Differential Effects of CC Chemokines on CC Chemokine Receptor 5 (CCR5) Phosphorylation and Identification of Phosphorylation Sites on the CCR5 Carboxyl Terminus

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The binding of CC chemokines to CC chemokine receptor 5 (CCR5) triggers cellular responses that, generally, are only transient in nature. To explore the potential role of G protein-coupled receptor kinases (GRKs) in the regulation of CCR5, we performed phosphorylation experiments in a rat basophilic leukemia cell line stably expressing CCR5. The ability of various CCR5 ligands to stimulate calcium mobilization in these cells correlated with their ability to induce receptor phosphorylation, desensitization, internalization, and GRK association with the receptor. Aminooxypentane-RANTES, a potent inhibitor of human immunodeficiency virus infection, has been proposed to act through enhanced CCR5 internalization and inhibition of receptor recycling. Aminooxypentane-RANTES profoundly induced CCR5 phosphorylation, but had no effect on CCR1. In permeabilized rat basophilic leukemia CCR5 cells, monoclonal antibodies with specificity for GRK2 or GRK3 inhibited RANTES-induced receptor phosphorylation. Consistent with a role for these kinases in CCR5 regulation, 1–2 × 10⁶ copies of GRK2 or GRK3 were found to be expressed in peripheral blood leukocytes. Phosphoamino acid analysis revealed that RANTES-induced CCR5 phosphorylation selectively occurs on serine residues. Our findings with receptor mutants indicate that serine residues at positions 336, 337, 342, and 349 represent amino acid residues within the CCR5 carboxyl terminus that are phosphorylated upon cellular stimulation with CC chemokines. This study demonstrates that chemokines differ in their ability to induce CCR5 phosphorylation and desensitization and provides a molecular mechanism for the agonist-induced attenuation of CCR5 signaling.

Chemokines are a large family of chemotactic cytokines that mediate leukocyte activation and recruitment to sites of inflammation (1). They are small polypeptides of 8–10 kDa, which can be subdivided into two major gene families depending on the relative position of four conserved cysteines, the first two of which are adjacent in CC chemokines or separated by one intervening residue in CXC chemokines. Both CC and CXC chemokines bind to heptahelical G protein-coupled receptors which transduce signals through members of the G₁ or Gq family of G proteins (2, 3). The ligand-binding repertoires of different chemokine receptors significantly overlap, as do the sets of receptors expressed by different leukocytes and other target cells. Further adding to the versatility of the chemokine system, multiple and distinct signaling pathways exist for individual receptors, which are differentially regulated depending on the ligand and cell type involved (2, 4).

Like many other G protein-coupled receptors, chemokine receptors demonstrate diminishing responsiveness upon repeated or prolonged exposure to agonist, a phenomenon generally referred to as receptor desensitization. Other regulatory processes are receptor sequestration and degradation, which summarily contribute to dampening the cellular response. Receptor phosphorylation by G protein-coupled receptor kinases (GRKs) has been shown to be crucial in the rapid agonist-induced desensitization of many G protein-coupled receptor systems (5). GRK-mediated receptor phosphorylation promotes the binding of regulatory arrestin proteins, which results in further uncoupling of receptor-G protein interactions. Agonist-induced desensitization is clearly important in the control of the cytotoxic potential of leukocytes in the response to chemoattractant agonists. Furthermore, this regulatory mechanism may be critical for chemokine receptors whose primary function is to continuously sense small changes in the gradients of chemoattractants and thereby direct cellular migration.

In the present study, we investigated early signaling events, which are initiated after ligand binding to CC chemokine receptor CCR5 and contribute to receptor desensitization. Under physiological conditions, CCR5 interacts with RANTES, MIP-1α, MIP-1β, or MCP-2 (6, 7). In addition, amino-terminal modifications of RANTES (Met-RANTES and AOP-RANTES) have been described, creating receptor ligands with antagonistic properties (8, 9). We show that these various CCR5 ligands differ in their abilities to stimulate the receptor-G protein-PLC system in RBL-2H3 cells and that the intrinsic activities of these compounds directly correlate with their abilities to induce CCR5 phosphorylation and desensitization through a GRK-mediated mechanism. Furthermore, we identify amino acid residues within the CCR5 carboxyl terminus that are phosphorylated upon cellular stimulation with CC chemokines.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture media and cell culture supplies were from Biochrom; RBL-2H3 and COS-7 cells were from the American Type Culture Collection; 12-myristate 13-acetate; RBL, rat basophilic leukemia; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein.

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§ The abbreviations used are: GRK, G protein-coupled receptor kinase; AOP-RANTES, aminooxypentane RANTES; RANTES, regulated on activation normal T cell expressed and secreted; CCR, CC chemokine receptor; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; Met-RANTES, methionylated RANTES; PAGE, polyacrylamide gel electrophoresis; Pipes, 1,4-piperazinediethanesulfonic acid; PKC, protein kinase C; PLC, phospholipase C; FMA, phorbol 12-myristate 13-acetate; RBL, rat basophilic leukemia; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein.
Phosphorylation Experiments—The agonist-induced phosphorylation of CCR5 in intact cells was determined as described previously (16). Briefly, RBL-CCR5 cells or COS-7 cells transiently transfected with CCR5-pEF-BOS were metabolically labeled with [32P]ATP. After treatment with different stimuli as indicated, cells were washed and solubilized in 0.5% Triton X-100, 50 mM HEPES (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% EDTA, 1% (w/v) Triton X-100, 0.05% SDS, with phosphatase and protease inhibitors as described (Ref. 16). Receptors were immunoprecipitated by incubating precleared cellular lysates with 12 μg of anti-FLAG M2 monoclonal IgG1 and protein G-Sepharose. Immune complexes were dissociated and resolved with SDS-PAGE using 10% gels. Radioactively labeled proteins were visualized by autoradiography and analyzed with a Molecular Dynamics PhosphorImager. *Receptor phosphorylation data were corrected for different receptor expression levels in transfected cells by multiplying the relative receptor expression as assessed by flow cytometry using receptor-specific antibodies (percentage of positive cells multiplied by the mean channel of fluorescence of positive cells) with the protein content of each immunoprecipitation mixture. The experimental protocol for determining receptor phosphorylation in permeabilized cells has been described (18). In brief, RBL-CCR5 cells were permeabilized by incubation for 5 min with 0.5 unit of streptolysin O in KG buffer (139 mM potassium glutamate, 5 mM glu- cose, 20 mM potassium salt of Pipes, 2.5 mM MgCl2, 1 mM EDTA, pH 7.4). This procedure resulted in the permeabilization of >95% of the cell population, as determined by trypan blue exclusion. Cells were incubated with various antibodies (500 μg/ml) for 10 min in KG buffer containing 10 μM ATP and 50 μM GTP in the presence or absence of staurosporine (500 nM). The concentration of 32P-labeled ATP (100 μCi/ml) was added, and cells were stimulated for 5 min with the indicated stimuli. Cells were lysed with detergent buffer, and receptors were immunoprecipitated using the same protocol as described for whole cell phosphorylation experiments.

Phosphoamino Acid Analysis—Labeled receptors were electro- phoretically transferred to polyvinylidene difluoride membranes (Im mobilon PVDF), excised and hydrolyzed in 6 N HCl for 1 h at 110 °C. The hydrolsates were lyophilized and resolubilized in pH 1.9 buffer (formic acid/acetate/H2O, 50/156/1794, (v/v/v)) containing phospho- amino acid standards. The samples were spotted on thin-layer cel- lulose plates and phosphoamino acids were separated by electrophoresis (900 V/1.5 h) at pH 1.9, followed by a second electrophoresis (900 V/45 min) at pH 3.5 (pyridine/acetate/H2O, 10/100/1890, (v/v/v)) in the orthogonal direction. After ninhydrin staining, thin layer plates were exposed to PhosphorImager screens.

Calcium Fluorometry and Internalization Assay—RBL-CCR5 or CHO-CCR1 cells were washed with buffer A (145 mM NaCl, 5 mM KCl, 1 mM CaCl2, 10 mM HEPES, pH 7.4) and 1 mM fluorescence (30 min/37 °C) to 105 cells/ml in the same buffer containing 0.1% bovine serum albumin and 8 μM Fluo3-AM. After washing the cells with buffer A, cells were resuspended at 5 × 105 cells/ml of buffer A. Chemokine-induced intracellular calcium mobilization was determined by spectrofluorometry using a Perkin Elmer MFP-44B fluorescence spectrophotometer with an excitation wavelength of 506 nm and an emission wavelength of 510 nm. Intracellular calcium levels were calculated using the equation \( [\text{Ca}^{2+}]_i = K_d \times (F_\text{max} - F) / (F_\text{max} - F) \) (19), where \( K_d \) is the dissociation constant of Fluo3-AM, \( F_\text{max} \) is the autofluorescence of cells that were incubated in the absence of calcium chelator and \( F_\text{max} \) is the maximal fluorescence determined with detergent-lysed cells.

The agonist-induced internalization of CCR5 in RBL-CCR5 cells was determined, in principal, as described previously (10). In brief, 5 × 105 RBL-CCR5 cells were incubated (30 min/37°C) in 100 μl of medium containing various concentrations of chemokines. Thereafter, cells were cooled to 4 °C and surface-expressed CCR5 were detected by flow cytom lex to the anti-CCR5 mAb MC-4 and a fluorescein isothiocyanate- labeled rabbit anti-mouse F(ab')2 fragment (Dako). The relative CCR5 surface expression was calculated as 100 × (mean channel of fluorescence [stimulated] – mean channel of fluorescence [negative control]) / (mean channel of fluorescence [stimulated] – mean channel of fluorescence [negative control]) [%].

GRK-specific mAb and ELISA Procedures—Synthetic peptides that correspond to amino acids Val-658 to Leu-689 of bovine GRK2 and Leu-658 to Leu-689 of bovine GRK3, respectively, were synthesized on an Applied Biosystems 430A peptide synthesizer using FastMoc chemistry, and the products were purified by high performance liquid chromatography. Peptides were coupled through an additional cysteine residue at the peptide amino terminus to bovine serum albumin using the heterobifunctional cross-linking reagent succinimidyl 4-(N-maleimido-ethyl)cyclohexane-1-carboxylic acid as described (20). Monoclonal antibodies were generated according to standard techniques following the immunization of BALB/c mice with peptide-bovine serum albumin con-
jugates. Hybridoma supernatants were screened by solid phase ELISA techniques or immunoblotting employing purified recombinant GRKs or cellular lysates from cells that overexpressed individual GRKs as the antigens. While several mAbs were obtained that specifically react with either GRK2 alone or both GRK2 and GRK3, no antibody was identified that solely recognizes GRK3. ELISA procedures were established that allowed the sensitive quantitation of GRK2 or GRK3 in cellular lysates. For the quantitation of GRK2, the mAb C5/1 (18), which recognizes a conserved sequence (Ala-480 to Phe-488) present in both GRK2 and GRK3, was adsorbed into wells of microtiter plates (5 μg/ml) in 50 mM carbonate, pH 10.6. Purified recombinant GRK2 as the protein standard or cellular lysates were diluted in phosphate-buffered saline-0.05% Tween and were allowed to bind for 2 h. GRK2 that had bound to the first mAb was detected by adding, in sequence, the biotinylated GRK2-specific mAb G7/5 (1 μg/ml; 1 h), a 4000-fold dilution of streptavidin-horseradish peroxidase (1 h) and 2,2-azino-di-(3-ethylbenzthiazoline sulfonate) as substrate. The quantitation of GRK3 by ELISA followed the same protocol except for the following modifications. Recombinant GRK3 as the protein standard or GRK3 present in cellular lysates was detected by adding the biotinylated GRK23-specific mAb G7/5 (1 μg/ml) in the presence of a 100-fold excess of mAb E23/8 (which blocks G7/5 binding to GRK2) to wells containing mAb C5/1 as the first antibody. Detection limits of these assays are 15 ng/ml GRK2 or GRK3. Purified recombinant GRK2 and GRK3 were kindly provided by Dr. Robert J. Lefkowitz (Duke University, Durham, NC).

**RESULTS**

**Chemokine-induced Receptor Activation, Desensitization, and Internalization**—To investigate the signaling properties of CCR5 in response to different chemokines, RBL-2H3 cells were stably transfected with a cDNA construct encoding human CCR5. Fig. 1 shows that RANTES, AOP-RANTES, Met-RANTES, MIP-1α, and MIP-1β induced calcium mobilization in RBL-CCR5 cells, albeit with different efficiencies. Dose-response curves indicated that the maximal MIP-1α or MIP-1β induced calcium mobilization amounted to only 30% of the signal seen in cells that were stimulated with AOP-RANTES or RANTES. Met-RANTES was the least effective of all CCR5 ligands tested. Half-maximal effects were observed at concentrations between 1.5 and 6 nM for the different chemokines, with AOP-RANTES being the most potent agonist.

**Other groups** using different cells and assay systems, reported rank orders of signaling efficacies of CC chemokines in the CCR5 system that partially deviate from our findings (6, 7, 10, 22). This may point to cell type-specific effects or to the differential activation of the various intracellular effectors by CCR5 signaling in response to chemokines (data not shown).

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alternative biological activities of the different preparations of recombinant chemokines may account for these discrepancies. We therefore compared chemokine effects on CCR5 with those on the closely related chemokine receptor CCR1. Here, RANTES and MIP-1α were essentially equally effective (Fig. 1). Interestingly, AOP-RANTES failed to activate intracellular calcium mobilization in CCR1-expressing cells.

RANTES-induced calcium mobilization was effectively desensitized in RBL-CCR5 cells by prior treatment with RANTES or with AOP-RANTES (Fig. 2). In contrast, pretreatment with MIP-1α, MIP-1β, or Met-RANTES reduced the subsequent RANTES-induced cellular stimulation by only 15–20%. Thus, the abilities of the different receptor ligands to activate calcium mobilization via CCR5 paralleled their abilities to induce receptor desensitization. This generally good fit between the efficacies of chemokines in receptor activation and desensitization was also observed in the CCR1 system (Fig. 2). Activation of PKC by treatment with PMA inhibited the subsequent RANTES-induced calcium mobilization via both CCR1 and CCR5.

Because of the importance of agonist-induced receptor internalization for receptor desensitization and resensitization in many G protein-coupled receptor systems, we also analyzed the effects of chemokines on CCR5 endocytosis in RBL-CCR5 cells. As shown in Fig. 3, AOP-RANTES was more effective than RANTES and Met-RANTES at receptor endocytosis. While 10 nM AOP-RANTES induced internalization of >80% of CCR5, treatment of RBL-CCR5 cells with equivalent concentrations of RANTES or Met-RANTES resulted in the down-regulation of surface-expressed CCR5 of only 60% or <20%, respectively. This result confirms previous findings with chemokine-induced CCR5 endocytosis in CHO-CCR5 cells (10).

Receptor Phosphorylation by Chemokines and PMA—To determine whether receptor phosphorylation correlated with the observed chemokine-induced receptor desensitization and internalization we immunoprecipitated CCR5 from RBL cells which were metabolically labeled with 32P and subsequently stimulated with various agonists. In a dose-dependent fashion, RANTES promoted within 5 min the phosphorylation of a 40-kDa band, which was absent in untransfected 32P-labeled control cells. At receptor-saturating concentrations, RANTES increased the phosphorylation of CCR5 in RBL cells 4.5-fold above basal levels (Fig. 4). After stimulation of these cells with MIP-1α or MIP-1β, maximal 32P incorporation into this receptor amounted to only 18 ± 4% or 12 ± 2% of the signal observed after RANTES stimulation. Yet, these signals were significantly (p < 0.01) different from basal values in the absence of stimulus. While Met-RANTES was the least effective of all CCR5 ligands tested, the maximal AOP-RANTES induced receptor phosphorylation was 2.8 ± 0.3-fold higher than in cells that had been stimulated with RANTES. Pretreatment of RBL-CCR5 cells with pertussis toxin reduced the maximal AOP-RANTES-induced receptor phosphorylation by 55% (data not shown). Again, MIP-1α and RANTES induced CCR1 phosphorylation equally well, while stimulation with AOP-RANTES and Met-RANTES did not result in significant CCR1 phosphorylation.

Since our calcium mobilization experiments had indicated that in RBL cells CCR5 is coupled to phospholipase C, we sought to determine the extent to which PKC might participate in the agonist-induced CCR5 phosphorylation. The phorbol es-
ter PMA significantly increased CCR5 phosphorylation above basal values (Fig. 5). However, pretreatment of RBL-CCR5 cells with the PKC inhibitor bisindolylmaleimide (BIM; 2 μM) for 10 min. Cells were then treated with either 250 nM PMA or with 100 nM AOP-RANTES for 10 min. Receptors were immunoprecipitated, resolved by SDS-PAGE, and subjected to autoradiography (A) and quantitative PhosphorImager analysis (B). Radioactive counts in the receptor bands were normalized to those in cells stimulated with 100 nM RANTES in the absence of PKC inhibitor. Data represent mean ± S.E. of three experiments; **, p < 0.001; *, p < 0.05 compared with samples without bisindolylmaleimide.

PLC (7). We therefore compared the RANTES-induced receptor phosphorylation in COS-7 cells that expressed CCR5 either alone or together with G_{q5o}. In G_{q5o}, the carboxy-terminal five amino acids of G_{αq}, which mediate receptor binding, replace those of G_{αq}. As shown in Fig. 6, CCR5 was rapidly phosphorylated even in the absence of G_{q5o}. Upon co-transfection of COS-7 cells with expression plasmids for CCR5 and G_{q5o}, agonist-promoted receptor phosphorylation was further enhanced up to 2-fold. This effect was, in part, sensitive to bisindolylmaleimide (data not shown). These results indicate that rapid chemokine-induced CCR5 phosphorylation derives principally from second messenger-independent kinases. Upon prolonged exposure to receptor ligand, however, PKC may significantly contribute to the agonist-induced receptor phosphorylation.

**GRK Expression Levels**—The rapid time course of RANTES-induced CCR5 phosphorylation by a second messenger-independent protein kinase is suggestive of a GRK-mediated mechanism. If CCR5 phosphorylation and desensitization under physiological conditions is to be attributed to the action of one or several GRK(s), GRKs need to be expressed in the same cells that normally express CCR5. We therefore determined expression levels of the widely expressed kinases GRK2, GRK3, GRK5, and GRK6 in human peripheral blood leukocytes and the various cell lines that were used in this study. First, we performed immunoblot analysis of concentrated cellular lysates using GRK-specific antibodies (data not shown). While GRK5 and GRK6 were readily detected in cells that overexpressed these kinases, they were absent (or below the detection limit of our assay) in leukocytes and cell lines relevant to this study. In contrast, GRK2/3-specific antibodies immunoprecipitated 80-kDa proteins from cellular lysates which comigrated with recombinant GRK2 or GRK3. To more accurately determine cellular expression levels of these two closely related receptor kinases, we established ELISA procedures for the quantitation of GRK concentrations in cellular lysates which are based on GRK-specific monoclonal antibodies. Human leukocyte subpopulations contain approximately 1 × 10^5 molecules GRK2 per cell and 2 × 10^5 molecules GRK3 per cell (Table I). GRK2 is the predominant receptor kinase in RBL cells, where it is expressed at high levels. In contrast, GRK
levels in COS-7 cells were at the lower detection limit (10 ng/mg of cellular protein) of the assays.

**Phosphorylation of CCR5 by GRK2 and GRK3 and Translocation of GRK2 to the Receptor—** If GRK2 or GRK3 are involved in RANTES-induced receptor phosphorylation, overexpression of these kinases can be expected to enhance CCR5 phosphorylation upon agonist stimulation. As shown in Fig. 7, overexpression of GRK3 together with CCR5 in COS-7 cells significantly (p < 0.05) enhanced receptor phosphorylation following cellular stimulation with chemokines. Among the different CCR5 ligands, overexpression of GRK3 had the least effect on the AOP-RANTES-induced receptor phosphorylation. This result suggests that, with AOP-RANTES, most of the CCR5 phosphorylation sites available to GRKs are phosphorylated by endogenous kinases. In contrast, receptor phosphorylation by MetRANTES, MIP-1α, or MIP-1β, which hardly induced any CCR5 phosphorylation in the absence of overexpressed GRKs, was enhanced up to 12-fold in the presence of high intracellular GRK3 levels. We also tested whether individual GRKs differ in their abilities to enhance CCR5 phosphorylation in response to agonist. Overexpression of GRK2, GRK3, GRK5, or GRK6 augmented RANTES-induced receptor phosphorylation 3–5-fold over that determined in control cells (data not shown). Significant differences between individual GRKs with regard to receptor phosphorylation were not observed.

To more directly address the question of which endogenously expressed protein kinases mediate CCR5 phosphorylation in RBL cells, we used monoclonal antibodies as intracellular inhibitors of endogenous GRKs in permeabilized cells. As shown in Fig. 8, the RANTES-stimulated receptor phosphorylation in permeabilized RBL-CCR5 cells was reduced by 60 ± 14% by antibodies (mAb C5/1) that block GRK2 and GRK3 while GRK4–6-specific antibodies (mAb A16/17) had no effect. A GRK2-specific mAb (E23/8), which is directed against an GRK2 carboxyl-terminal epitope and does not inhibit GRK2 activity, also did not affect CCR5 phosphorylation in permeabilized cells. In the presence of staurosporine, which suppressed basal levels of receptor phosphorylation, the inhibitory effect of anti-GRK2/3 antibodies was even more pronounced (92 ± 11% inhibition compared with control cells).

Translocation of cytosolic kinases to the membrane is an essential step in the agonist-induced receptor phosphorylation by GRK2 or GRK3. Given the relatively high expression levels of GRK2 and GRK3 in RBL cells, we asked whether these kinases can be found associated with CCR5 after chemokine stimulation. We therefore co-immunoprecipitated endogenous GRKs together with CCR5 from stimulated RBL-CCR5 cells. The result from one representative Western blotting experiment is depicted in Fig. 9. Only small amounts of GRK2/3 were found to be associated with CCR5 in cells that had been treated with medium or with MIP-1α. In contrast, stimulation of RBL-CCR5 cells with AOP-RANTES or RANTES led to a significant increase in receptor-associated GRK2/3. Thus, at least for these three agonists, ligand-induced association of GRKs with CCR5 appears to correlate with receptor phosphorylation and desensitization.

**Phosphoamino Acid Analysis and Identification of Phospho-**

**TABLE I**

*Expression levels of GRK2 and GRK3 in human leukocytes and in cell lines*

| GRK2 | GRK3 |
|------|------|
| Molecules/cell | Protein | Molecules/cell | Protein |
| × 10⁶ | ng/mg | × 10⁶ | ng/mg |
| Lymphocytes (n = 15) | 128 ± 74 | 50 ± 28 | 205 ± 80 | 76 ± 29 |
| Monocytes (n = 15) | 126 ± 22 | 18 ± 27 | 268 ± 31 | 49 ± 46 |
| Granulocytes (n = 7) | 102 ± 30 | 14 ± 6 | 222 ± 17 | 30 ± 24 |
| RBL cells | 645 ± 66 | 44 ± 3 | 195 ± 17 | 13 ± 8 |
| COS cells | ND | <10 | ND | <10 |
| COS-GRK2 | ND | 1670 ± 326 | ND | 1830 ± 186 |
| COS-GRK3 | ND | ND | ND | ND |

**Fig. 7.** Chemokine-induced phosphorylation of CCR5: augmentation by overexpressed GRK3. COS-7 cells were transfected with plasmids encoding CCR5 along with expression plasmids encoding either no protein (Control) or GRK3 (+GRK3). [32P]Labeled cells were stimulated with 100 nM of the indicated chemokines for 5 min. Receptor immunoprecipitation and analysis proceeded as described for Fig. 3. A, shown is an autoradiogram representative of three experiments. B, data (mean ± S.E.) from quantitative PhosphorImager analysis were normalized to values obtained from RANTES-stimulated cells in the absence of overexpressed GRK3.
mechanisms occurs exclusively on serine residues (Fig. 10).

We used a mutational approach to define the precise location of those serines that are phosphorylated upon cellular stimulation with RANTES or AOP-RANTES. First, a CCR5 truncation mutant was generated by introducing a stop codon at position CCR5–334. This truncated version of CCR5 was not phosphorylated upon agonist stimulation (not shown), indicating that Ser-325 is not a GRK phosphorylation site. The remaining four serine residues at positions 336, 337, 342, and 349 were substituted with alanine either individually or in various combinations. As shown in Fig. 11, alanine-mutagenesis of any single or any two serines did not significantly affect the RANTES-induced CCR5 phosphorylation as compared with wild type receptor. In contrast, AOP-RANTES-stimulated receptor phosphorylation was reduced 50–70% relative to CCR5 wild type, and alanine substitution of all four serines completely abrogated agonist-stimulated receptor phosphorylation.

Furthermore, we observed a stepwise shift in the electrophoretic mobility of phosphorylated receptor variants, depending on the number of Ser to Ala mutations (Fig. 12, left). The immunoblot (Fig. 12, right) revealed the presence of additional receptor bands in lanes B-D with the same electrophoretic mobility as found in non-stimulated cells. These bands probably correspond to intracellular receptors, which form a significant portion of the total cellular receptor complement in transiently transfected cells and which are, consequently, neither exposed to ligand nor phosphorylated by receptor kinases. The observed stepwise shift in the electrophoretic mobility of phosphorylated CCR5 suggests that AOP-RANTES stimulates incorporation at three to four acceptor sites per receptor. In contrast, RANTES apparently induces receptor phosphorylation by GRKs at a lower stoichiometry with no preference for any specific residue(s) among the four serines.

To directly investigate whether each of the four serines at positions 336, 337, 342, and 349 is a GRK substrate, we co-expressed GRK3 together with CCR5 triple mutants in COS-7 cells (Fig. 13). Overexpression of GRK3 resulted in a significant increase in the RANTES-induced phosphorylation not only of CCR5 wild type, but also of every of these four receptor variants. Since GRK3 overexpression had no effect on the CCR5 phosphorylation in permeabilized RBL-CCR5 cells by GRK-specific antibodies. Cells were permeabilized by streptolysin O (0.5 unit/ml) and incubated with anti-glutathione S-transferase mAb O3/5 (control), anti-GRK2/3 mAb C5/1, anti-GRK2 mAb E23/8, or anti-GRK4–6 mAb A16/7 (0.5 mg/ml) in buffer containing [γ-32P]ATP with or without staurosporine. After stimulation with RANTES (100 nM/5 min), cells were lysed and receptors were immunoprecipitated. A, the autoradiogram from one representative experiment is shown. B, data are mean ± S.E. from three experiments. *, p < 0.005 compared with RANTES-stimulated cells.

FIG. 8. Inhibition of RANTES-induced CCR5 phosphorylation in permeabilized RBL-CCR5 cells by GRK-specific antibodies. Cells were permeabilized by streptolysin O (0.5 unit/ml) and incubated with anti-glutathione S-transferase mAb O3/5 (control), anti-GRK2/3 mAb C5/1, anti-GRK2 mAb E23/8, or anti-GRK4–6 mAb A16/7 (0.5 mg/ml) in buffer containing [γ-32P]ATP with or without staurosporine. After stimulation with RANTES (100 nM/5 min), cells were lysed and receptors were immunoprecipitated. A, the autoradiogram from one representative experiment is shown. B, data are mean ± S.E. from three experiments. *, p < 0.005 compared with RANTES-stimulated cells.
mutant that had all four serines mutated to alanine, these residues represent the only sites on CCR5 available to GRK3 for agonist-induced phosphorylation.

**DISCUSSION**

Several lines of evidence from the current study point to a central role for GRK2 and/or GRK3 in the agonist-induced phosphorylation of CCR5. First, CCR5 transiently expressed in COS-7 cells was still rapidly phosphorylated upon stimulation with RANTES despite the fact that COS-7 cells apparently lack signaling elements that would allow efficient coupling of CCR5 to PLC (7, 24). This suggests that homologous CCR5 phosphorylation, at least after short term exposure to agonist, is largely independent of second messenger-dependent protein kinases. Our finding of a significant reduction of receptor phosphorylation in pertussis toxin-treated RBL-CCR5 cells does not preclude a major role for GRKs in receptor phosphorylation. ADP-ribosylation of Gαi prevents receptor-induced dissociation of heterotrimeric G proteins and thereby interferes with Gαi-mediated targeting to membrane-bound receptors of GRK2 and GRK3 (25). Second, the time course of RANTES-induced CCR5 phosphorylation conforms with the rapid kinetics of GRK-mediated receptor phosphorylation observed in previous studies (16, 26). Second, overexpression of GRKs in COS-7 cells was shown to significantly enhance chemokine-induced CCR5 phosphorylation. While this finding shows that agonist-occupied CCR5 is a potential substrate for GRKs, it does not directly address the question of which endogenously expressed kinases actually mediate receptor phosphorylation in RBL cells or in leukocytes. We therefore adapted a previously described method for the intracellular inhibition of receptor kinases by anti-GRK antibodies in permeabilized cells (18) to the CCR5 system in RBL cells. These experiments revealed that GRK2 and/or GRK3 are the major kinases that mediate RANTES-induced CCR5 phosphorylation in RBL cells. Consistent with a major role for GRK2 and GRK3 in CCR5 phosphorylation under physiological conditions, we determined high intracellular levels of these kinases in human leukocytes and in RBL cells. In this study we provide, to our knowledge for the first time, quantitative estimations of intracellular GRK concentrations in biological materials. These data not only support a role for GRKs in the regulation of chemotactic leukocyte receptors, they also shed light on the stoichiometry of signaling components that participate in the regulation of G protein-coupled receptors in native cells. With 1–2 × 10⁵ molecules of GRK2 or GRK3 per cell, these kinases are expressed in leukocytes in numbers that are roughly equivalent to expression levels of many G protein-coupled receptors, but much lower than concentrations of Gα subunits (27). The relative abundance of signaling elements that transduce signals compared with levels of receptor kinases may ensure that ligand binding will still lead to cellular activation until receptor inactivation by phosphorylation and arrestin binding gradually takes over.

Although GRK2 and/or GRK3 are the protein kinases that are mainly responsible for the rapid agonist-induced phosphorylation of CCR5 in RBL cells and possibly in human peripheral blood leukocytes, this receptor is also a substrate for PKC, the kinase that is activated by signaling pathways downstream of CCR5. Inhibitor studies using bisindolylmaleimide or anti-GRK antibodies revealed that PKC only modestly contributes to rapid RANTES-induced receptor phosphorylation in RBL-CCR5 cells. In COS-7 cells, restoration of the receptor-PLC-PKC system by co-transfection with Gqα resulted in a significant increase in CCR5 phosphorylation only upon prolonged (>10 min) stimulation with agonist. The kinetics of GRK- versus PKC-mediated CCR5 phosphorylation resembles the time course of GRK- and second messenger-dependent kinase-mediated phosphorylation and desensitization in other receptor systems (16, 26). By analogy with these earlier studies, functional

**FIG. 11. Effects of Ser → Ala mutagenesis on agonist-induced CCR5 phosphorylation.** COS-7 cells were transfected with expression plasmids encoding CCR5 wild type or CCR5 mutants which contained Ser → Ala mutations of four carboxyl-terminal serine residues (Ser-336, Ser-337, Ser-342, Ser-349) in various combinations (e.g. A--- denotes Ala mutation of Ser-336). Receptor phosphorylation was monitored after cellular stimulation with 100 nM RANTES or 50 nM AOP-RANTES. Agonist-induced receptor phosphorylation was calculated by subtracting the values of basal phosphorylation in the absence of stimulus for each receptor variant and was normalized to that obtained in CCR5 wild type cells. Shown are data (mean ± S.E.) from experiments performed in triplicate.
differentiation of PKC-mediated from GRK-mediated phosphorylation of CCR5 seems to relate to their differing time courses of action. Additional significance for our finding of PKC-mediated CCR5 phosphorylation derives from the fact that any agent that activates PKC will induce heterologous phosphorylation of CCR5.

Serine or threonine residues of the CCR5 carboxyl terminus most likely represent phosphorylation sites for GRKs. By using a combination of phosphoamino acid analysis and alamethicin scanning mutagenesis, we identified residues Ser-336, Ser-337, Ser-342, and Ser-349 as the phosphoacceptor sites on CCR5. After electrophoretic separation (10% SDS-PAGE) of cellular lysates and electrophoretic separation (10% SDS-PAGE) of cellular lysates and "\[\text{FIG. 12. AOP-RANTES-induced phosphorylation and shift in electrophoretic mobility of CCR5 (Ser \rightarrow Ala) mutants. COS-7 cells were transfected with expression plasmids encoding CCR5 wild type (B), or CCR5 mutants containing Ser \rightarrow Ala mutations at positions 336 (C), 336 and 337 (D), 336, 337, and 342 (E), or 336, 337, 342, and 349 (F). A are empty vector-transfected control cells. }\]

anti-CCR5

Immunoblot

32P-C-CRR5

Autoradiogram

(kDa)

94 -

67 -

43 -

30 -

A B C D E F

A B C D E F

of receptor phosphorylation sites may determine whether GRKs phosphorylate receptors in a sequential or non-sequential manner.

Third, this study contributes to the characterization of consensus phosphorylation site motifs for GRKs. Phosphorylation studies using synthetic peptides have indicated that GRK2 is an acidotropic kinase that prefers acidic amino acids juxtaposed to serines or threonines (33). Interestingly, of the four CCR5-carboxyl-terminal serines that were identified as GRK phosphorylation sites in this study, only Ser-349 is located in close proximity to an upstream glutamic acid residue. Yet, experiments with overexpression of GRK3, which is closely related to GRK2, indicated that this kinase phosphorylates all four serines equally well. These findings support the view that the overall structure of an activated receptor rather than a specific amino acid sequence determines whether a serine or threonine residue will be phosphorylated by GRKs.

Fourth, the four serine residues that constitute phosphorylation sites on CCR5 for GRKs are highly conserved in several members of the CC chemokine receptor family (6). Thus, the results of this study may also be relevant to the identification of phosphorylation sites on closely related members of this receptor family. Of note, alanine mutation of 7 out of the 10 carboxyl-terminal Ser/Thr residues in the CCR2b receptor, including 4 amino acids homologous to the CCR5 phosphorylation site, almost completely prevented the inhibitory effect of GRK3 on the MCP-1-induced calcium response in Xenopus oocytes (34).

Rapid and dose-dependent receptor phosphorylation has been linked to homologous and heterologous desensitization in several CC and CXC chemokine receptor systems (22, 34–36). Experiments with overexpression of GRK2 and/or GRK3 together with CCR2b in either the Xenopus oocyte expression system (34) or in HEK-293 cells (37) have directly implied a role for GRKs in the phosphorylation and desensitization of this receptor. Using a similar approach, Aramori et al. have recently demonstrated that CCR5 in HEK293 cells are rapidly phosphorylated and desensitized upon cellular stimulation with MIP-1β (22). However, this effect was only observed in cells that overexpressed receptor kinases. The authors hypothesized that MIP-1β-induced phosphorylation and desensitization of CCR5 may still be physiologically relevant in cells that express high cellular levels of GRKs, such as leukocytes. In this study we show that, in RBL cells which express GRK2 and GRK3 at levels comparable to those determined in human leukocytes, MIP-1β has only a marginal effect on CCR5 phosphorylation and desensitization. We conclude that for CCR5 regulation under physiological conditions intracellular levels of receptor kinases are a much less important determinant than the nature of the respective CCR5 ligand.

The finding that various ligands which bind to CCR5 with comparable affinities (7) differ largely in their abilities to induce cellular signaling as well as receptor desensitization and phosphorylation was unexpected. The results of our study imply that these molecules may function as full or partial agonists with a wide range of ligand efficacies. Most interestingly, the amino-terminal modification of RANTES with either an additional methionine (Met-RANTES) or with the structurally closely related aminoxyopentane group (AOP-RANTES) drastically affected ligand efficacy in opposite directions. Our findings support the concept that the amino-terminal region of RANTES is an important triggering domain which is involved in chemokine receptor activation (38, 39). In general, we observed a close correlation between the efficacies of receptor ligands in CCR5 activation, phosphorylation and desensitization, with rank order AOP-RANTES > RANTES > MIP-1α,
MIP-1\(\beta\) > Met-RANTES. Our results conform with a previous study in the \(\beta\)-adrenergic receptor system using purified components, which showed that the abilities of partial agonists to stimulate adenylate cyclase activity directly correlated with their abilities to promote receptor phosphorylation by GRKs (40).

By co-immunoprecipitation of CCR5 together with GRKs, we find that GRK2 forms a macromolecular complex with CCR5 after chemokine stimulation. A similar approach was previously used to demonstrate receptor specificities among different GRKs in the endothelin and angiotensin II receptor systems (21). We show here that differences in ligand efficacies are also reflected in the abilities of chemokines to induce GRK association with the receptor. Mechanistically, this may be the result of varying numbers of dissociated \(\beta\)y subunits that are released from the \(\alpha\) subunit after agonist-induced receptor activation. \(\gamma\) protein \(\beta\)y subunits are essential in the translocation of GRK2 and GRK3 to the membrane (25). Alternatively, full agonists such as AOP-RANTES may induce conformational changes of CCR5 that allow a more stable association of GRKs with the receptor.

The results of our study suggest a molecular mechanism that may explain the differential effects of CC chemokines on CC chemokine receptor internalization, observed previously (10, 17) and confirmed for the RBL-CCR5 cell system in this study. Mack et al. reported that natural CC chemokines and the amino-terminal modifications of RANTES induced varied degrees of CCR5 down-regulation from cellular surfaces (10), which followed the same rank order of ligand efficacy as was determined in the present study. According to this concept binding of the different ligands to CCR5 leads to conformational changes of the receptor that activate \(\gamma\) proteins as well as GRKs with varying efficacies. Arrestin binding to the phosphorylated receptor precludes \(\gamma\) protein interaction with the receptor, leading to functional desensitization. Furthermore, as with other receptors, arrestin-like proteins may be instrumental in the removal of CCR5 from the cell surface via clathrin-coated pits (41). The finding that overexpression of \(\beta\)-arrestin 2 together with GRK2 results in a significant increase in the MIP-1\(\beta\)-induced CCR5 sequestration in COS-7 cells (22) lends support to this notion. Clearly, more work needs to be done to establish the functional significance of GRK-mediated receptor phosphorylation for the agonist-induced endocytosis of CCR5.

By comparison of chemokine effects on CCR1 and CCR5, we show that different rank orders of ligand efficacies exist within these two closely related receptor systems. This is most strikingly illustrated by the different effects of AOP-RANTES on CCR1 and CCR5. While AOP-RANTES has the highest potency on CCR5 among the various CC chemokines used in this study, it did not induce CCR1 signaling or phosphorylation. Therefore, the original observation that AOP-RANTES acts as a chemokine antagonist of monocyte chemotaxis (9) may well be due to the predominant expression of CCR1 over CCR5 in these cells. This study demonstrates that CC chemokines largely differ in their agonistic efficacies within the CCR5 and CCR1 systems. The differential ability of chemokines to induce receptor activation, phosphorylation, desensitization, and internalization provides yet another mechanism that allows chemokines to fine-tune the inflammatory response.

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FIG. 13. Effect of GRK3 overexpression on the phosphorylation of CCR5 (Ser \(\rightarrow\) Ala) mutants. COS-7 cells were transfected with expression plasmids encoding CCR5 wild type or CCR5 (Ser \(\rightarrow\) Ala) mutants alone (open bars) or together with DNA encoding GRK3 (filled bars). \(3^P\)-Labeled cells were exposed to 100 nM RANTES for 5 min; receptors were immunoprecipitated and resolved by SDS-PAGE. RANTES-induced receptor phosphorylation was quantitated by PhosphorImager analysis and was calculated by subtracting basal levels in the absence of stimulus. Results are mean \(\pm\) S.E. of three experiments. *, \(p < 0.05\) compared with cells with a normal GRK complement.
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