cell surface after incubation of wild type or mutant cells with chemokines for 45 min and subsequent fluorescence-activated cell sorting analysis. Since, in Chinese hamster ovary cells, a significant fraction of CCR5 recycles back to the cell surface during prolonged incubation with RANTES or AOP-RANTES, alterations in cell surface expression of CCR5 do not directly reflect receptor endocytosis (37). Interestingly, we find that differences in the uptake of radiolabeled RANTES between wild type and palmitoylation-deficient CCR5 are most pronounced at early time points, suggesting that the mutation primarily affects kinetics, rather than overall efficiency of ligand endocytosis. Alternatively, differences between these two studies might be related to different cellular milieus in which the receptors were expressed and which potentially differ in GRK/ $\beta$ -arrestin expression levels and the preferential utilization of distinct endocytic pathways.

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Signaling and Receptor Internalization**

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