Differential Desensitization, Receptor Phosphorylation, β-Arrestin Recruitment, and ERK1/2 Activation by the Two Endogenous Ligands for the CC Chemokine Receptor 7*

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Trudy A. Kohout‡‡, Shelby L. Nicholas‡, Stephen J. Perry¶, Greg Reinhart‡, Sachiko Junger¶, and R. Scott Struthers‡‡

From the Departments of ‡‡Exploratory Discovery and ¶Molecular Biology, Neurocrine Biosciences Inc., San Diego, California 92121

Numerous G protein-coupled receptor (GPCR)† systems have been identified where multiple endogenous ligands can interact with a shared cognate receptor. These ligand clusters can be derived from different gene transcripts or from differential processing of a common transcript or precursor protein. The variety of pro-opiomelanocortin-derived peptides active at melanocortin receptors is an example of the latter (1), whereas the various families of chemokines may provide the most extensive example of the former (2). In the chemokine family, despite numerous receptors that recognize multiple endogenous chemokines, functional differences between ligands have yet to be well characterized.

The CC chemokine receptor 7 (CCR7) is expressed on T lymphocytes and is the central regulator of homing and trafficking of lymphocytes into and within the secondary lymphoid tissues (3). CCR7 has two endogenous ligands, CCL19 (ELC and MIP3β) and CCL21 (SLC, Exodus-2, 6-Ckine and TCA-4), both of which are full agonists with similar receptor binding affinities and chemotactic potencies (4–6). Thus, the reason for the existence of two endogenous ligands for CCR7 is not clear. However, the possibility that they have different regulatory effects on CCR7 has been largely unexplored.

Like all chemokine receptors, CCR7 initiates signal transduction through activation of members of the G family of heterotrimeric G proteins (7). When CCL19 or CCL21 binds CCR7 the heterotrimeric Gi complex is activated and dissociates into its α and βγ subunits. The released Go subunit inhibits adenylyl cyclase, and the Gβγ dimer activates two major signaling enzymes, phospholipase Cβ (8) and phosphatidylinositol 3-kinase (9), which initiate the activation of downstream targets, such as calcium mobilization and Akt, respectively.

In addition to activation of signaling pathways, GPCRs also undergo the regulatory process of desensitization. Desensitization results in diminished responsiveness of GPCRs to repeated or prolonged exposure to agonist. This process requires phosphorylation of the receptor tail by G protein-coupled receptor (GRKs) to promote the binding of β-arrestins, which uncouple the receptor from its cognate G protein (10). Recent evidence also suggests that β-arrestins contribute to GPCR signaling by functioning as scaffolds for the recruitment of signaling molecules into a complex with the agonist-occupied receptors (11). Several members of the c-Src family of tyrosine kinases such as c-Src, Hck, c-Fgr, and Yes are translocated to

N-terminal kinase; AT1_R, angiotensin type 1A receptor; PAR-2, protease-activated receptor-2; RBL, rat basophilic leukemia cells; HEK, human embryonic kidney cells; siRNA, small interference RNA; FACS, fluorescence-activated cell sorting; PBS, phosphate-buffered saline; GTP-S, guanosine 5’-3-O-thiotriphosphate; BSA, bovine serum albumin; ANOVA, analysis of variance; PKA, protein kinase A; FKC, protein kinase C.
CCL19 and CCL21 Differentially Regulate CCR7

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—All cell lines were obtained from the American Type Culture Collection. H9 T cell lymphoma cells were maintained in RPMI medium 1640 supplemented with 15% fetal bovine serum, 2 mM l-glutamine, 10 mM HEPES, pH 7.4, and penicillin-streptomycin. Rat basophilic leukemia (RBL) cells and human embryonic kidney (HEK293) cells were maintained in Dulbecco’s modified eagle medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 10 mM HEPES, pH 7.4, and penicillin-streptomycin. Transfections of HEK293 cells on 10-cm dishes were performed with 5 μg of plasmid and 15 μl of FuGENE6 (Roche Applied Science), according to the manufacturer’s instructions. Stable clones were selected and maintained with 1 mg/ml Geneticin (Invitrogen). Transfections of HEK293 cells plated on 10-cm dishes with β-arrestin-2 small interfering RNA (siRNA) (Qiagen) were performed with 20 μM siRNA and 50 μl of GeneSilencer (Gene Therapy Systems) as previously described (17).

Plasmid Construction—The CCR7 cDNA (4) was subcloned into pcDNA3.1 (Invitrogen) with a FLAG tag added to the carboxyl terminus. The CCR7-SI (FLAG), CCR7–SS/S (SS/S), and CCR7-ALL (ALL) mutations were introduced into the pcDNA3.1-CCR7-FLAG clone using the QuikChange II site-directed mutagenesis kit (Stratagene). All mutations were verified by DNA sequence analysis. The pcDNA3.1-β-arrestin-2 construct was prepared by amplifying the β-arrestin-2 coding sequence from a human brain cDNA library (BD Biosciences Clontech) with primers that also encoded the restriction sites for EcoRI and BamHI; the PCR product was digested and ligated into the equivalent sites of the pcDNA3.1 vector (Invitrogen).

Fluorescence-activated Cell Sorting (FACS) Analysis of Cell Surface CCR7 Expression—CCR7 expression on stably transfected HEK293

GPCRs by binding to β-arrestin (12). Activation of the CXC-chemokine receptor-1 by interleukin 8 induces the formation of a β-arrestin complex with Hck and c-Fgr, which is essential for granule release in neutrophils (13). Similarly, β-arrestins can scaffold various members of the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) pathways (11, 12, 14). For example, β-arrestins directly interact with c-Raf-1 and ERKs 1 and 2 (ERK1/2) and promote ERK1/2 activation (15). Several GPCRs have been shown to activate ERK1/2 in this manner, including the angiotensin type 1A receptor (AT1AR) (15) and the protease-activated receptor-2 (PAR-2) (16).

In the work presented here, we investigate whether CCL19 and CCL21 differentially regulate CCR7 by studying the early signaling events initiated after ligand binding, including an analysis of the mechanism involved in the desensitization of the receptor and its activation of ERK1/2 MAPK.

Fig. 1. CCL19 stimulation, but not CCL21, promotes CCR7 desensitization. H9 cells endogenously expressing CCR7 were pretreated with 100 nM CCL19, 100 nM CCL21, or left untreated (control) for 10 min. The residual agonist was removed from the cells with two sequential acid washes, then further washed three times with phosphate-buffered saline. Membranes were prepared from these cells and the ability of CCR7 to activate GTP exchange in response to a second stimulation with various doses of CCL19 (A) and CCL21 (B) was measured as GTP-γS binding. GTP-γS binding was plotted as agonist-stimulated activation over basal (non-stimulated GTP-γS binding). Data are the mean ± S.E. of four experiments. Two-way ANOVA analyses were performed on the GTP-γS binding curves of pretreated membranes and compared with control, untreated membranes (*, p < 0.001). There was no statistical difference between the CCL19-pretreated and the control, untreated membranes. Furthermore, the −log EC50 (EC50) values for CCL19- and CCL21-stimulated G protein loading in naive membranes were 7.55 ± 0.15 (28.0 nM) and 7.68 ± 0.11 (20.9 nM), respectively, and in CCL21-pretreated membranes were 7.18 ± 0.10 (66.4 nM) and 7.29 ± 0.21 (51.5 nM), respectively. The apparent differences in EC50 between the control and CCL21-pretreated membranes are not statistically significant. To assess the efficiency of ligand removal with the acid washes, the basal GTP-γS binding of the CCL19- and CCL21-stimulated membranes were compared with that of the untreated membranes. The basal levels of the CCL19-pretreated and CCL21-pretreated membranes were 1.06 ± 0.08 and 0.92 ± 0.04, respectively, compared with the untreated membranes, which was set at 1.00.

Fig. 2. CCL19 and CCL21 are equipotent for the activation of CCR7-G protein coupling and Ca2+ mobilization. A, membranes prepared from H9 cells were stimulated with increasing concentrations of CCL19 or CCL21, and GTP-γS binding was measured. The calculated −log EC50 (EC50, nM) for CCL19- and CCL21-stimulated GTP-γS binding were 7.63 ± 0.18 (23.4 nM) and 7.87 ± 0.32 (13.5 nM), respectively. Data are the mean ± S.E. of four experiments. B, RBL cells stably expressing CCR7 were stimulated with various concentrations of CCL19 and CCL21, and the resulting Ca2+ flux was measured as described under “Experimental Procedures.” The calculated −log EC50 (EC50, nM) for CCL19- and CCL21-stimulated Ca2+ flux were 7.27 ± 0.18 (53.7 nM) and 7.27 ± 0.16 (53.7 nM), respectively. Data are the mean ± S.E. of six experiments. GTP-γS binding and Ca2+ mobilization data were plotted as the -fold stimulation by dividing the agonist-stimulated response by the basal response.
cells were determined by FACS analysis. The CCR7 was detected with a phycoerythrin-conjugated rat anti-human CCR7 antibody and normalized to the isotype control, phycoerythrin-conjugated rat IgG2a, κ (BD Pharmingen). Samples were acquired and analyzed using a FACSCalibur flow cytometer (BD Biosciences).

**Ligands**—CCL19 was synthesized by solid-phase methodology on a CS536 Peptide Synthesizer (CS Bio), as previously described (6). The synthesized CCL19 contained a substitution at Met-72 for norleucine to enhance the stability of the peptide. This substitution was shown to be silent in its binding affinity for CCR7 and in multiple functional assays (6). CCL21 was obtained from ID Laboratories.

**Receptor Desensitization Assay**—1.5–2.0 × 10⁶ H9 cells were centrifuged and resuspended in serum-free media (RPMI 1640 supplemented with 2 mM L-glutamine and 10 mM HEPES, pH 7.4). The cells were transferred to three 10-cm plates and stimulated with either 100 nM CCL19 or 100 nM CCL21 for 10 min at 37 °C, or left untreated. After stimulation the cells were washed twice for 5 min with 20 ml of ice-cold acid wash buffer (90 mM NaCl and 50 mM sodium citrate, pH 4.5) to remove bound ligand. The cells were then washed three times in phosphate-buffered saline (PBS) and membranes were prepared.

**Membrane Preparation**—Membranes from H9 cells were prepared as previously described (6). Briefly, H9 cells were washed with PBS, resuspended in ice-cold membrane buffer (20 mM HEPES, 6 mM MgCl₂, 1 mM EDTA, pH 7.2), and homogenized by Dounce homogenization (60 strokes). The homogenate was centrifuged at 1,200 rpm and 4 °C for 10 min. The resulting supernatant was subsequently centrifuged at 20,000 rpm and 4 °C for 45 min. The membrane pellet was resuspended in membrane buffer, and protein concentration was determined by BCA Protein assay (Pierce).

**[35S]GTP·S Binding Assays**—Guanine nucleotide exchange assays were performed, as previously described (6). Briefly, membranes were incubated in 50 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 0.1% BSA, 10 μM GDP, and 500 pM [35S]GTP·S with various concentrations of CCL19 and CCL21 for 30 min at 37 °C. Incorporated [35S]GTP·S was measured in a liquid scintillation counter.

**Intracellular Calcium Assay**—RBL cells stably expressing CCR7 (RBL-CCR7) were grown overnight at 8 × 10⁶ cells per well in a 96-well poly-lysine-coated plate. After 24 h, 100 μl of Calcium 3 Assay Reagent solution (1× Hanks’ balanced salt solution, 20 mM HEPES, pH 7.4, 5 mM probenecid, and FLIPR Calcium 3 Assay Reagent (Molecular Devices)) was added directly to the cells. The assay plate was incubated for 1 h at 37 °C. A 96-well plate with various concentrations of CCL19 and CCL21 diluted in 1× Hanks’ balanced salt solution containing 0.1% BSA, and 20 mM HEPES was prepared. Both the assay plate and the peptide dilution plate were run on an ImageTrak Epi-Fluorescence system (PerkinElmer Life Sciences).

![Fig. 3. CCL19 and CCL21-induced CCR7 phosphorylation.](image1) HEK-CCR7-FLAG cells or HEK293 cells stably transfected with the empty vector (pC3) were metabolically labeled with [32P] orthophosphate ([32P]O) incorporation to the total CCR7 protein. The phosphorylation was calculated by normalizing the [32P] incorporation to the isotype control, phycoerythrin-conjugated rat IgG2a, κ (BD Pharmingen). Samples were acquired and analyzed using a FACSCalibur flow cytometer (BD Biosciences).

![Fig. 4. Effects of PKA and PKC inhibition on CCL19-mediated CCR7 phosphorylation.](image2) HEK-CCR7-FLAG cells were incubated overnight with 2 μM GF109203X or incubated for 2 h with 10 μM H89. Cells were metabolically labeled with [32P] orthophosphate ([32P]O) incorporation to the total CCR7 immunoprecipitated for each sample. The phosphorylation was calculated by normalizing the [32P] incorporation to the total CCR7 immunoprecipitated for each sample. The data graphed are the mean ± S.E. of three to six experiments.
Cells were stimulated with the appropriate ligand for 10 min at 37 °C, or left untreated. Cells were washed twice with PBS, and cell extracts were prepared by lysing cells in 500 μl of glycerol lysis buffer (50 mM HEPES, pH 7.4, 0.5% Nonidet P-40, 250 mM NaCl, 2 mM EDTA, 10% glycerol, 100 μM Na3VO4, 10 mM NaF, 100 mM microcystin-LF, and protease inhibitor tablet (Roche Applied Science)). After clarification CCR7-FLAG was immunoprecipitated from 2.0 mg of protein with 50 μl of M2 anti-FLAG-agarose conjugate for 16 h with constant rotation. Immunoprecipitates were washed five times with glycerol buffer and eluted with 50 μl of 2× SDS sample buffer (Invitrogen) supplemented with 200 mM NaCl, 1,4-dithiothreitol. Equal volumes of the immunoprecipitates were resolved on two 4–15% polyacrylamide gels (Invitrogen). One gel was dried to measure 32P incorporation using a phosphorimaging device (Bio-Rad); the second gel was transferred onto a nitrocellulose filter and immunoblotted with anti-FLAG-horseradish peroxidase antibody conjugate. Bands were quantitated on a VersaDoc3000 Imaging System (Bio-Rad). Stimulation with CCL19 but Not CCL21 Induces Desensitization of CCR7. HEK293 cells stably expressing CCR7 or CCR7 mutants were serum-starved for 1 h and stimulated with the corresponding ligands for 5 min at 37 °C. Cells were washed once with PBS and harvested directly into 2× SDS sample buffer. Cell extracts were sonicated, heated at 55 °C for 5 min, and subjected to SDS-PAGE. Resolved proteins were transferred onto nitrocellulose membranes. The activated phosphorylated form of ERK1/2 was detected using an anti-phosphoMAPK p42/44 antibody (Cell Signaling Technology) diluted at 1:3000 in 1% nonfat dried milk in TBST. Detection of immunoreactive proteins was performed with SuperSignal West Pico reagent (Pierce) and quantified on the VersaDoc3000 Imaging System (Bio-Rad).

Data Analysis—Data are expressed as mean ± S.E. Dose-response data were plotted and analyzed (± log EC50 determination) with GraphPad Prism software. Statistical significance between dose-response curves was determined with a two-way ANOVA test. Statistical significance between single doses was determined by an unpaired, two-tailed t test. When comparing -fold stimulation with basal stimulation, statistical significance was determined by a paired, two-tailed t test.

RESULTS

Stimulation with CCL19 but Not CCL21 Induces Desensitization of CCR7 in H9 Cells—GPCRs become desensitized after stimulation, resulting in a reduction in their ability to couple to G proteins following subsequent exposure to their ligands. Because CCR7 has two endogenous ligands, we compared the ability of CCL19 and CCL21 to desensitize CCR7. H9 cells (a human T cell lymphoma cell line that expresses...
CCR7 endogenously) were pretreated for 10 min with either 100 nM CCL19 or CCL21, or left untreated. Membranes were prepared and the ability of a second stimulation with CCL19 or CCL21 to mediate GTP exchange on G proteins was measured. Cells that were not pretreated with agonist stimulated GTP exchange equally efficiently after receptor activation with either CCL19 or CCL21 (Fig. 1). Membranes prepared from cells pretreated with CCL19, however, were significantly impaired in CCR7-G protein coupling when rechallenged with either CCL19 or CCL21. There was a 58.1 ± 7.4% decrease in the maximum GTP exchange in membranes pretreated with CCL19 when restimulated with CCL19 (Fig. 1A), and 57.9 ± 5.3% after restimulation with CCL21 (Fig. 1B), compared with membranes that received no pretreatment. However, pretreatment with CCL21 had no effect on the ability of either CCL19 (Fig. 1A) or CCL21 (Fig. 1B) to promote GTP loading, indicating that CCL21 cannot efficiently desensitize CCR7. Similar desensitization profiles were obtained in HEK293 cells expressing CCR7-FLAG (data not shown). This difference would be explained if the two agonists differed in their potency. To test this, we compared the ability of CCL19 and CCL21 to activate CCR7 using both G protein loading assays and downstream signaling assays. Fig. 2A shows that CCL19 and CCL21 stimulate equivalent levels of GTP-8 loading of H9 cell membranes. Furthermore, CCL19 and CCL21 are equipotent at promoting calcium mobilization in RBL cells that are stably transfected with CCR7 (Fig. 2B). Taken together, these data show that, despite CCL19 and CCL21 being equally potent at activating CCR7, only CCL19 is capable of inducing receptor desensitization.

**CCR7 Phosphorylation Is Induced by CCL19 but Not CCL21**—Phosphorylation of most GPCRs is a necessary step for their desensitization. Therefore, we compared the ability of CCL19 and CCL21 to induce CCR7 phosphorylation to determine whether this correlated with the difference we observed in the receptor desensitization profiles of the two ligands. HEK293 cells expressing FLAG-tagged CCR7 (HEK-CCR7-FLAG cells) were metabolically labeled with 32P and stimulated with various concentrations of the two agonists to induce receptor phosphorylation. Receptor molecules were then isolated by immunoprecipitation, and the amount of 32P incorporated was determined. A substantial level of basal phosphorylation of CCR7 was evident prior to stimulation with ligand (Fig. 3A). However, this level of phosphorylation increased in a dose-dependent manner following treatment with CCL19, but was almost unchanged when the receptor was stimulated with CCL21: phosphorylation of CCR7 increased by 83% over basal levels with CCL19 stimulation, whereas CCL21 promoted a maximal 32P incorporation of only 18% (Fig. 3, A and B).

To identify which kinases affect CCR7 phosphorylation, cells were pretreated with specific inhibitors of the second messenger-dependent kinases, aCAM-dependent protein kinase (PKA) and protein kinase C (PKC). Whereas the PKA inhibitor H89 had no effect on either the basal or the CCL19-stimulated phosphorylation of CCR7, the PKC inhibitor GF109203X (bisindolylmaleimide I) significantly inhibited the basal and the agonist-stimulated phosphorylation of the receptor (Fig. 4, A and B).

**Identification of Phosphorylation Sites on CCR7**—The carboxyl-terminal domain of CCR7 contains several serine and threonine residues that represent potential sites for GPCR kinase (GRK) and second messenger kinase-mediated receptor phosphorylation. To identify which of these sites are phosphorylated on CCR7, three CCR7 mutants were constructed in which various groups of serines and threonines were mutated to alanines (Fig. 5A): CCR7(-S/4T) lacks the carboxyl-terminal cluster, Ser-378 and Thr-373/374/375/376; CCR7(-SS/SS) lacks the PKA and PKC consensus phosphorylation sites of Ser-365/364/357/356; CCR7(-All) lacks both sets of sites mutated in the (-S/4T) and (-SS/SS) receptors. These mutant receptors were stably expressed in HEK293 cells at equivalent levels, and the effect the mutations had on basal and CCL19-stimulated phosphorylation of the receptor was examined. Fig. 5 (B and C) shows that CCL19-mediated phosphorylation was reduced by 74% in CCR7(-S/4T) as compared with wild type (WT) CCR7, whereas its basal phosphorylation was unaffected (Fig. 5D); CCR7(-SS/SS) displayed a 63% inhibition of basal phosphorylation compared with WT CCR7 (Fig. 5, B and D), whereas its ability to undergo agonist-mediated phosphorylation was unchanged (Fig. 5, B and C). Interestingly, the phosphorylation pattern of the (-SS/SS) mutant paralleled that of the WT receptor when treated with a PKC inhibitor (Fig. 4), indicating that the basal phosphorylation by PKC occurs at one or more of these sites. Lastly, the CCR7(-All) mutant, which contains both sets of mutations, was defective in both its basal and CCL19-mediated phosphorylation (Fig. 5, B–D). These data suggest that most of the sites of CCL19-stimulated phosphorylation reside within the string of residues in the distal portion of the tail of the receptor, and basal phosphorylation by PKC occurs at one or more serines at positions 356, 357, 364, and 365.

**β-Arrestin 2 Is Recruited to CCR7 by CCL19 Stimulation but Not by CCL21**—Agonist-stimulated phosphorylation of GPCRs
CCL19 and CCL21 Differentially Regulate CCR7

Fig. 7. CCL19 and CCL21 induce ERK1/2 MAPK activation. A. HKE-
CCR7-FLAG cells were serum-starved for at least 1 h, and then stimulated with various concentrations of CCL19 and CCL21, or 10 ng/ml EGF for 5 min. Representa-
tive phospho-ERK1/2 and total ERK immunoblots are shown. B, the lev-
els of phospho-ERK1/2 were quantitated and normalized to the total ERK1/2 in
each sample. Because basal activation of ERK1/2 cannot be quantitated, the data
are presented as the percentage of the total activable ERK1/2 in the cells as de-
termined by EGF stimulation of the cells. Data graphed are the mean ± S.E. of four
experiments. Two-way ANOVA analysis was performed. Results showed that
CCL19-stimulated ERK activation is statistically different from the CCL21 activa-
tion of ERK1/2 (*, p < 0.01).

typically results in the rapid recruitment of β-arrestins to the receptor. To determine whether β-arrestins are translocated to CCR7 upon stimulation by CCL19 and CCL21, HEK293 cells expressing FLAG-tagged CCR7 and β-arrestin-2 were stimu-
lated with 100 nM CCL19 or CCL21 and recruitment of β-arrestin-2 to CCR7 was assessed by the amount of β-arrestin-2 that co-immunoprecipitated with the receptor. Stimulation with CCL19 resulted in the time-dependent translocation of β-arrestin-2 (Fig. 6, A and B) to CCR7 (+4.1-fold over unstimu-
lated cells). As expected, because stimulation with CCL21 re-

cults in minimal CCR7 phosphorylation, β-arrestin-2 recruit-
ment to the receptor by CCL21 was also significantly attenuated. Thus, the level of recruitment of β-arrestin-2 to CCR7 by the two ligands correlated with the previously deter-
mined ability of the ligands to induce receptor phosphorylation.

CCL19 Activates ERK1/2 MAP Kinases in a CCR7 Phospho-
rylation- and β-Arrestin-2-dependent Manner—Besides the well charac-
terized role of β-arrestins in receptor desensitization,
β-arrestins also facilitate activation of ERK1/2 by scaffolding
components of the ERK1/2 MAPK cascade to the activ-
ted receptor. Thus, we sought to determine if stimulation of
CCR7 with CCL19 and CCL21 resulted in ERK1/2 activation and whether the pattern of activation paralleled the β-arrestin-2 translocation pattern to the receptor. HEK-CCR7-FLAG
cells were stimulated with increasing concentrations of either CCL19 or CCL21. The extent of ERK1/2 phosphorylation by these agonists was measured as a percentage of the maximal possible ERK1/2 activation, determined by stimulation with 10 ng/ml EGF. Fig. 7 shows that a 5-min stimulation with a maximal dose of CCL19 (100 nM) resulted in a 3.7-fold greater activation of ERK1/2 than did a maximal dose (100 nM) of CCL21; the difference in ERK1/2 activation by CCL19 and CCL21 correlates with their abilities to recruit β-arrestin-2 to CCR7. Stimulation of endogenous CCR7 in human peripheral blood lymphocytes with CCL19 resulted in a similar enhance-
ment in the activation of maximal ERK1/2 compared with the ERK1/2 activated by CCL21 (data not shown). We further tested the role of β-arrestin in CCR7-mediated ERK1/2 activation by utilizing the technique of small interfering RNA (siRNA) to reduce the expression of endogenous β-arrestin-2 (17), and then examined ERK1/2 activation by CCL19. Transfection with a siRNA specific for β-arrestin-2 resulted in a 42% reduction in β-arrestin-2 expression compared with cells trans-

fected with a control siRNA (Fig. 8B). Down-regulation of β-arrestin-2 expression decreased the dose-dependent activation of ERK1/2 of CCL19 by 72% compared with control siRNA-
treated cells (Fig. 8, A and B). These findings suggest that recruitment of β-arrestin-2 to CCR7 is important in its ability to
activate the ERK1/2 MAPK pathway.

Because β-arrestins are recruited to the activated, phospho-
ylated receptor, we sought to establish whether the phospho-
ylation state of the receptor was important in its ability to
activate the ERK1/2 pathway. For this purpose we utilized the phosphorylation-deficient CCR7 mutants that not only allowed us to determine if CCR7 phosphorylation is necessary for ERK1/2 activation but also to determine whether basal and/or agonist-activated phosphorylation is important. CCR7(-S/4T) and CCR7(-ALL) exhibited a 56 and 70% reduction in maximal ERK1/2 activation, respectively, when compared with WT re-
ceptor, indicating that agonist-induced phosphorylation is nec-

cessary for full activation of ERK1/2 (Fig. 9, A and B). In con-
trast, the CCR7(-SS/SS) mutant, which is impaired in its basal
but not agonist-induced phosphorylation, was capable of acti-
vating ERK1/2 to a similar magnitude as the WT receptor.

These findings indicate that agonist-stimulated phosphoryla-
tion of CCR7 is required for ERK1/2 activation, but its agonist-

independent basal phosphorylation is dispensable.

DISCUSSION

CCR7 initiates diverse cellular behaviors at temporally and
spatially separate stages in the recruitment of T lymphocytes
to peripheral lymph nodes; these include T cell homing, teth-
ering, and rolling on the endothelium of high endothelial
venules, extravasation, and migration to the T cell zones (3).

A single receptor can produce such a diversity of effects
cannot easily be explained by a simple paradigm involving one
receptor and one ligand. The existence of two ligands for CCR7
provides one mechanism by which multiple responses can be
elicited, because the two ligands are differentially expressed in
high endothelial venules (CCL21) and T cell zones (CCL19) (3).
Here we provide evidence that CCR7 also undergoes differen-
tial regulation by CCL19 and CCL21, resulting in a divergent
signaling of CCR7 to downstream effectors. In the present
study we document that stimulation of CCR7 by CCL19, but
not CCL21, results in the desensitization of the receptor, because CCL19 alone possesses the ability to place CCR7 in a conformation that can undergo agonist-dependent phosphorylation. The phosphorylated receptor then recruits β-arrestin, which interdicts further association of the receptor with its cognate G protein, preventing further signaling. Using mutational analysis of CCR7 we determined that the CCL19-mediated phosphorylation mostly occurs within residues 373 and 376 of the carboxyl terminus. This region of the receptor contains a stretch of 4 threonines and 1 serine that could potentially be a site of phosphorylation by GRKs. Similar sites on the carboxyl tails of the vasopressin 2 and the angiotensin II type 1A (AT_1R) receptors have been shown to be GRK phosphorylation sites and be important for the formation of stable receptor-β-arrestin complexes (18, 19). We also discovered that CCR7 is basally phosphorylated, probably by PKC, within serines 356, 357, 364, and 365. The physiological function of this phosphorylation is currently unknown. PKC may also play a role in CCL19-stimulated phosphorylation of CCR7, because 63% of the stimulated phosphorylation of the receptor is eliminated when PKC is inhibited with GF109203X. This may be due in part to PKC phosphorylation of sites within the tail and intracellular loops of the receptor. Alternatively, PKC activity may be required for the efficient recruitment of GRKs to agonist-occupied receptors. Such a mechanism has been demonstrated previously for GRK2, whereby its phosphorylation by either PKC or PKA is necessary for its efficient recruitment to the plasma membrane (20–22).

Interestingly, the intrinsic potencies of CCL19 and CCL21 to activate signaling do not correlate with their abilities to induce CCR7 phosphorylation and desensitization. This contrasts with other chemokine receptors that have multiple ligands, such as CCR4 and CCR5, where the ability of each ligand to stimulate receptor-G protein coupling is directly proportional to its ability to desensitize the receptor. Oppermann et al. (23) observed a close correlation between the efficacies of various CCR5 ligands, CCL3, CCL4, and CCL5, in their receptor activation and desensitization profiles. Similarly, D’Ambrosio et al. (24) found that CCL22 is more potent than CCL17 at activating and phosphorylating the receptor, and that this is consistent with the higher binding affinity and calcium mobilization potency of CCL22 (25).

Our results suggest that a dissociation exists between the chemokine-mediated activation of CCR7 coupling to G proteins and the regulation of the receptor by each ligand. That is, each chemokine ligand induces a distinct receptor conformation, each of which has similar G protein-coupling properties but differ significantly in their ability to interact with phosphorylating kinases. Multiple active conformations for GPCRs have been identified in kinetic models and by functional studies. Using fluorescence lifetime spectroscopy, Swaminath et al. (26) have described a sequential binding model for the activation of the β2-adrenergic receptor that comprises at least two distinct conformations. One state of the receptor is capable of activating G protein coupling, and a second subsequent state is necessary for receptor internalization. In a second study, Wei et al. (27) observed that stimulation of the AT_1R activates a G protein-independent and β-arrestin-dependent pathway that is distinct from that used to activate G protein signaling. In a manner similar to the CCR7 desensitization abilities of CCL19 and CCL21, the µ-opioid ligands [N-α-Ala², N-methyl-Phe⁴, Gly⁵]-enkephalin and morphine also differ in their receptor-desensitizing abilities, whereas they are equipotent in their signaling abilities (28). However, it should be noted that, while CCL19 and CCL21 are endogenous physiological agonists for CCR7, both [N-α-Ala², N-methyl-Phe⁴, Gly⁵]-enkephalin and morphine are synthetic ligands for the µ-opioid receptor.

Following agonist binding, GPCRs assume a conformation that is recognized as a substrate for the GRKs, allowing the receptor to be phosphorylated, bind β-arrestins, and become desensitized. Several GPCRs, including the AT_1R and the PAR-2, have also been shown to activate ERK1/2 through a pathway that depends on the recruitment of a β-arrestin-Raf1-ERK1/2 complex to the activated and phosphorylated receptor (15, 16). In this study we show that siRNA-mediated depletion of β-arrestin-2 leads to a partial inhibition of the CCL19-stimulated ERK1/2 pathway, thus demonstrating that CCR7 can also activate ERK1/2 in a β-arrestin-dependent fashion. GPCRs can activate MAPKs through multiple signaling pathways, most of which are independent of β-arrestin, thus it may not be possible to completely abolish ERK1/2 activation by ablating this single pathway (29, 30). Because CCL21 can also activate ERK1/2, albeit to a much lesser extent than CCL19, it

FIG. 8. β-Arrestin-2 mediates ERK1/2 activation induced by CCL19. A, HEK-CCR7-FLAG cells were transfected with control and β-arrestin-2 siRNA. After 3 days, cells were serum-starved for 1 h and stimulated with various doses of CCL19 and 10 ng/ml EGF for 5 min. Representative phospho-ERK1/2 and total ERK immunoblots are shown. B, the levels of phospho-ERK1/2 were quantitated and normalized to the total ERK1/2 in each sample. Because basal activation of ERK1/2 cannot be quantitated, the data are presented as the percentage of the total activable ERK1/2 in the cells as determined by EGF stimulation of the cells. EGF stimulation was unaffected by siRNA treatment. The extent of β-arrestin-2 depletion in the β-arrestin-2 siRNA treated cells is 42 ± 2% compared with control siRNA-treated cells. Data graphed are the mean ± S.E. of three experiments. Two-way ANOVA analysis was performed. Results showed that ERK1/2 activation in β-arrestin-2 siRNA-treated cells is significantly different from control siRNA treated cells (*, p < 0.05).

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is tempting to speculate that both ligands can weakly activate ERK1/2 through a β-arrestin-dependent pathway, but the majority of the CCL19-mediated activation is through a β-arrestin-dependent mechanism. Finally, we demonstrated that maximal β-arrestin-dependent ERK1/2 activation also requires the receptor to become phosphorylated, probably by GRKs, because elimination of the agonist-dependent phosphorylation sites at the end of the carboxyl tail of CCR7 also eliminates most of the ERK1/2 activation. In an analogous manner, elimination of the phosphorylation sites in the tails of the PAR-2 (16) and neurokinin1 receptor (Substance P receptor) (31) ablates the β-arrestin-dependent activation of ERK1/2. Furthermore, phosphorylation of the CXCR1 receptor in neutrophils has been shown to be necessary to permit the recruitment and activation of Hck and c-Fgr to β-arrestin, a required step in the process of neutrophil degranulation (13).

The β-arrestin-mediated activation of the ERK1/2 pathway can occur either in a G protein-dependent (11) or -independent fashion (27, 32). Our studies showed that CCR7-mediated ERK activation is completely dependent on Gq coupling and PKC activation, because pertussis toxin and GF109203X abolish all ERK1/2 phosphorylation (data not shown). These results suggest a mechanism whereby β-arrestin recruitment is triggered by GRK2/3-mediated receptor phosphorylation, which requires G protein activation to generate free Gβγ for the translocation of the kinase(s) to the receptor. Furthermore, this mechanism is also consistent with our data demonstrating the requirement of receptor phosphorylation for CCR7-mediated ERK1/2 activation.

The functional consequences of activating ERK1/2 through the formation of receptor-β-arrestin-ERK1/2 complexes have also begun to be elucidated. Two groups have reported that by recruiting ERK1/2 to the AT1R and the PAR-2, the activated kinase is prevented from translocating to the nucleus, diminishing its effects on gene transcription and cell proliferation (16, 33). In contrast, DeFea et al. (31) have shown that ERK1/2 activation by the neurokinin1 receptor is also β-arrestin-dependent, but this pathway is required for the proliferative effects of the receptor. Furthermore, Ge et al. (34) have recently described a role for the formation of a PAR-2-β-arrestin-ERK1/2 complex in pseudopod formation in fibroblasts during wound healing. The complex is localized at the pseudopodia where it promotes actin rearrangement.

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Differential Desensitization, Receptor Phosphorylation, β-Arrestin Recruitment, and ERK1/2 Activation by the Two Endogenous Ligands for the CC Chemokine Receptor 7

Trudy A. Kohout, Shelby L. Nicholas, Stephen J. Perry, Greg Reinhart, Sachiko Junger and R. Scott Struthers

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