Analysis of Ligand-stimulated CC Chemokine Receptor 5 (CCR5) Phosphorylation in Intact Cells Using Phosphosite-specific Antibodies

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Human CC chemokine receptor 5 (CCR5), a member of the superfamily of G protein-coupled receptors, regulates the activation and directed migration of leukocytes and serves as the main coreceptor for the entry of R5 tropic strains of human immunodeficiency viruses. We have previously shown that RANTES/CCL5 binding to CCR5 induces GPCR kinase (GRK) and protein kinase C (PKC)-mediated phosphorylation of four distinct C-terminal serine residues. To study these phosphorylation events in vivo, we have generated monoclonal antibodies, which specifically react only with either phosphorylated or nonphosphorylated CCR5. These phosphosite-specific antibodies reveal that following ligand stimulation of the receptor serine 337 is exclusively phosphorylated by a PKC-mediated mechanism, while GRKs phosphorylate serine 349. GRK-mediated receptor phosphorylation proceeds in a regular time-dependent manner (t_1/2 ~ 2 min) with an apparent EC_50 of 5 nM. In contrast, PKC phosphorylates serine 337 at 50-fold lower concentrations and in a very rapid, albeit transient manner. Protein phosphatases that are active at neutral pH and are inhibited by okadaic acid rapidly dephosphorylate phosphoserine 337, but less efficiently phosphoserine 349, in intact cells and in an in vitro assay. Immunofluorescence microscopy demonstrates that phosphorylated receptors accumulate in a perinuclear compartment, which resembles recycling endosomes. This study is the first to analyze in detail the spatial and temporal dynamics of GRK- versus PKC-mediated phosphorylation of a G protein-coupled receptor and its subsequent dephosphorylation on the level of individual phosphorylation sites.

G protein-coupled receptors (GPCR) comprise the largest known family of signal-transducing proteins and respond to a large variety of external stimuli (1, 2). The receptors relay the information encoded by the ligand through the activation of heterotrimeric guanine nucleotide-binding proteins and intracellular effector molecules. Many GPCR undergo a process of rapid desensitization, which involves ligand-induced phosphorylation of serine and threonine residues located in the third intracellular loop or C-terminal domain by two different families of protein kinases. (i) GRK kinases (GRKs) specifically phosphorylate only the agonist-occupied GPCR and thus mediate agonist-specific or homologous receptor phosphorylation (3, 4). (ii) In contrast, the second messenger-activated kinases, such as cyclic AMP-dependent protein kinase and protein kinase C (PKC), potentially phosphorylate both the ligand-bound GPCR and multiple other receptors in a heterologous manner. Receptor phosphorylation enhances the affinity of the agonist-occupied receptor for interaction with arrestin which interdicts signal transduction between the receptor and G proteins by steric mechanisms. The nonvisual arrestins, β-arrestin-1 and β-arrestin-2, also promote clathrin-mediated endocytosis of phosphorylated receptors and have been implicated in cross-talk with other signaling pathways. Once internalized, GPCR are targeted to recycling or degradative pathways (5). Some GPCR are dephosphorylated by membrane-associated G protein-coupled receptor phosphatases and recycled rapidly to the plasma membrane where they can again respond to agonists while other receptors appear to be retained within the cell.

Although reversible receptor phosphorylation is a well-recognized mechanism that plays important roles in multiple aspects of GPCR signaling, with few exceptions the exact sites of second messenger kinase- and GRK-mediated receptor phosphorylation have not been identified. Most insights into receptor phosphorylation derive from in vitro assays with purified proteins in reconstituted systems or from mutagenesis studies with elimination of the presumed consensus sites for receptor phosphorylation under experimental conditions of protein over-expression. Results obtained by these various methods can sometimes prove misleading, as illustrated by studies with the β2-adrenergic receptor (6–8). The observed discrepancies in the outcome of these studies are probably explained by unspecific effects of receptor mutagenesis and by the poor substrate specificity of receptor kinases in reconstituted systems. Furthermore, very little is known about the different kinetics and intracellular localization of agonist-induced GPCR phosphorylation, which is mediated by second messenger-dependent kinases versus GRKs at normal levels of protein expression.

In the present study we have taken a new approach to the analysis of receptor phosphorylation in intact cells. By using phosphosite-specific monoclonal antibodies we determined with high temporal and spatial resolution the ligand-induced

PFS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; HPLC, high performance liquid chromatography; Pipes, 1,4-piperazinediethanesulfonic acid.
phosphorylation and dephosphorylation of CC chemokine receptor CCR5 at two separate phosphorylation sites. This receptor is well suited as a model protein for this kind of analysis since its function as co-factor for the entry of R5 tropic strains of human immunodeficiency viruses (HIV) has stimulated detailed investigations into the mechanisms that regulate CCR5 signaling and cell surface expression in recent years. On the other hand, this receptor was shown to be a potential substrate for two major GRK isoforms (9, 10), and inhibitor studies revealed that PKC as well as GRK2 and/or GRK3 are responsible for the chemokine-induced receptor phosphorylation in rat basophilic leukemia (RBL) cells, which stably express CCR5 (10). Two-dimensional phosphoamino acid analysis in combination with site-directed mutagenesis identified four serine residues that are located in the CCR5 C terminus as the only potential phosphorylation sites on this receptor (10). Intact C-terminal phosphorylation sites were found to be necessary for β-arrestin binding as well as efficient receptor desensitization and internalization, but not for CCR5-mediated chemotaxis (11, 12). HIV-1 entry does not require receptor phosphorylation (13), yet a fully phosphorylation-deficient CCR5 mutant is largely resistant to the inhibitory effect of CC chemokines on in vitro HIV infection (14).

It was previously unknown whether phosphorylation of different serine residues can be attributed to particular kinases or whether they compete for the same phosphoacceptor sites. The current report demonstrates that agonist-induced CCR5 phosphorylation at two distinct sites proceeds with different kinetics and characteristic intracellular distribution in a dynamic manner that involves different protein kinases and phosphatases.

**EXPERIMENTAL PROCEDURES**

**Materials—** Tissue culture reagents were from Biochrom; RBL-2H3, HEK-293, and X68-Ag6.635 cells were from the American Type Culture Collection; LipofectAMINE was from Invitrogen; genitinin, detergents, potato acid phosphatase, activators and inhibitors of protein kinase C, proteases, and phosphatase inhibitors were from Calbiochem; synthetic phospho-peptides were from Jerini; enhanced chemiluminescence (ECL) Western blotting reagents and protein G-Sepharose were from Amersham Biosciences; the CCR5 antagonist TAK-779 was kindly provided by Takeda; anti-phosphotyrosine antibody PY20 was from Becton Dickinson Transduction Laboratories; horseradish-conjugated and FITC-conjugated anti-mouse antibodies were from Dako; streptavidin–horseradish peroxidase was from Jackson ImmunoResearch; all other reagents were from Sigma-Aldrich.

**Cell Culture and Transfection—** Rat basophilic leukemia cells, which stably express wild-type CCR5 (RBL-CCR5 (10)) or CCR5 mutants with alanine exchange of three serine residues at amino acid positions 336, 317, and 342 (RBL-CCR5-AAAS, Ref. 12) or of all four C-terminal serine phosphorylation sites (RBL-CCR5(P)-, Ref. 11) were maintained in 80:20-10 medium (80 parts RPMI 1640, 20 parts medium 199, supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 IU/ml), streptomycin, and 600 μg/ml gentamicin) in a 5% CO2 incubator at 37 °C. Human embryonic kidney (HEK-293) cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. CCR5 cells were seeded at 3 × 105 cells per 600-mm dish and transfected using LipofectAMINE with pEF-BOS expression vectors (1.5 μg/dish), which encode various CCR5 Ser/Ala mutants (10). Transfection efficiencies as determined by flow cytometry with an anti-CCR5 mAb (Q10/19) ranged between 43 and 68%.

**Generation of Phospho-specific Antibodies—** A phosphopeptide (CEAPERA(pS)pS(VGL) corresponding to the TGEQI(T) of intact C-terminal CCR5 peptides at 1:5:5 molar ratios using SMCC as a cross-linking reagent. Results were expressed in arbitrary units (1 AU equals 1 ng of BSA-peptide per ml).

**Immunoblotting—** RBL-2H3 cells which express wild-type or phosphorylation-deficient CCR5 (7 × 106 cells per 60-mm dish) were stimulated with varying concentrations of RANTES for 10 min at 37 °C, washed once with PBS, and solubilized in detergent buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.005% SDS with phosphatase and protease inhibitors as described, Ref. 16) on ice. Experiments that focused on the detection of tyrosine phosphorylation were performed in the presence of 10 μM sodium orthovanadate. Receptors were immunoprecipitated by the incubation of 2 haemolyzed peripheral blood leukocytes with 20 μg of anti-CCR5 R22/7 and protein G-Sepharose. Receptors were eluted by incubation at 37 °C for 30 min in SDS sample buffer containing 2% SDS and 5% 2-mercaptoethanol and subjected to 10% SDS-polyacrylamide gel electrophoresis. Immunoblots were performed using monoclonal anti-pS337 V14/2 (5 μg/ml), anti-pTyr PY20 (0.1 μg/ml), or polyclonal anti-pY127 (10 μg/ml) antibodies in Tris-buffered saline containing 0.1% Tween 20/5% nonfat dry milk. Enhanced chemiluminescence detection of antigens was achieved when horseradish peroxidase-conjugated secondary antibodies. Afterward membranes were stripped and reprobed for total cellular receptors with anti-CCR5 mAb R22/7 (110 μg/ml).

**Enzyme Immunoassay—** Immunofluorescence Microscopy—RBL-CCR5 cells were incubated with 30 nM RANTES (10 min) and 200 nM PMA (5 min) to induce maximal receptor phosphorylation. Cells were scraped in lysis buffer and filtered directly into wells of ELISA plates, which were pre-coated with anti-CCR5 mAb T21/8 (5 μg/ml in 50 mM carbonate, pH 10.6) as the capture antibody. The same cellular lysates were probed, in parallel, with several biotinylated mAb anti-pS337 E11/19, anti-pS337 V14/2, anti-pTyr PY20 (1 μg/ml in PBS/Tween 20), which detect phosphorylation states and sites on the CCR5 C terminus. After a 2-h incubation period (non-phosphorylated CCR5 were detected by adding a 4000-fold dilution of streptavidin peroxidase (Jackson ImmunoResearch) in PBS/Tween for 1 h and 2-azino-di-(3-ethyl-benzthiazoline sulfonate) as substrate. Incubation periods were terminated by 3 wash cycles with PBS/Tween, respectively. The assays were calibrated with a standard protein, which was obtained by the conjugation of bovine serum albumin with synthetic N-terminal and non-phosphorylated C-terminal CCR5 peptides at 1:5:5 molar ratios using SMCC as a cross-linking reagent. Results were expressed in arbitrary units (1 AU equals 1 ng of BSA-peptide per ml).

**Immunofluorescence Microscopy—** RBL-CCR5 cells were incubated with 30 nM RANTES (10 min) and 200 nM PMA (5 min) to induce maximal receptor phosphorylation. Cells were scanned in lysis buffer and filtered directly into wells of ELISA plates, which were pre-coated with anti-CCR5 mAb T21/8. Receptors bound to the solid phase were incubated for up to 1 h at 37 °C with potato acid phosphatase (0.6 U/ml) and either phosphatase-free buffer or buffer containing casein kinase II inhibitors. Alternatively, RBL-2H3 cells were scanned in 50 mM Tris-HCl, pH 7.0, 0.1 mM EDTA, 50 mM 2-mercaptoethanol with protease inhibitors and homogenized by sonication (four 10-s bursts at 100 watts). Nuclei were removed by centrifugation (10 min; 300 g) and the supernatant was incubated for 1 h at 37 °C with receptors bound to the ELISA plate in the presence of 200 nM RANTES (10 min).
were placed on ice, washed with cold medium, and fixed with an ice-cold solution of 3% paraformaldehyde, pH 7.4 in PBS for 20 min. Free aldehyde groups were quenched with 50 mM NH₄Cl in PBS for 30 min. After permeabilization with cold PBS containing 0.05% saponin and 0.2% gelatin for 15 min, cells were washed with the same buffer and stained with anti-CCR5 (T21/8), anti-pS337 (V14/2), or anti-pS349 (E11/19) antibodies (5 μg/ml in PBS/saponin) for 1 h on ice. After washing with PBS/saponin/gelatin, secondary antibody staining was carried out with a FITC-conjugated goat anti-mouse IgG (1:100 dilution) for 1 h. After further washes in PBS, coverslips were mounted in Mowiol containing 0.1% p-phenylenediamine. The samples were analyzed by confocal laser-scanning microscopy utilizing a Leica TCS SP2 system, images were assembled in Corel Draw.

RESULTS

Characterization of Phosphospecific Antibodies Directed against CCR5—Synthetic (phospho-)peptides that encompass the four C-terminal serine phosphorylation sites of CCR5 were used to generate monoclonal antibodies which differentially bind to phosphorylated or nonphosphorylated versions of the immunogenic peptides. Two hybridomas (E11/19, IgG1/κ; V14/2, IgG1/κ) were identified, which selectively react only with ligand-activated CCR5 in a phosphorylation-dependent manner as shown by several independent immunological techniques. Another mAb (R-C10, IgG1/κ) was derived from a separate fusion after immunization with a nonphosphorylated C-terminal receptor peptide which exclusively recognizes non-activated CCR5. The immunoblot shown in Fig. 1 demonstrates that the pCCR5-specific mAb V14/2 reacts with CCR5, which migrates as a broad 40-kDa protein in SDS-PAGE analysis typical of a glycosylated G protein-coupled receptor, but only in its ligand-activated, i.e. phosphorylated form. Under the experimental conditions used in this study we did not observe higher molecular weight forms of CCR5. Despite RANTES activation this mAb did not recognize a phosphorylation-deficient CCR5 mutant, which was generated by alanine replacement of all four C-terminal serine phosphorylation sites. Similar results were obtained with the mAb E11/19, whereas the mAb R-C10 displayed reverse reactivity and lost its ability to react with CCR5 upon ligand activation in a dose-dependent manner.

It has been proposed that chemokine binding to their receptors exposes the tyrosine residue within the highly conserved Asp-Arg-Tyr motif in the second intracellular receptor loop which is then rapidly phosphorylated by Janus kinases (JAKs) (15, 17). We tested this hypothesis by generating phosphopeptide-specific antibodies, which specifically recognize phospho-Tyr-127 and probed the ligand-activated CCR5 with this and anti-phosphotyrosine antibodies, in parallel, by immunoblotting and enzyme-linked immunosorbent assay. We did not observe phosphorylation of either this specific or any other tyrosine residue of this receptor after incubation of RBL-CCR5 cells with saturating concentrations of RANTES (30 nM) within a time frame of 30 s up to 20 min (Fig. 2).

To exactly map the epitopes that are recognized by the various phospho-CCR5-specific mAbs we determined ELISA reactivities of RBL cellular lysates containing maximally phosphorylated CCR5 Ser/Ala mutants (Fig. 3). Substitution of serine 337 by alanine either alone or in combination with any other mutation resulted in the complete loss of V14/2 binding to the receptor, whereas the mutation of serine 349 eliminated E11/19 reactivity. This indicates that V14/2 and E11/19 recognize phosphosерines at positions 337 and 349, respectively, and independent of the phosphorylation state of any of the other three serine residues. Using the same cellular lysates in the absence of receptor activation we found that the mAb R-C10 exclusively reacts with nonphosphorylated serine 337 (not shown). Epitope mapping with monophosphorylated CCR5 peptides independently confirmed these results.

Phosphorylation of Ser-337 and Ser-349 by Different Protein Kinases—Exposure to CC-chemokines produces a rapid increase in CCR5 phosphorylation, which is mediated by protein kinases belonging to the PKC and GRK families. We tested the hypothesis that both protein kinases phosphorylate distinct CCR5 C-terminal serine residues. To this end ELISA procedures were established that are based on a capture antibody with specificity for a CCR5 N-terminal epitope and different
Kinetic Analysis of CCR5 Phosphorylation at Different Agonist Concentrations—Previous studies that employed receptor mutants (18) or various kinase inhibitors (19, 20) suggested that second messenger-dependent kinases and GRKs differently contribute to GPCR phosphorylation upon exposure to different agonist concentrations and at different time intervals. Phosphosite-specific mAb now allowed us to monitor the kinetics and dose-dependence of the RANTES-induced phosphorylation of non-mutated CCR5 by different protein kinases under close to physiological conditions. When RBL-CCR5 cells were stimulated with increasing concentrations of agonist for various time intervals half-maximal phosphorylation of Ser-349 was observed at 5 nM RANTES and ~4-fold higher concentrations were required to achieve maximal phosphorylation (Fig. 5B). The concentration dependence of RANTES-induced phosphorylation paralleled ligand binding to the receptor (11) and thus conformed to the characteristics of a GRK-mediated mechanism. In contrast, phosphorylation of Ser-337 by the second messenger-activated kinase (PKC) was detected at 50-fold lower concentrations when cells were exposed to agonist for 1 min (Fig. 5A). This indicates significant signal amplification downstream of the receptor under these experimental conditions. Unexpectedly, the dose dependence of this effect shifted to higher concentrations when cells were exposed to RANTES for longer incubation times. A detailed kinetic analysis revealed that stimulation with low RANTES concentrations results in the phosphorylation of Ser-337 in a very rapid, albeit transient manner (Fig. 5C). Maximal CCR5 phosphorylation at this site was obtained by stimulation for 40 s, and thereafter the receptor was rapidly dephosphorylated. In the presence of receptor-saturating concentrations of ligand dephosphorylation of phospho-Ser-337 was significantly retarded.

When lysates of RBL-CCR5 cells that had been stimulated with 0.5 nM RANTES for various times were probed with a mAb (R-C10), which specifically recognizes only nonphosphorylated Ser-337 the results represented a mirror image of the values obtained with anti-phospho-Ser-337 mAb (V14/2) in parallel. Data (means ± S.D.) were normalized to values obtained with cells expressing wild-type CCR5.

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phosphorylation of CCR5 proceed independently and in a non-
hierarchical manner.

**Site-specific Regulation of Receptor Dephosphorylation**—
Since different kinases appear to independently phosphorylate
distinct sites on CCR5 with characteristic time courses we
investigated the possibility that this also applies to the mech-
anisms involved in receptor dephosphorylation. We therefore
determined the kinetics of Ser-337 and Ser-349 dephosphoryl-
ation in intact cells after a short pulse (2 min) of stimulation
with a saturating concentration (30 nM) of RANTES. Thereaf-
fter, agonist was quenched by adding a large excess (2 μM) of the
CCR5 antagonist TAK-779 (26). As illustrated in Fig. 8, Ser-
337 is rapidly and completely dephosphorylated within 3 min
(t_{1/2} ≈ 90 s) after withdrawal of the agonist. In contrast, dephos-
phorylation of Ser-349 proceeded much slower (t_{1/2} ≈ 12 min).
This indicated that either phospho-Ser-337 is a much better
substrate for the same receptor phosphatase, which also de-
phosphorylates phospho-Ser-349, or that different protein
phosphatases are involved in receptor dephosphorylation at the
two sites.

To address this question, we established an in vitro CCR5
dephosphorylation assay that allowed determining site-specific
receptor dephosphorylation after the exposure of fully phos-
phorylated CCR5 to phosphatases from various sources. Under
these experimental conditions purified acid phosphatase (0.6
units/ml; at 37 °C) completely dephosphorylated both phos-
phoserine residues. Incubation with RBL-2H3 cell lysates at
pH 7.0 eliminated more than 50% of phosphate associated with
Ser-337 (Fig. 9A) and 30% of phosphate associated with Ser-
349 (Fig. 9B). Preincubation of cell lysates with 200 nM okadaic
acid, a potent inhibitor of protein phosphatases 1 (PP1) and 2a
(PP2A), significantly (p < 0.05) inhibited dephosphorylation of
the phosphoserine located at amino acid position 337 and had
little effect on the dephosphorylation of phospho-Ser-349.

**Intracellular Localization of Phosphorylated Receptors**—To
identify the subcellular compartments in which CCR5 is either phosphorylated or dephosphorylated by the various protein kinases and phosphatases we determined the intracellular localization of phosphorylated receptors in RBL-CCR5 cells by immunofluorescence. In untreated cells the majority of receptors were present at the cell surface in saponin-permeabilized cells (Fig. 10) when we used an anti-CCR5 mAb (T21/8) with specificity for an N-terminal epitope to detect CCR5. No phosphorylated receptors were detected in the absence of ligand. Within 30 s after exposure to a saturating concentration of RANTES (25 nM) punctate areas of fluorescence formed at or close to the membrane, which corresponded to CCR5 that was phosphorylated by PKC at serine 337. After 10 min of incubation at 37°C the fluorescence was predominantly in small diffusely distributed cytoplasmic vesicles which contained receptors phosphorylated at both serine 337 and serine 349. By 30 min most of the receptors had concentrated in a region of the cell adjacent to the nucleus. A faint fluorescent signal, which corresponded to an extracellular CCR5 epitope was visible at the cell surface after 30 min or longer stimulation times, but these receptors were never stained by phosphosite-specific mAb.

**DISCUSSION**

Receptor phosphorylation by two major classes of protein kinases, the second messenger-dependent kinases and GRKs, is of critical importance in the homologous and heterologous desensitization of many G protein-coupled receptors and promotes their internalization through clathrin-coated vesicles by a β-arrestin-dependent mechanism (3, 5). In addition to terminating the cellular response, receptor phosphorylation was also shown to initiate β-arrestin-mediated signaling pathways and to switch coupling of the β2-adrenergic receptor away from Gs in favor of enhanced coupling to Gi (27, 28). Despite its significance for many aspects of GPCR signaling, little is known about the dynamic regulation of receptor phosphorylation and dephosphorylation at distinct sites by different protein kinases and phosphatases. Previously, regulation of receptor phosphorylation by second messenger-dependent and -independent kinases could be described only through removal of presumptive consensus sites of receptor phosphorylation or under experimental conditions of overexpressed kinases. Accurate mapping of in vivo phosphorylation sites of a GPCR under physiological conditions has been achieved only for rhodopsin and, more recently, the bradykinin B2 receptor (29, 30). In the case of rhodopsin two C-terminal serine residues were differentially phosphorylated by rhodopsin kinase (GRK1) after a bright flash or under constant illumination. Receptor dephosphorylation at these two sites proceeded with markedly different kinetics and appeared to be spatially controlled within rod outer segments (29).

In the current study we use phosphosite-specific antibodies to analyze in intact cells the ligand-induced phosphorylation of the CC chemokine receptor CCR5 by two different protein kinases in a cellular environment which resembles natural conditions. We show that in RBL cells, which express physiological levels of the protein kinase, PKC exclusively phosphorylates Ser-337, while GRKs phosphorylate Ser-349. The evi-
ence for PKC-mediated phosphorylation of Ser-337 is derived from experiments with selective PKC activators and inhibitors. Reagents that selectively modulate GRK activity in whole cells are currently not available. Our conclusion that GRKs are responsible for Ser-349 phosphorylation is therefore indirect and primarily based on the lack of effect of a large number of different protein kinase inhibitors, including staurosporine. Ligand-induced phosphorylation at this site was also not affected by pertussis toxin, which indicates that the kinase that phosphorylates Ser-349 is independent of G protein activation and second messengers. The concentration dependence of RANTES-induced phosphorylation of Ser-349 paralleled ligand binding to the receptor, which further supports our conclusion that GRKs phosphorylate this particular site (10). Previously, we concluded from radiolabeling experiments on the phosphorylation of CCR5 serine to alanine triple mutants in GRK-overexpressing cells that GRKs participate in the ligand-induced phosphorylation of all four C-terminal serine residues (31). These divergent results emphasize that phosphorylation experiments with receptor mutants in the presence of unphysiological concentrations of protein kinases reveal, at best, potential phosphorylation sites and the identity of protein kinases, which are likely to be involved in substrate phosphorylation. The unequivocal identification of phosphorylation sites and corresponding kinases requires that experiments are performed with non-mutated proteins at physiological intracellular concentrations of protein kinases.

The observation that mutation of tyrosine 139 within the conserved DRY sequence motif in the second intracellular loop of the CCR2 chemokine receptor to phenylalanine eliminated functional activity of the receptor has lead to the hypothesis that this critical tyrosine residue is phosphorylated by JAKs following ligand binding to this or other related chemokine receptors (17). By using phosphosite-specific or anti-phospho-tyrosine antibodies and in accord with earlier experiments with two-dimensional phosphoamino acid analysis (10) we found no evidence that this mechanism may operate in RBL-CCR5 cells.

In the present study we observed that GRK-mediated receptor phosphorylation after RANTES stimulation was significantly slower compared with phosphorylation caused by PKC. Half-maximal phosphorylation of Ser-349 was achieved after 1.5 to 2 min. The kinetics of GPCR phosphorylation by GRKs versus second messenger-dependent kinases has been investigated only in relatively few receptor systems. In permeabilized A431 cells the GRK-mediated phosphorylation and desensitization of $\beta_2$-adrenergic receptors was reported to transpire with a $t_{1/2}$ of less than 20 s, whereas phosphorylation and desensitization by the cyclic AMP-dependent protein kinase (PKA) had a $t_{1/2}$ of at least 2 min (19). The desensitization and phosphorylation of odorant receptors from detached olfactory cilia preparations proceeded even more rapidly, within 0.2–1 s, and both GRK3 and second messenger-activated kinases appeared to function in series in this particular receptor system (32). A limitation of these early studies is that they were performed with permeabilized cells and kinase inhibitors such as heparin, which lack specificity. Nonetheless, these varying results indicate that the kinetics of GPCR phosphorylation largely differ between receptor systems, depending on such variables as different receptor substrates and cellular expression levels of receptor kinases. Variability in the mechanisms which regulate GPCR phosphorylation is also illustrated by the finding in the present study that GRK- and PKC-mediated CCR5 phosphorylation proceed independently and in a non-hierarchical manner, in contrast to other receptor systems (23–25).

An unexpected finding of the present work related to the kinetics of PKC-mediated CCR5 phosphorylation. Upon RANTES stimulation Ser-337 was rapidly phosphorylated by PKC and half-maximal phosphorylation was observed after 10 s. In the presence of low concentrations of ligand Ser-337 phosphorylation was a transient event and dephosphorylation proceeded with a calculated $t_{1/2}$ of 1 min. In contrast, treatment of cells with PMA induced a sustained phosphorylation of Ser-337, which was maximal after 5 min. Several PKC isoenzymes undergo reversible membrane translocation in response to GPCR activation. Our data show that the kinetics of ligand- or phorbol ester-induced CCR5 phosphorylation by these kinases closely follows the time course of membrane recruitment of PKC$\beta$ after GPCR activation, as reported in other receptor systems which are coupled to the activation of phospholipase C (33, 34). Whereas PMA induced the persistent redistribution of the kinase from the cytosol to the membrane after 2–5 min, $G_{q/11}$-coupled receptor signaling was reported to lead to only one rapid and brief peak of PKC membrane translocation (33). Within 1 min after translocation the kinase returned to the cytoplasm by a mechanism that involves PKC autophosphorylation (35). Agonist stimulation of intact cells that express the substance P receptor induced a very rapid and transient trans-
location of a green fluorescent protein (GFP)-PKCβ construct, whereas GFP-GRK2 was recruited to the plasma membrane shortly after PKC and persisted over a longer time period (34).

PKC-mediated receptor phosphorylation after stimulation with low concentrations of RANTES is tightly controlled by protein phosphatases, which rapidly dephosphorylate phospho-Ser-337. The kinetics and dose-dependence of Ser-337 de-phosphorylation suggest that these phosphatases are different from the GPCR phosphatase (GRP), a latent endosome-associated form of PP2A, which was previously identified as the major phosphatase that dephosphorylates the GRK-phosphorylated β2-adrenergic receptor (36). Whereas GRP dephosphorylates receptors only in the acidic milieu of endosomal vesicles and, thus, requires receptor endocytosis, the phosphatases that dephosphorylate Ser-337 are active at neutral pH and efficiently dephosphorylate receptors at the cell surface that have been phosphorylated by a heterologous mechanism. At higher ligand concentrations a larger fraction of receptors at the cell membrane are present in the ligand-bound form, which then leads to GRK-mediated receptor phosphorylation, β-arrestin binding, and receptor endocytosis. We have previously shown that β-arrestin binding to CCR5 strictly follows ligand occupancy and is maximal after stimulation of receptors with RANTES for 3–10 min (12). Our data are consistent with the hypothesis that at high concentrations of ligand a stable complex between β-arrestin and phosphorylated CCR5 is formed, which prevents the phosphatase from dephosphorylating Ser-337. This notion is supported by our observation that treatment of cells that express maximally phosphorylated CCR5 with a receptor antagonist, which quickly dissociates receptor-β-arrestin complexes, leads to the rapid dephosphorylation of Ser-337. An inhibitory effect of arrestin on the dephosphorylation of rhodopsin, a GPCR that does not internalize, by a retinal protein phosphatase 2A was previously suggested by Palczewski et al. (37). In this study we show that stimulation with a low concentration of agonist induces the PKC-mediated reversible phosphorylation of a significant fraction of all CCR5 receptors in a heterologous manner. We suggest that a protein phosphatase which efficiently dephosphorylates PKC-phosphorylated GPCR at the plasma membrane fulfills an important role in maintaining cell membrane receptors in a nonphosphorylated state. In contrast to the endosome-associated GPCR, this phosphatase does not require receptor endocytosis. Whether protein phosphatases that either directly (38) or indirectly through anchoring proteins (39) bind to receptors also contribute to this mechanism remains to be investigated in future studies. A different mechanism appears to be responsible for the dephosphorylation of GRK-phosphorylated Ser-349. In chase experiments with the CCR5 antagonist TAK-779 phosphoserine 349 was much slower dephosphorylated compared with phosphoserine 337 and the phosphatase(s) present in RBL cell lysates that dephosphorylated phosphoserine 337 in vitro at neutral pH less efficiently dephosphorylated phospho-Ser-349.

The ligand-induced endocytosis and recycling of CCR5 was previously described in detail using immunofluorescence and immune electron microscopy (40, 41). These studies revealed that after RANTES stimulation the intracellular distribution of CCR5 largely overlaps with that of the transferrin receptor, a marker of early and recycling endosomes. Immunofluorescence with phosphosite-specific antibodies allowed us to assign receptor phosphorylation and dephosphorylation at the GRK and PKC sites as defined by biochemical assays to these intracellular compartments. Shortly (30 s) after treatment with a high concentration of RANTES we observed strong staining with the antibody, which recognizes PKC-phosphorylated Ser-337 at the plasma membrane and in small punctate vesicles close to the cell surface. Vesicular staining in the periphery of the cell was observed after 10 min with both antibodies, which recognize GRK- or PKC-phosphorylated CCR5. At later time points phosphorylated receptors accumulated in perinuclear clusters, which are probably equivalent to recycling endosomes as previously described in CHO cells (41). We showed by flow cytometry that prolonged stimulation of RBL-CCR5 cells with RANTES reduces receptor expression at the cell surface by no more than 60% (10). Accordingly, CCR5 immunofluorescence at the cell surface was reduced, but still detectable even after agonist stimulation for more than 30 min. Since these receptors were not detected by phospo-CCR5-specific antibodies they appear to be dephosphorylated by phosphatases within the perinuclear recycling compartment. It has been hypothesized that dephosphorylation of a GRK-phosphorylated C-terminal retention domain within this perinuclear compartment precedes and even may be required for the return of intracellular receptors to the plasma membrane (42, 43). Our data in the CCR5 system do not prove, but are compatible with this concept. In summary, we have shown that phosphosite-specific antibodies provide unique and sensitive means for studying GPCR phosphorylation and dephosphorylation in a native cellular environment.

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Analysis of Ligand-stimulated CC Chemokine Receptor 5 (CCR5) Phosphorylation in Intact Cells Using Phosphosite-specific Antibodies
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