G Protein-coupled Receptor Kinases Promote Phosphorylation and β-Arrestin-mediated Internalization of CCR5 Homo- and Hetero-oligomers*

Expression levels of the chemokine receptor, CC chemokine receptor 5 (CCR5), at the cell surface determine cell susceptibility to HIV entry and infection. Cellular activation by CCR5 itself, but also by unrelated receptors leads to cross-phosphorylation and cross-internalization of CCR5. This study addresses the underlying molecular mechanisms of homologous and heterologous CCR5 regulation. As shown by bioluminescence resonance energy transfer experiments, CCR5 formed constitutive homo- as well as hetero-oligomeric complexes together with C5aR but not with the unrelated AT1aR in living cells. Stimulation with CCL5 of RBL cells, which co-expressed CCR5 together with an N-terminally truncated CCR5-ΔNT mutant, resulted in both protein kinase C (PKC)- and G protein-coupled receptor (GPCR) kinase (GRK)-mediated cross-phosphorylation of the mutant unligated receptor, as determined by phosphosite-specific monoclonal antibody. Similarly, both PKC and GRK cross-phosphorylated CCR5 in a heterologous manner after C5a stimulation of RBL-CCR5/C5aR cells, whereas AT1aR stimulation resulted only in classical PKC-mediated CCR5 phosphorylation. Co-expression of CCR5-ΔNT together with a phosphorylation-deficient CCR5 mutant that neither binds β-arrestin nor undergoes internalization partially restored the CCL5-induced association of β-arrestin with the homo-oligomeric receptor complex and augmented cellular uptake of 125I-CCL5. Co-expression of C5aR, but not of AT1aR, promoted CCR5 co-internalization upon agonist stimulation by a mechanism independent of CCR5 phosphorylation. Co-internalization of phosphorylated CCR5 was also observed in C5a-stimulated macrophages. Finally, co-expression of a constitutively internalized C5aR-US28CCT, mutant led to intracellular accumulation of CCR5 in the absence of ligand stimulation. These results show that GRKs and β-arrestin are involved in heterologous receptor regulation by cross-phosphorylating and co-internalizing unligated receptors within homo- or hetero-oligomeric protein complexes.

Leukocytes express multiple pertussis toxin-sensitive chemoattractant receptors that may be engaged simultaneously or sequentially as these cells are recruited into tissue sites of inflammation. Interaction of such receptors with their cognate ligands results, among other cellular functions, in directional cell movement, integrin activation, and release of granular contents (1). Upon ligand binding, receptors also undergo adaptive changes, which include desensitization and internalization. Two major mechanisms of rapid receptor regulation have been discriminated, namely homologous (agonist-specific) and heterologous (agonist-nonspecific) desensitization, and both mechanisms are believed to be important in fine tuning leukocyte responses (2).

Homologous desensitization involves phosphorylation of ligand-occupied receptors by members of the GPCR kinase (GRK) family, with, according to the current paradigm, essentially no effect on other receptors expressed in the same cell (3, 4). These kinases often phosphorylate serine/threonine residues present in the C-terminal domains of chemo-tactic leukocyte receptors, which then induce association of the phosphorylated receptors with β-arrestins. β-Arrestins bind to receptors physically interfere with further G protein coupling, initiate endocytosis through clathrin-coated vesicles, and act as scaffolding proteins for various signaling molecules (5). In contrast, heterologous desensitization is traditionally defined as a state of cellular refractoriness to multiple agonists following phosphorylation of receptor sites different from GRKs by second messenger-activated protein kinases, which have been activated by other receptors or signaling pathways. Mechanisms downstream of the receptor that involve decreased activation of phosphoprotein C-β also contribute to heterologous receptor desensitization (2). Whereas second messenger-activated protein kinases affect homologous desensitization through their ability to phosphorylate GPCR, which modulates their kinase activities in different ways (6), GRKs are generally thought not to be involved in heterologous regulation of unligated GPCR.

According to the traditional view of GPCR regulation, these membrane proteins operate as monomeric entities. However, recent evidence based on biochemical and biophysical studies show that GPCR form homo- or heterodimeric (oligomeric) complexes in vivo (7). Despite the large number of studies that demonstrate dimerization of different GPCR, some of which also address functional aspects, the implications of these findings for GPCR regulation by receptor kinases and β-arrestins have not been fully investigated. In the present study, we chose to address this aspect of GPCR regulation using the CC chemokine receptor 5 (CCR5) as a model. A better understanding of the mechanisms that regulate the function and cell surface expression of CCR5, one of the two major co-receptors for the human immunodeficiency viruses, is of particular interest because down-modulation of CCR5 can

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The abbreviations used are: GPCR, heterotrimeric GTP-binding protein-coupled receptor; ABS, antibody binding sites; AT1aR, type 1a angiotensin II receptor; AU, arbitrary unit; BRET, bioluminescence resonance energy transfer; CCR5, CC chemokine receptor 5; CCL5, also known as RANTES (released on activation normal T cell expressed and secreted); ELISA, enzyme-linked immunosorbent assay; GRK, GPCR kinase; HEK-293 cells, human embryonic kidney 293 cells; HIV, human immunodeficiency virus; mAb, monoclonal antibody; pCCR5, phosphorylated CCR5; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RBL-2H3 cells, rat basophilic leukemia cells; PIPES, 1,4-piperazinediethanesulfonic acid; GFP, green fluorescent protein; TM, transmembrane; NT, N-terminal; WT, wild type; PBS, phosphate-buffered saline; QFACS, quantitatitve fluorescent-activated cell sorter; HA, hemagglutinin; FITC, fluorescein isothiocyanate; MCF, mean channel of fluorescence.

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prevent HIV entry into target cells (8, 9). Several recent studies have unanimously shown that CCR5 forms homo- and heterooligomeric protein complexes, but conflicting results have been reported for whether CCR5 oligomerization is important for signaling or HIV coreceptor function of CCR5 (10–13).

By combining a functional complementation approach with the use of phosphosite-specific antibodies that allow us to monitor GRK–versus PKC-mediated phosphorylation of the CCR5 in whole cells, we provide here the first direct evidence that GRKs can also cross-phosphorylate unligated GPCR if they form homo- or heterooligomeric complexes with other receptors that undergo GRK-mediated phosphorylation. These findings suggest an alternative model of heterologous receptor desensitization and cross-internalization, which also involves GRKs and β-arrestins.

EXPERIMENTAL PROCEDURES

Materials—Most reagents have been reported before (14, 15). Restriction enzymes, T4 ligase, and calf intestine alkaline phosphatase were from MBI Fermentas; recombinant human CCL5/RANTES was from PeproTech, anti-CD55/CD98 mAb 55/1 and P12/1 were from Serotec; recombinant human C5a was prepared as described before (16). 125I-CCL5 and 125I-C5a were from PerkinElmer Life Sciences, 3H125I, and 32Pi and 125I were from Amersham Pharmacia Biotech; 35S were from MBI Fermentas; recombinant human CCL5/RANTES was from R&D Systems; anti-CCR5 T21/8, anti-human CD14 P12/1, and anti-human C5aR/CD88 S5/1 and P12/1 were from Sero-Lab; αtubulin and β-tubulin mAb E11/19 and V14/2 (17) and anti-C5aR P12/1 mAb in Tris-buffered saline containing 0.1% protease inhibitors as described, Ref. 17) for 30 min on ice. Following centrifugation (12,000 × g for 10 min) receptors were immunoprecipitated (2 h at 4 °C) with 12 g of anti-CCR5 R22/7, anti-C5aR S5/1, or anti-HA 12CA5 antibodies and protein G-Sepharose. After four wash steps, receptors were eluted by incubation at 37 °C for 30 min in SDS sample buffer containing 0.2% SDS and 5% 2-mercaptoethanol and subjected to 10% SDS-polyacrylamide gel electrophoresis. Immunoblots were performed using phospho-CCR5-specific monoclonal anti-pSer337 V14/2 or anti-pSer339 E11/19 (5 μg/ml) antibodies (15) or anti-C5aR P12/1 mAb in Tris-buffered saline containing 0.1% Tween 20/5% nonfat dry milk. Enhanced chemiluminescence detection of antigens was achieved with horseradish peroxidase-conjugated secondary antibodies. Afterward membranes were stripped and reprobed for total cellular receptors with anti-HA 12CA5 peroxidase conjugate (1:5000).

CCR5 Phosphorylation Assays—Ligand-induced phosphorylation of CCR5 C-terminal serine residues at positions 337 and 349 were determined by immunoblotting or enzyme-linked immunosorbent assays (ELISA), which are based on different phosphosite-specific mAbs (15). For ELISA, 1.5 × 106 RBL cells expressing CCR5 and C5aR variants were incubated in 40-mm wells for various times at 37 °C with stimulus and scraped in 0.7 ml of lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% SDS with phosphatase and protease inhibitors). Following centrifugation (3,000 × g for 10 min), supernatants were applied (1 h at room temperature) into wells of microtiter plates coated with the anti-CCR5 mAb T21/8 (5 μg/ml). After washing twice, wells were incubated (1 h at room temperature) with biotinylated mAb E11/19 and V14/2 (1 μg/ml PBS-0.5% Tween with specificities for phosphorylated Ser339 and Ser349, respectively). Immune complexes were detected with streptavidin-horseradish peroxidase and 2,2′-azino-di-(3-ethylbenzthiazoline) sulphonate as substrate. The assays were calibrated with a synthetic bovine serum albumin–CCR5-(phospho)peptide standard protein. Results were expressed in relative units (r.u.) (1 r.u. equals 1 ng of bovine serum albumin-peptide per ml). For immunoblotting, receptors were immunoprecipitated as described above, except that cells were lysed in lysis buffer, and samples were resuspended in sample buffer containing 5% SDS.

* M. Sgodda and M. Oppermann, unpublished data.
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Whole cell phosphorylation experiments using $^{32}$P incorporation into CCR5 or C5aR were performed, in general, as described before (18). RBL-CCR5 cells were labeled with $^{32}$P$_i$ (150 μCi/ml) in phosphate-free medium for 45 min in the presence of 0–2 μM bisindolylmaleimide (15 min). After incubation with stimulus, cells were washed with ice-cold PBS and lysed in 700 μl of lysis buffer. Insoluble material was pelleted (5 min, 20,000 × g), and receptors were immunoprecipitated with 12 μg of CCR5-specific mAb R22/7 and protein G-Sepharose. Receptors were solubilized in sample buffer containing 5% SDS and resolved by SDS-PAGE with equal amounts of receptor protein in each lane. $^{32}$P incorporation into receptors was visualized by autoradiography and quantitated by phosphorimager analysis (Molecular Dynamics). For two-dimensional phosphoamino acid analysis, receptors were electrophoretically transferred to a polyvinylidene difluoride membrane, excised, and hydrolyzed in 6 N HCl (2 h at 110 °C). Hydrolysates were resolubilized in pH 1.9 buffer (formic acid/acetic acid/H$_2$O, 50:156:1794 (v/v/v)) and spotted on a thin layer cellulose plate. Phosphoamino acids were separated by electrophoresis (900 V for 1.5 h) followed by a second electrophoresis (900 V for 45 min) at pH 3.5 (pyridine/acetic acid/H$_2$O, 10:100:1890 (v/v/v) and 0.5 mM EDTA) in the orthogonal direction. After ninhydrin staining of phosphoamino acid standards, thin layer plates were exposed to autoradiographic screens.

Quantitative FACS (QFACS) Analysis and Immunofluorescence Microscopy—Receptor expression levels on transfected cell lines and on monocytes were determined by quantitative FACS analysis. QFACS was performed by converting the mean channel of fluorescence (MCF) into antibody binding sites (ABS) per cell by using a standardized microbeads kit (Quantum Simply Cellular kit, Sigma). This kit consists of a mixture of four separate microbead populations with incremental capacities to bind mouse Ig. Briefly, beads (10⁵ per sample) were incubated with the same saturating concentrations of antibodies as the samples and treated like the samples being quantitated. FITC-conjugated anti-mouse Ig was used as secondary detecting reagents. The binding capacities of the stained microbeads were then assessed against the corresponding MCF of each bead population, and the MCF of the receptor epitope on the sample cells was converted to ABS per cell by comparison with regression curve (QuickCal software).

Monocyte-derived macrophages were grown on glass coverslips in 24-well plates. After fixation with 3% paraformaldehyde, pH 7.4 in PBS for 20 min on ice, free aldehydes were quenched with 50 mM NH$_4$Cl in PBS for 30 min. Cells were permeabilized with cold PBS containing 0.05% saponin, 0.2% gelatin for 15 min, washed once with the same buffer, and stained with anti-CCR5 (T21/8), anti-CCR5-pSer$^{349}$ (E11/19), or anti-C5aR (SS5/1) antibodies (10 μg/ml in PBS-saponin) for 1 h on ice. After washing with PBS-saponin-gelatin, goat anti-mouse Ig FITC-conjugate (Dako) (1:100 dilution) was added 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, and images were assembled in Adobe Photoshop.

BRET Assay—Two days after transfection, HEK-293 cells were suspended in buffer (0.1 g/liter CaCl$_2$, 0.1 g/liter MgCl$_2$, 1 g/liter D-glucose in PBS) and washed once in the same buffer. Cells were distributed in duplicate into 96-well microplates (white Optiplate, PerkinElmer Life Sciences) at a density of $\approx 5 \times 10^5$ cells per well. Deep Blue C (PerkinElmer Life Sciences) was added at a final concentration of 5 μM, and readings were collected immediately after addition of the coelenterazine (Molecular Probes) using a Mithras LP940 microplate analyzer (Berthold). The BRET signal is expressed as the ratio of light emitted by the GFP$^3$ construct (500–530 nm) over the light emitted by the hRluc construct (370–450 nm), corrected for Rf, which corresponds to the signal in cells that express only the hRluc construct in the same experiment. Total GFP fluorescence and luminescence signals were determined, in parallel, for all samples using coelenterazine H, 5 μl, and the Mithras plate reader to assess expression levels of the GFP$^3$ and Rluc conjugates.

Functional Assays—The CCL5-induced N-acetyl-β-d-glucosaminidase release from CCR5-expressing RBL-2H3 cells was determined as described (20). Values were expressed as a 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 with the Ligand Binding module of Sigma-Plot software (SPSS).

Agonist-dependent intracellular calcium mobilization was measured in transfected RBL-2H3 cells as described (18). Calcium decays from 80% of the peak height to basal levels were fitted to an exponential ($\alpha + be^{-\tau}$) where the time constant τ reflects the ability of CCR5 variants to evoke a more or less sustained calcium response (21).

Internalization Assays—The internalization of $^{125}$I-C5a by transfected RBL-2H3 cells was determined as described before (19). In brief, cells were incubated with binding medium (BM; RPMI1640 without bicarbonate, 0.2% bovine serum albumin, 10 mM HEPES, pH 7.4) containing $^{125}$I-C5a (90 min at 4°C). Unbound ligand was removed by washing at 4°C. Cells were incubated at 37°C for different times (0, 3, 10, 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 at pH 2.5. 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 nonspecific binding at time 0.

The agonist-induced down-modulation of CCR5 co-expressed at the RBL cell surface together with C5aR variants or AT$_1$R was measured using radiolabeled antibodies. To this end, T21/8 anti-CCR5 mAb was iodinated by the $^{125}$I-Bolton-Hunter reagent to a specific activity of 1100 Ci/mmol. Adherent cells were treated with 100 nM C5a in BM for up to 2 h at 37°C and then transferred to ice. After washing with ice-cold BM, cells were incubated with $^{125}$I-T21/8 (0.2 μg/ml BM) for 1.5 h. Cell-bound activity was measured by scintillation counting after four final washes with cold BM. The same protocol was used to determine homologous internalization of C5aR (100 nM C5a; $^{125}$I-SS5/1), CCR5 (100 nM CCL5; $^{125}$I-T21/8), or AT$_1$R (100 nM Ang II; $^{125}$I-L2CA5).

The agonist-induced internalization of CCR5 and C5aR in macrophages was determined by quantitative FACS analysis. Monocyte-derived macrophages (5-10$^6$ in 100 μl) were incubated (2 h at 37°C) with medium containing either 100 nM C5a or 100 nM CCL5. Thereafter, cells were cooled to 4°C and T21/8 (8 μg/ml BM) for 1 h. Cell-bound activity was measured by scintillation counting after four final washes with cold BM. The same protocol was used to test homologous internalization of C5aR (100 nM C5a; $^{125}$I-SS5/1), CCR5 (100 nM CCL5; $^{125}$I-T21/8), or AT$_1$R (100 nM Ang II; $^{125}$I-L2CA5).

The agonist-induced translocation of CCR5 and C5aR from the cytosol to the plasma membrane was determined in RBL cells by subcellular fractionation and immunoblotting (14). In brief, C5aR- or CCR5-expressing RBL cells were incubated in the presence of 10 nM CCL5 or 20 nM C5a for 3 min at 37°C. The cells were placed on ice and scraped into 3 ml of buffer A (10 mM PIPES, 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl$_2$, pH 7.0) containing protease inhibitors. After homogenization and centrifugation (1000 × g for 20 min) the supernatant was loaded onto a discontinuous sucrose gradient and centrifuged at 160,000 × g (4°C for 2 h). The 35%/50% sucrose interphase (membrane fraction) was collected, diluted in 3 ml of buffer A and re-centrifuged (160,000 × g for 15 min). The pellet was resuspended in detergent buffer (19), and equal amounts
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RESULTS Characterization of CCR5 Homo-oligomers Expressed in RBL Cells—To examine the significance of receptor oligomerization for homologous receptor regulation, we generated a N-terminally truncated CCR5-ΔNT mutant with substitution of an essential N-terminal Asp¹⁷ ligand binding domain (22) by an HA tag sequence and a phosphorylation-deficient CCR5-Ser/Ala mutant (P⁻), which lacks the four C-terminal serine phosphorylation sites of this receptor (18). Both receptor variants were transfected, either separately or together, into RBL cells and were (co-)expressed at comparable levels at the cell surface, as determined by quantitative FACS analysis (QFACS; TABLE ONE). In this assay we used antibodies (12CA5, anti-HA; T21/8, anti-CCR5-NT) that specifically react with N-terminal epitopes on the respective receptor constructs. In accordance with previous reports (19, 22), the treatment of RBL-CCR5-ΔNT cells with CCL5 induced no intracellular calcium mobilization or glucosaminidase release, whereas in CCL5-stimulated RBL-CCR5-P⁻ cells a prolonged calcium response and enhanced enzyme release was observed, consistent with the previously documented desensitization and β-arrestin binding defect of this mutant (19). In cells that co-expressed CCR5-ΔNT and CCR5-P⁻, the time constant for CCL5-induced calcium decay was intermediate between cells that express CCR5-WT and CCR5-P⁻.

Previous studies, based on immunoprecipitation assays and BRET technology, indicated that CCR5 receptors form constitutive homo-oligomers as well as complexes with truncated CCR5-Δ32 mutants (12, 23). When the CCR5-ΔNT mutant was immunoprecipitated with HA affinity beads and separated by SDS-PAGE using low concentrations of SDS in the sample buffer, the truncated receptor migrated as a monomer with an apparent molecular mass of approximately 35 kDa. An additional ~70-kDa band was consistently observed when low SDS concentrations were present in the SDS-PAGE sample buffer (Fig. 1) but were absent under more denaturing conditions. This indicates that this N-terminally truncated receptor forms oligomers in the absence of ligand. As shown by bioluminescence resonance energy transfer (BRET), CCR5-ΔNT associates with intact or phosphorylation-deficient CCR5 in living cells with the same affinity compared with complexes that are formed between wild-type receptors (Fig. 2A). BRET detects molecules in close proximity to each other based on nonradioactive energy transfer between bioluminescent donors (Renilla luciferase; Rluc) and fluorescent acceptors (GFP²). BRET was previously used to demonstrate constitutive multimerization of CCR5 (12, 25) as well as of several other GPCR (26–28). These results show that neither an intact N terminus nor the presence of C-terminal phosphorylation sites appear to be required for the formation of CCR5 homo-oligomers. GRK-mediated Phosphorylation of CCR5 Homo-oligomers—We hypothesized that CCR5-ΔNT/CCR5-P⁻ complex formation may
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rescue GRK-mediated phosphorylation of the ligand binding-deficient receptor mutant. Different RBL cell transfecants were therefore exposed to PMA or CCL5 and CCR5 phosphorylation at either the PKC site (Ser\(^{337}\)) or the GRK site (Ser\(^{349}\)) was documented using phosphosite-specific antibodies. Neither CCR5-ΔNT, which does not bind ligand, nor CCR5-ΔNT/P, which lacks C-terminal phosphorylation sites, undergo chemokine-induced and GRK-mediated phosphorylation if expressed alone on separate RBL cell clones (Fig. 3). However, when both receptor mutants were expressed in the same cells, CCR5-ΔNT was clearly phosphorylated on PKC as well as on GRK sites upon CCL5 stimulation. Treatment of RBL-CCR5 cells with mastoparan, a peptide toxin from wasp venom and known GRK activator (24), did not induce GRK-mediated receptor phosphorylation (data not shown). This indicates that an unligated receptor can undergo GRK-mediated phosphorylation, but only if the substrate is in close proximity to another agonist-occupied receptor at the plasma membrane.

CCL5-induced β-Arrestin Translocation to CCR5-ΔNT/CCR5-P and Internalization of Receptor Complexes—β-Arrestin binding to ligand-activated CCR5 involves both phosphorylation-dependent as well as phosphorylation-independent interaction domains on the receptor (14). We therefore asked whether the formation of CCR5-ΔNT/CCR5-P complexes may restore the β-arrestin binding defect of the two individual CCR5 mutants. RBL-2H3 cells express high endogenous levels of β-arrestin 1 and β-arrestin 2 and thus allow us to monitor ligand-induced recruitment of these important adaptor proteins to receptors in the absence of β-arrestin overexpression. As depicted in Fig. 4, stimulation with CCL5 did not result in the translocation of the two β-arrestin isoforms to membrane fractions of RBL cells that express phosphorylation-deficient or N-terminally truncated receptor variants alone. In contrast, β-arrestins translocated to the plasma membrane in cells that co-express the two defective receptors upon CCL5 stimulation. Membrane recruitment of β-arrestins in these cells was less efficient as compared with cells that express wild-type CCR5. No significant difference in CCL5-induced β-arrestin membrane translocation was observed in cells that express wild-type CCR5 alone (not shown) or together with CCR5-ΔNT. The observed differences in β-arrestin1/2 recruitment to the cell membrane may also be determined by the stoichiometry of subunits within the oligomers. Different receptor expression levels in RBL cell clones are less likely to have an effect in our experiments, because receptors are expressed in excess over endogenous β-arrestins.

Previously, we observed that CCL5 is endocytosed by RBL-CCR5 cells in a rapid (<10 min), β-arrestin-dependent manner as well as in a more prolonged fashion that may involve intracellular uptake of the chemokine by membrane bulk flow (14). To measure the β-arrestin-dependent internalization of oligomeric receptors we determined the uptake of radiolabeled CCL5 in the different RBL cell clones during the first 10 min of agonist exposure (Fig. 5). Because of the lack of chemokine binding, RBL-CCR5-ΔNT cells did not significantly take up radiolabeled CCL5 compared with nontransfected cells and are therefore not shown. As reported before (14), phosphorylation-deficient CCR5-P receptors are internalized much slower than wild-type CCR5. The rate of CCR5-P internalization in a complex together with CCR5-ΔNT was moderately, yet significantly (\(p < 0.005\)), enhanced by a factor of 3 (3 min) or 1.4 (10 min) compared with cells that expressed CCR5-P.
cells that co-express both receptors and were not caused by artifactual alone. This indicates that CCR5/C5aR hetero-oligomers pre-existed in immunoprecipitates prepared under identical conditions from untrans-
tated with antibodies against one N-terminal receptor epitope and then observed. Together, these results indicate that 
ences between the endocytosis of the different receptor variants was 
ary—According to a classical model of monomeric GPCR regulation, receptors are phosphorylated by second messenger-activated kinases such as PKC in a heterologous, ligand-independent manner, whereas GRKs bind to and specifically phosphorylate only ligand-occupied receptors. To determine whether these receptor kinases also phosphorylate CCR5/C5aR heterodimers, we assessed CCR5 phosphorylation in whole RBL-CCR5/C5aR cells in response to either C5a or CCL5. The disruption of cell membranes by lysis buffer in the presence of 5% SDS results in the dissociation of receptor complexes (12), and thus allowed us to selectively document phosphorylation of the resulting receptor monomers. As shown in Fig.
Equal numbers of RBL cells (6 experiments with similar results is shown. The CCR5. Pretreatment with 2 sites except for Ser349, a known GRK site (15). This mutant, when co-mutant was generated that lacks all C-terminal CCR5 phosphorylation including H-89, KN-93, staurosporine, and genistein did not diminish the C5a-induced CCR5-P phosphorylation. Several lines of evidence indicated that this kinase may represent a member of the GRK family (data not shown). First, a CCR5-P (Ser349) mutant that was generated that lacks all C-terminal CCR5 phosphorylation sites except for Ser349, a known GRK site (15). This mutant, when co-expressed with C5aR in RBL cells, was also phosphorylated upon stimulation with CCL5 and C5a, but not with phorbol ester, thus reproducing the results obtained with BIM-treated RBL-CCR5/C5aR cells. Second, a wide range of commercially available kinase inhibitors, including H-89, KN-93, staurosporine, and genistein did not diminish the C5a-induced CCR5-P (Ser349) phosphorylation. Finally, the dose-dependent phosphorylation of CCR5-P (Ser349) after stimulation with C5a for 12 min exactly paralleled C5aR phosphorylation by this ligand in the same cells (EC$_{50}$ ~ 10 nM). C5aR was previously shown to be phosphorylated by GRKs in this concentration range (29, 30). Together, these findings suggest that GRKs phosphorylate CCR5 not only in a homologous manner, but also after heterologous stimulation via C5aR.

To directly test this hypothesis in the absence of kinase inhibitors or receptor mutagenesis we monitored C5a-induced CCR5 phosphorylation in RBL-CCR5/C5aR cells using phosphosite-specific antibodies. As shown in Fig. 7B, treatment with increasing concentrations of C5a for 3 min resulted in CCR5 phosphorylation by both PKC- (EC$_{50}$ ~ 0.7 nM) as well as GRK-mediated (EC$_{50}$ ~ 30 nM) mechanisms. When cells were exposed to C5a for longer periods of time, the dose response curve of PKC-mediated CCR5 phosphorylation significantly shifted to higher concentrations of C5a. A kinetic analysis showed that C5a-mediated CCR5 phosphorylation at these two sites proceeds with distinct kinetics (Fig. 7C). Whereas PKC phosphorylates CCR5 rapidly (t$_{1/2}$ < 0.5 min) but transiently, especially at low agonist concentrations, C5a-stimulated CCR5 phosphorylation at the GRK site is slower (t$_{1/2}$ ~ 3 min) but sustained. Overall, the dose dependence and the kinetics of PKC- and GRK-mediated heterologous phosphorylation of CCR5 closely resembles our earlier findings in homologous CCL5-induced CCR5 phosphorylation (15), and thus support our conclusion that GRKs phosphorylate unligated receptors within activated hetero-oligomers. To exclude the possibility that C5a exerts its effects by direct binding to CCR5, we applied specific receptor antagonists (data not shown). These experiments revealed that an inhibitory anti-C5aR mAb (S5/1) completely blocked C5a-induced CCR5 cross-phosphorylation at both the PKC and GRK sites, but did not affect homologous CCL5-mediated phosphorylation. TAK-779 (1 μM) fully blocked CCL5 induced CCR5-phosphorylation but did not inhibit PMA-induced phosphorylation of CCR5-Ser337. C5a-induced and GRK-mediated cross-phosphorylation on CCR5-
Ligand-induced and Constitutive Co-internalization of CCR5/C5aR Heterodimers—We next examined whether heterodimer formation facilitates CCR5 co-internalization together with C5aR after heterologous receptor activation. First, we confirmed that stimulation with saturating concentrations of C5a, CCL5, and Ang II resulted in homologous down-modulation of a significant fraction (50–60%) of C5aR, CCR5, and AT1aR from the cell surface of transfected RBL cell lines (Fig. 8A). Despite the very rapid and efficient Ang II-mediated internalization of AT1aR, only 23% of CCR5 were co-internalized after heterologous stimulation (100 nM Ang II for 2 h) of RBL-AT1aR/CCR5 cells (Fig. 8B). In contrast, stimulation of RBL-C5aR/CCR5 cells with C5a resulted in the down-modulation of up to 60% of CCR5 from the cell surface. Activation of PKC by phorbol ester was previously shown to induce down-modulation of CXCR4 through phosphorylation of a conserved Ser-(Ile/Leu) motif (31), and other chemotactic receptors have been reported to internalize through clathrin-coated vesicles following receptor phosphorylation by second messenger-activated kinases (32). To determine the significance of C-terminal phosphorylation sites on CCR5 for heterologous internalization of this receptor, we co-expressed C5aR together with CCR5 mutants, which lack either only PKC phosphorylation sites (CCR5-P−(Ser349)), or which are completely phosphorylation-deficient (CCR5-P−). Notably, these receptors were co-internalized upon C5aR stimulation as efficiently as wild-type CCR5 (Fig. 8F). Together, these results show that cross-internalization of CCR5 does not depend on intact PKC or GRK phosphorylation sites on this receptor, but requires co-expression of another agonist-activated receptor that oligomerizes with CCR5.

To further substantiate the hypothesis that CCR5 co-internalizes together with C5aR through physical interaction of the two receptors, but not as a consequence of signaling cross-talk between the two receptors, we studied the intracellular localization of receptors in RBL cells that co-express CCR5 together with either wild-type C5aR or a constitutive endocytic variant (C5aR-US28CT) of this receptor. In these experiments we took advantage of the fact that the C terminus of the human cytomegalovirus-encoded US28 chemokine receptor represents a transposable endocytic element that can confer constitutive, ligand-independent internalization to other GPCR (33). We therefore constructed a chimeric C5aR-US28CT mutant with replacement of the 46 amino acids that follow the conserved NPIIY motif in transmembrane domain VII, i.e., the entire C5aR C terminus, by the 59 C-terminal residues of US28. In various cell lines that transiently or stably overexpress this mutant, C5aR-US28CT was located almost exclusively in intracellular compartments. As shown by flow cytometry of intact and saponin-permeabilized cells, in RBL-CCR5/C5aR cells both receptors are mainly expressed at the cell surface, whereas in RBL cells that co-express CCR5 together with C5aR-US28CT, a significantly higher fraction of CCR5 can be detected within the cell (TABLE THREE). In comparison to up to 60% CCR5 co-internalization in C5a-stimulated RBL-CCR5/C5aR cells, these changes may appear modest, but RBL-C5aR-US28CT cells also express much less C5aR. Immunofluorescence microscopy revealed co-localization of CCR5 together with the C5aR mutant in intracellular vesicles adjacent to cell nuclei, which also contain transferrin receptors (not shown). Taken together, these results do not formally prove that the predominantly intracellular localization of CCR5/C5aR-US28CT is caused by constitutive endocytosis of receptor hetero-oligomers. How-

Ser349 was reduced in the presence of TAK-779 by ~40–50%. This partial inhibition may be caused by stabilization of an inactive receptor conformation by the receptor antagonist. TAK-779 was recently reported to act as an inverse CCR5 agonist (55). No CCR5 phosphorylation was observed when a mixture of two different RBL cell lines that separately expressed C5aR or CCR5 was stimulated with C5a. This rules out the possibility that C5a indirectly phosphorylates CCR5 via soluble mediators.

To further examine the significance of hetero-oligomer formation for receptor cross-phosphorylation, we applied synthetic CCR5 peptides that correspond to TM domains I and IV of CCR5, which reportedly block receptor homodimerization and function (11). In our initial experiments (not shown), these compounds were found not to inhibit C5a-induced cross-phosphorylation of CCR5; neither did they affect CCR5 dimerization (as determined by BRET) nor function (CCL5-induced calcium release). In a separate approach, we generated an RBL cell line that co-expresses CCR5 (1.9 × 106± 2.6 × 105 copies per cell; n = 3) together with AT1aR (5.8 × 106± 1.4 × 105 copies per cell; n = 3). This receptor pair does not engage in hetero-oligomer formation (Fig. 2B). Whereas stimulation of RBL-CCR5/AT1aR cells with Ang II resulted in phosphorylation of CCR5-Ser337 with kinetics and dose dependence that is consistent with a PKC-mediated mechanism, no phosphorylation at the GRK site (Ser349) was detected (Fig. 7, B and C). This suggests that GRKs phosphorylate only receptors that are directly adjacent to the ligand-activated receptor, whereas PKC also phosphorylates receptors at more distant locations within the cell membrane.

**FIGURE 8. Co-internalization of CCR5 upon stimulation of C5aR, but not of AT1aR, homologous receptor internalization. RBL cells that express either C5aR, AT1aR, or CCR5 were stimulated with 100 nM of the corresponding ligands (C5a, Ang II, or CCL5) for up to 2 h at 37 °C. Receptor expression at the cell surface was detected with 125I-labeled 55/1 (anti-C5aR), T21/8 (anti-CCR5), or 12CA5 (anti-HA-AT1aR) antibodies.** A, co-internalization of CCR5 upon heterologous stimulation. RBL-AT1aR/CCR5 cells were stimulated with 100 nM Ang II for up to 2 h, and CCR5 surface expression was determined with 125I-labeled S5/1 (anti-CCR5), T21/8 (anti-CCR5), or 12CA5 (anti-HA-AT1aR) antibodies. B, co-internalization of CCR5-Ser337 with kinetics and dose dependence.
ever, they do show that the intracellular trafficking of these two receptors is tightly interconnected.

**Significance of C5αR Phosphorylation for CCR5 Cross-phosphorylation and Cross-internalization**—Agonist-activated C5αR are internalized in a phosphorylation- and β-arrestin-dependent manner similar to CCR5 (34, 35). According to these earlier reports C5αR is phosphorylated on six C-terminal serine residues upon C5α stimulation and serine phosphorylation is required for both high affinity binding of β-arrestin to the receptor and efficient internalization. In our own studies we confirmed by two-dimensional phosphoamino acid analysis that in RBL-C5αR cells the C5α-stimulated receptor is primarily phosphorylated on serine residues (Fig. 9A), but we also observed additional 32P incorporation into threonine residues, albeit to a much lesser degree. This phosphothreonine signal was preserved in the ligand-activated C5αR-S(T) mutant with alanine replacement of all six serine residues. In agreement with the earlier work we also observed that upon agonist stimulation of C5αR-S(T) β-arrestin is recruited to the cell membrane (Fig. 9B), but this alone is not sufficient for (rapid) receptor internalization (Fig. 9C). A C5αR mutant that lacks C-terminal threonine as well as serine residues was not efficiently internalized and also failed to induce β-arrestin translocation to the cell membrane upon C5α stimulation. This suggests that one or more C-terminal threonine residues participate in recruiting β-arrestins to the ligand occupied C5αR.

We next asked whether phosphorylation-deficient C5αR are capable of cross-phosphorylating CCR5 and whether this heterologous phosphorylation may restore β-arrestin binding and internalization of mutant C5αR by a similar mechanism that we had identified in the regulation of CCR5 homo-oligomers. BRET analysis (Fig. 2B) confirmed that the absence of C-terminal serine and/or threonine phosphorylation sites did not impair the ability of C5αR to associate with CCR5. As shown in Fig. 10A, stimulation of cells with high concentrations of CCL5 or C5α for 5 min resulted in similar (maximal) levels of PKC site phosphorylation of all receptors at the cell surface. Treatment of RBL-CCR5/C5αR cells with a saturating concentration of the cognate ligand CCL5 results in GRK phosphorylation of all receptors at the cell surface. Treatment of RBL-CCR5/C5αR-S–T cells with C5α reproducibly, but less efficiently, induced GRK-mediated cross-phosphorylation of CCR5. Phosphorylation of Ser349 after heterologous stimulation amounted to only 13% of the signal, which was obtained after homologous receptor activation. This difference in CCR5 cross-phosphorylation could be because of the impaired ability of the mutant C5αR to recruit/activate GRKs or may reflect different expression levels of C5αR, the active subunit of the hetero-oligomer. C5αR is present in 3-fold excess over CCR5 in RBL-CCR5/C5αR cells, but is expressed at lower levels in RBL-CCR5/C5αR-S–T cells.

As shown in Fig. 10C, co-expression of wild type, but not of mutant C5αR that lack serine and/or threonine phosphorylation sites, led to CCR5 co-internalization in C5α-stimulated cells. Likewise, co-expres-

### TABLE THREE

| Cells                  | CCR5 expression [MCF] \(\dagger\) in: | C5αR expression [MCF] \(\dagger\) in: |
|------------------------|--------------------------------------|--------------------------------------|
|                        | Intact cells | Permeabilized cells | n | Intact cells | Permeabilized cells | n |
| RBL-CCR5/C5αR          | 27.5 \pm 3.3 | 31.0 \pm 2.4 | 4 | 124 \pm 20 | 139 \pm 21 | 5 |
| RBL-CCR5/C5αR-US28CT   | 20.0 \pm 4.1 | 32.5 \pm 6.8 \(\ddagger\) | 5 | 1.8 \pm 0.6 | 32.3 \pm 6.4 \(\ddagger\) | 5 |

\(\dagger\) As determined by flow cytometry with anti-CCR5-NT mAb T21/8. The MCF in non-transfected RBL-2H3 cells was 0.7 (intact cells) and 1.2 (permeabilized cells).  
\(\ddagger\) As determined by flow cytometry with anti-C5αR mAb S5/1. The MCF in non-transfected RBL-2H3 cells was 0.8 (intact cells) and 1.3 (permeabilized cells).

\(p < 0.05\) compared to intact cells.
GRKs and β-Arrestin Regulate CCR5 Homo- and Heteromers

**DISCUSSION**

In the present study we confirmed earlier observations that the CCR5 associates as dimer, both as homodimer and as heterodimer together with a related chemotactic receptor (C5aR). The first evidence that expression of CCR5 together with C5aR−S−T− did not restore the β-arrestin translocation defect of the mutant receptor upon C5a stimulation (Fig. 10B). Because only a small fraction of all CCR5 expressed in these cells is present in the GRK-phosphorylated form that facilitates β-arrestin binding, these negative results can be explained by the low number of active hetero-oligomeric receptor complexes in these cells. Alternatively, the findings may indicate different structural requirements for β-arrestin binding to and internalization of receptor hetero-oligomers compared with CCR5 homo-oligomers.

C5a-induced. Heterologous CCR5 Phosphorylation and Endocytosis in Human Macrophages—The experiments presented so far were done in transfectants that express high levels of receptors. To investigate whether GRK-mediated cross-phosphorylation and cross-internalization also occurs under physiological conditions we examined the C5a-mediated heterologous regulation of CCR5 in human monocyte-derived macrophages. Monocytes express relatively high endogenous levels of C5aR and further upregulate this receptor and CCR5 during their in vitro differentiation into macrophages. Both receptors were homogenously expressed in monocytes after in vitro incubation for 5 days as determined by quantitative FACS analysis, although C5aR are expressed at ∼20-fold higher levels compared with CCR5 (Fig. 11A and TABLE TWO). Stimulation of the cells with 100 nM CCL5 for 2 h resulted in the loss of 46% of CCR5 at the cell surface, but had little effect on C5aR expression levels (Fig. 11B). In contrast, treatment with 100 nM C5a resulted in the down-modulation of more than 50% of both C5aR and CCR5 receptors from the cell surface. As shown by immunofluorescence microscopy (Fig. 11C), C5aR and CCR5 in unstimulated cells were detected at the plasma membrane. After exposure to 20 nM C5a for 2 h, a significant fraction of C5aR were internalized and located within perinuclear clusters. When C5a-treated cells were stained for CCR5 using E11/19, a mAb that specifically reacts with a GRK phosphorylation site (pSer349) on CCR5, these receptors co-localized to the same endosomal compartment that also contained internalized and phosphorylated CCR5 after treatment of macrophages with CCL5. In the absence of any direct evidence for receptor hetero-oligomerization in these cells it is not possible to draw further conclusions on the underlying mechanisms of receptor co-internalization. Nonetheless, these experiments show that heterologous stimulation of human macrophages with C5a leads to GRK-mediated cross-phosphorylation and cross-internalization of CCR5 in a native system.
GRKs and β-Arrestin Regulate CCR5 Homo- and Heteromers

CCR5 forms homodimers derived from experiments with epitope-tagged receptors (23). Later it was shown by BRET that CCR5 homodimerization and hetero-oligomerization occurs in living cells at physiological levels of receptor expression (12, 25). Moreover, receptor oligomerization is constitutive, i.e. independent of receptor activation, and does not require the N-terminal extracellular domain (36). By using a functional complementation approach we demonstrate that a CCR5ΔNT mutant deficient in ligand binding undergoes GRK-mediated phosphorylation if this receptor is co-expressed together with a different receptor mutant that recruits receptor kinases to the CCR5 homooligomer at the plasma membrane (Fig. 12A). These findings support an alternative model of homologous GPCR regulation, which originally was proposed to involve GRK-mediated phosphorylation specifically of agonist-occupied receptor monomers. Our results show that unligated receptors may also form GRK substrates if they are part of ligand-activated homo- or hetero-oligomeric receptor complexes. Interactions of GRKs with GPCR substrates at sites distinct from their sites of phosphorylation was previously known from studies that showed that synthetic peptides derived from the first intracellular loop of the β2-adrenergic receptor effectively inhibited GRK-mediated phosphorylation of sites located on different domains of the same receptor (37). The observation that GRK1-mediated phosphorylation of peptide substrates is significantly enhanced by a factor of >100 in the presence of activated rhodopsin is compatible with the notion that ligand-bound GPCR enhance GRK activity in an allosteric manner (24). However, receptor activation alone is not sufficient for GRK-mediated phosphorylation, because mastoparan, which displays properties of ligand-activated GPCR (38), could not substitute for agonist-occupied receptors in CCR5 phosphorylation experiments. Thus, GRK-mediated GPCR phosphorylation appears to be confined to the immediate vicinity of the ligand/receptor complex. If GPCR homodimerization is even necessary for GRK-mediated receptor phosphorylation, as one might infer from earlier work (39), requires further investigation. In any case, the published crystallographic structure of GRK2 in complex with a Gβγ subunit is compatible with the interaction of the receptor kinase with a GPCR oligomer (40).

Detailed analysis of the binding of β-arrestin to ligand-activated CCR5 has revealed that at least two distinct interaction sites on the receptor are involved, namely the phosphorylated C terminus and a separate phosphorylation-independent site on the second intracellular loop of CCR5 (14). Receptors that are mutated at either one of the two critical β-arrestin binding sites are not capable of recruiting β-arrestin to the plasma membrane upon agonist stimulation. The results presented herein suggest that the two interaction sites may not necessarily have to be present within a single monomeric receptor, but that they can be provided by separate proteins that upon co-expression can complement their respective defects in β-arrestin binding. In this respect our study extends recent findings by Hansen et al. (41) who show by BRET technology that co-expression of the AT1 wild-type receptor can rescue β-arrestin 2 recruitment to signaling deficient AT1-K199A mutant. Alternatively, agonist-induced activation of one of the monomers within the receptor dimer may induce conformational changes in the unligated monomer via their dimerization interface, which leads to expression of all necessary binding sites on a single protomer. Evidence for such cooperative conformational changes within a GPCR dimer was recently obtained using a leukotriene B4 receptor dimer where one of the monomers displays lower affinity for the agonist (42). Both models are consistent with the crystal structure of β-arrestin that contains two structurally homologous seven-stranded β-sandwich, possible binding sites for two different GPCR (43, 44). Modeling of the rhodopsin-arrestin interface, which is based on crystal structures of the two proteins, suggests that, indeed, one arrestin monomer may interact with one rhodopsin dimer (45).

The internalization experiments indicated that β-arrestin bound to the CCR5 dimer is functionally active, although the increase in endocytosis rates of CCR5-P' in cells that co-express CCR5ΔNT was small and detectable only during early time points after agonist stimulation. The desensitization rate of the CCL5-induced calcium response in RBLCCR5-P'/CCR5ΔNT cells was also not fully restored compared with cells expressing CCR5-WT. One has to take into account that probably only a minor fraction of the entire population of receptors forms CCR5-P'/CCR5ΔNT heterodimers, and this also explains the lower efficiency of β-arrestin translocation to the plasma membrane compared with cells that express wild-type CCR5. Because this dimer consists of two receptor mutants, only one of which is capable of chemokine binding, this implies that ligand binding to one component of the receptor dimer is sufficient for membrane recruitment and activation of β-arrestins. In contrast, Novi et al. (46) conclude from their study using two different co-expressed adrenergic and muscarinic receptor chimeras that β-arrestin-mediated activation of the ERK1/2 pathway requires simultaneous activation of the two components of the receptor dimer. We did not examine chemokine-induced activation of MAP kinase pathways, which in RBL cells is independent of β-arrestins (19). Nonetheless, co-activation of more than one partner of the CCR5-containing dimer is unlikely, since cross-competition experiments with cells expressing CCR5 together with CCR2b indicated that such a receptor dimer can only bind a single chemokine ligand (25).
The data herein are also relevant for the cross-regulation of chemokine receptors by heterologous stimuli. Treatment of RBL-CCR5/C5aR cells with C5a induced cross-phosphorylation of CCR5 not only by well-established PKC-mediated mechanisms, but also by GRKs. The evidence for this is based on a combination of receptor mutagenesis studies, the use of specific kinase inhibitors, as well as the direct analysis of CCR5 phosphorylation in whole cells using phosphosite-specific antibodies. Analogous to the regulation of CCR5 homodimers by GRKs, these data suggest that unligated CCR5 receptors are also substrates for GRKs if they form hetero-complexes together with other agonist-activated receptors, such as C5aR (Fig. 12B). Whether GRK-mediated cross-phosphorylation of the unligated receptor can rescue β-arrestin binding and internalization if this receptor forms hetero-complexes together with a different mutant receptor, is less clear. The results of β-arrestin binding and co-internalization experiments in cells that co-express CCR5 and mutant C5aR suggest that this may not be the case and, thus, hetero-oligomeric receptor complexes interact with components of the endocytic machinery differently from homo-oligomers. This interpretation is more in keeping with earlier observations that receptor heterodimers that consist of two protomers with different trafficking patterns are internalized as stable oligomeric receptor complexes, but the endocytic routes of these complexes are determined by the identity of the ligand-activated receptor and its ability to stably interact with β-arrestins (47). On the other hand, the efficiency of cross-regulation between the two subunits of such a receptor complex depends on the relative expression levels of both receptors at the cell surface, as evidenced by the low level of GRK-mediated cross-phosphorylation of CCR5 in cells that co-express mutant C5aR. A minor C5a-induced β-arrestin membrane translocation and receptor internalization in these cells may have escaped detection by the methods that were applied in this study.

Several recent studies have demonstrated cross-phosphorylation and β-arrestin-mediated cross-internalization of receptor heterodimers (39, 48–50). Using a synthetic bivalent dimerizing reagent that promotes the heterodimerization of cyclophilins fused to β-arrestin 2 and vasopressin (V1a/V2) receptors, respectively, Terrillon and Bouvier (51) elegantly demonstrated that binding of β-arrestins to either of the two receptors is sufficient to induce co-internalization of both receptors in the complete absence of agonist-mediated receptor activation. Congruent with this concept, we also observed that a significant fraction of CCR5 is localized intracellularly in cells that co-express a constitutively internalized C5aR-US28CT mutant even in the absence of agonist stimulation. Moreover, elimination of PKC and/or GRK phosphorylation sites on CCR5 did not inhibit C5a-induced cross-internalization in cells that co-express C5aR. Together, these results suggest that cross-internalization of CCR5 after heterologous stimulation does not require cross-signaling via PKC or other pathways, but rather depends on physiological interaction between the two subunits. This is in contrast to another report that favors a major role for PKC in the interleukin-8-induced cross-internalization of CCR5 in RBL cells that co-express CXCR1 (32). We have not tested whether or not CXCR1 forms hetero-oligomeric complexes with CCR5 and thus may regulate its activity in a different manner as described here for C5aR. However, PKC appears not to be solely responsible for heterologous CCR5 internalization because, in addition to the results presented herein, neither PKC activation by phorbol esters nor its inhibition has a major effect on membrane expression and ligand-induced trafficking of this receptor (31).

GRK-mediated cross-phosphorylation and co-internalization of CCR5 was also detected in C5a-stimulated human monocyte-derived macrophages. In these cells, C5aR exceeds CCR5 expression by a factor of ~20. This constellation favors C5aR effects on CCR5 and may explain why C5a down-modulates CCR5 from the cell surface at least as efficient as the homologous ligand CCL5, whereas CCL5 stimulation did not change C5aR expression. However, the magnitude of heterologous, C5a-induced CCR5 cross-internalization in these cells was unexpected, because macrophages also express other chemotactic receptors such as CCR2, which are known to form hetero-oligomers and may thus compete with C5aR for CCR5 binding. Alternatively, different chemotactic receptors may co-exist in the plasma membrane of leukocytes as even larger protein aggregates that are together phosphorylated and internalized, rather than as exclusive heterodimers. Co-immunoprecipitation studies of differently tagged forms of the M2 muscarinic receptor revealed evidence for oligomeric receptors at least as large as trimers (56). Despite growing evidence for the existence and functional relevance of heterodimer formation in vitro of other GPCR (57), this has yet to be shown for chemotactic receptors.

Our results are also potentially relevant to ongoing efforts to modify the function and membrane expression of HIV co-receptors by therapeutic agents. The ability of modified chemokine ligands to suppress HIV infection in vitro and in vivo by inducing endocytosis and intracellular sequestration of CCR5 through homologous receptor stimulation is well established (9, 52). Several other GPCR ligands that are unrelated to chemokines have also been shown to attenuate the capacity of CCR5 to act as HIV coreceptors in a heterologous manner. Both PKC-mediated cross-desensitization and cross-internalization have been implicated in this heterologous regulation of coreceptor functions (32, 53, 54). Further work is required to better understand the structural parameters that determine the formation of CCR5 hetero-oligomers and their interaction with β-arrestins, as they are described in the present work. Nonetheless, the GRK-promoted phosphorylation of CCR5 containing homo- and heterocomplexes defines a new and functionally relevant mechanism how chemotactic leukocyte receptors interact with each other.

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