Similarities and Differences in RANTES- and (AOP)-RANTES–triggered Signals: Implications for Chemotaxis

José M. Rodríguez-Frade,* Antonio J. Vila-Coro,* Ana Martín,* Marta Nieto,‡ Francisco Sánchez-Madrid,‡ Amanda E.I. Proudfoot,§ Timothy N.C. Wells,§ Carlos Martínez-A.,* and Mario Mellado*†

*Department of Immunology and Oncology, Centro Nacional de Biotecnología, CSIC/UAM, Campus de Cantoblanco, E-28049 Madrid, Spain; ‡Servicio de Inmunología, Hospital de la Princesa, Universidad Autónoma de Madrid, Diego de León 62, E-28006 Madrid, Spain; and §Serono Pharmaceutical Research Institute, Plan-les-Ouates, Geneva CH-1228, Switzerland

Abstract. Chemokines are a family of proinflammatory cytokines that attract and activate specific types of leukocytes. Chemokines mediate their effects via interaction with seven transmembrane G protein–coupled receptors (GPCR). Using CCR5-transfected HEK-293 cells, we show that both the CCR5 ligand, RANTES, as well as its derivative, aminooxypentane (AOP)-RANTES, trigger immediate responses such as Ca\(^{2+}\) influx, receptor dimerization, tyrosine phosphorylation, and G\(_{\text{ai}}\) as well as JAK/STAT association to the receptor. In contrast to RANTES, (AOP)-RANTES is unable to trigger late responses, as measured by the association of focal adhesion kinase (FAK) to the chemokine receptor complex, impaired cell polarization required for migration, or chemotaxis. The results are discussed in the context of the dissociation of the late signals, provoked by the chemokines required for cell migration, from early signals.

Key words: chemokines • receptor dimerization • inflammation • HIV-1

RANTES is a member of the continuously growing family of chemoattractant cytokines (chemokines). The members of this group of low molecular weight proinflammatory cytokines are characterized by their ability to induce migration and activation of specific leukocyte subsets (Schall, 1994; Schall and Bacon, 1994; Baggiolini, 1998). There are two major chemokine subfamilies, the CXC or \(\alpha\) chemokines (in which the two cysteines nearest the NH\(_2\) terminus are separated by a single amino acid) and the CC or \(\beta\) chemokines (in which these two cysteines are adjacent). The structural similarities, chromosomal location, and overall specificity for different leukocyte populations concur in these families (Baggiolini et al., 1994). Two exceptions to the CC/CXC classification rule are as follows: lymphotoxin, a chemokine lacking one of the two conserved cysteines (Kelner et al., 1994); and fractalkine, an integral membrane protein with a chemokine domain in its NH\(_2\) terminus (Bazan et al., 1997).

RANTES is a CC chemokine, and interacts with several chemokine receptors (CCR1, CCR3, CCR4, and CCR5) (Rollins, 1997). It is a potent chemoattractant for CD4\(^+\) and CD8\(^+\) lymphocytes, as well as for monocytes, NK cells, and eosinophils (Schall et al., 1990; Taub et al., 1995; Ugoccioni et al., 1995). RANTES can prevent infection by macrophage-tropic HIV-1 strains (Cocchi et al., 1995; Alkhatib et al., 1996), and its overexpression has been demonstrated in various chronic pathologies. For example, in experimental autoimmune encephalomyelitis, RANTES overexpression is followed by the appearance of infiltrating cells in the central nervous system (Godiska et al., 1995). Furthermore, in asthmalike inflammation models, T cell and eosinophil accumulation in the lungs of sensitized mice are associated with RANTES and eotaxin expression (Gonzalo et al., 1996; Jia et al., 1996). Due to the possible relevance of this chemokine in diverse pathologies, therapy based on administration of chemokine receptor antagonists is a strategy for many of these diseases (Baggiolini and Moser, 1997). Hence, RANTES derivatives have been developed that lack chemotactic and leukocyte-activating properties, and act as functional antagonists (Simmons et al., 1997). This is the case for (AOP)-RANTES, a RANTES analogue lacking the first eight NH\(_2\)-terminal amino acids (Arenzana-Seisdedos et al., 1996). This analogue binds to several CC chemokine receptors (CCR1, CCR3, CCR4, and CCR5), but does not induce chemotaxis or enzyme release, thus acting as a

Address correspondence to Carlos Martínez-A., Department of Immunology and Oncology, Centro Nacional de Biotecnología, CSIC/UAM, Campus de Cantoblanco, E-28049 Madrid, Spain. Tel.: 34-91-585-4559. Fax: 34-91-372-0493. E-mail: cmartineza@cnb.uam.es
RANTES antagonist (Gong et al., 1996). In addition, (AOP)-RANTES is a potent inhibitor of macrophage-tropic HIV-1 strains that use CCR5 as a coreceptor (Arenzana-Seisdedos et al., 1996; Simmons et al., 1997). (AOP)-RANTES drives the internalization of CCR5 in recombinant cell lines and in primary cells (Mack et al., 1998).

Chemokines activate leukocytes through interaction with single-chain seven transmembrane G protein–coupled receptors (GPCR) (Murphy, 1994) that are grouped in two families: CC and CXC receptors, depending on the chemokines they bind (Murphy, 1994). To date, nine CC and five CXC receptors have been reported, as have orphan receptors whose ligands remain to be elucidated. The extracellular loops of these receptors act in concert to bind the chemokine ligand, whereas different intracellular regions are involved in transducing the chemokine signal. Chemokine receptors are generally coupled to Go, proteins (Bokoch, 1995), rendering cellular responses pertussis toxin (PTX)-sensitive, although there are several examples of chemokine receptors that couple to both PTX-insensitive or -sensitive G proteins, as is the case of the C5a receptor (Amatruda et al., 1993). Ligand binding triggers a number of biochemical pathways (Ward et al., 1998) that lead to changes in intracellular cAMP levels (Ahmed et al., 1995); phospholipase activation (Bacon et al., 1995a); increases in tyrosine phosphorylation (Bacon et al., 1995b); including the Src substrates focal adhesion kinase (FAK) (p125 FAK) and ZAP-70 (Bacon et al., 1996); increased association of Src family kinases with p110ras (Worthen et al., 1994); and MAPK cascade activation (Jones et al., 1995; Ganju et al., 1998).

We have recently demonstrated that the initiation of chemokine signaling through the CCR2 chemokine receptor involves ligand-triggered receptor association to members of the Janus associated kinase (JAK) family of tyrosine kinases (Mellado et al., 1998), as is the case for the cytokine receptors (O’Shea, 1997). Thus, MCP-1 association to the CCR2 chemokine receptor induces CCR2 dimerization (Rodriguez-Frade et al., 1999) and activation of the JAK2/STAT3 signaling pathway (Mellado et al., 1998). RANTES and other chemokines implicated in regulating T cell functions, such as MIP-1α, also rapidly activate some of the STAT family transcription factors (Wong and Fish, 1998), suggesting similar dimerization-triggered responses.

Here we analyze early RANTES and (AOP)-RANTES-activated signaling events following binding to CCR5. Both RANTES and its modified form, (AOP)-RANTES, trigger tyrosine phosphorylation and activation of the JAK1/STAT5 pathway as well as promoting dimerization and tyrosine phosphorylation of the CCR5 receptor. While both promote Ca2+ influx, only RANTES induces sustained cell polarization and chemotaxis, indicating the activation of different signaling events. These data indicate that JAK/STAT pathway activation is a general mechanism for chemokine receptor activity and suggest that receptor dimerization and internalization may be key features in chemokine-induced signaling.

### Materials and Methods

#### Biological Materials

HEK-293 cells (ATCC TIB202) were obtained from the American Type Culture Collection, and CCR5-transfected HEK-293 cells were kindly donated by Dr. J. Gutierrez (Dept. of Immunology and Oncology, Centro Nacional de Biotecnología, Madrid, Spain). Antibodies used include rabbit anti-JAK1 and anti-JAK2 (Upstate Biotechnology, Inc.), anti-Goαi and anti-STAT5 (Santa Cruz Biotechnology); monoclonal anti-PTyr (4G10; Upstate Biotechnology Inc.), anti-PTyr (PY20) (Transduction Laboratories), and anti-β2-microglobulin (PharMingen). Anti-CCR5 mAb, CCR5-02, CCR5-03, and anti-CXCR4 mAb were generated in our laboratory as described (Mellado et al., 1997). EGF-stimulated A-431 cell lysates were obtained from Upstate Biotechnology Inc. and recombinant human RANTES and SDF-1α were from Peprotech Inc.

#### Flow Cytometry Analysis

Cells were centrifuged (250 g, 10 min, room temperature), plated in V-bottom 96-well plates (2.5 × 10^3 cells/well), and incubated with 50 μl/well biotin-labeled mAb (5 μg/ml, 60 min, 4°C). Cells were washed twice in PBS with 2% BSA and 2% FCS, and centrifuged (250 g, 5 min, 4°C). FITC-labeled streptavidin (Southern Biotechnologies Associates, Inc.) was added, incubated (30 min, 4°C) and plates were washed twice. Cell-bound fluorescence was determined in a Profile XL flow cytometer at 525 nm (Coulter Electronics).

#### Calcium Determination

Changes in intracellular calcium concentration were monitored using a fluorescent probe (Fluo-3-AM; Calbiochem). Cells (2.5 × 10^5 cells/ml), were resuspended in RPMI containing 10% FCS, 10 mM Hepes, and incubated with 10 μl/10^6 cells of Fluo-3-AM (300 μM in DMSO, 15 min, 37°C) (Frade et al., 1997a). After incubation, cells were washed, resuspended in complete medium containing 2 mM CaCl₂, and maintained at 4°C until just before chemokine addition to minimize membrane trafficking and to eliminate spontaneous Ca2+ entry. Calcium mobilization in response to 10 nM RANTES or (AOP)-RANTES and 20 nM SDF-1α was determined at 37°C in an EPICS XL flow cytometer at 525 nm. It includes background level stabilization and determination of the probe loading level for each sample. Only samples with a low level of background staining, as indicated by ionophore-induced Ca2+ mobilization, were used (5 μg/ml ionomycin; Sigma Chemical Co.).

#### Cell Migration

CCR5-transfected HEK-293 cells (0.5 × 10^5 cells/ml) were starved for 120 min at 37°C, 5% CO₂ in RPMI 1640 containing 0.1% BSA. Cells (0.25 × 10^5 cells in 0.1 ml) were placed in the upper well of 24-well, 8-μm pore size transmigration chambers (Transwell; Costar Corp.) and RANTES or (AOP)-RANTES (diluted in 0.6 ml RPMI containing 0.25% BSA) were added to the lower well. Plates were incubated (240 min, 37°C, 5% CO₂) and the cells that migrated to the lower chamber were counted, as described (Frade et al., 1997a).

#### Immunoprecipitation, SDS-PAGE, and Western Blot Analysis

RANTES- or (AOP)-RANTES-stimulated cells (20 × 10^6) were lysed in a detergent buffer (20 mM triethanolamine, pH 8.0, 300 mM NaCl, 2 mM EDTA, 20% glycerol, 1% digitonin, with 10 μM sodium orthovanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotonin) for 30 min at 4°C with continuous rocking and then centrifuged (15,000 g, 15 min). Immunoprecipitations were performed essentially as described earlier (Mellado et al., 1997). Protein extracts preclarified by incubation with 20 μg of anti-mouse IgM-agarose (Sigma Chemical Co.) or protein A-Sepharose (60 min, 4°C) were centrifuged (15,000 g, 1 min), immunoprecipitated with the appropriate antibody (5 μg/sample, 120 min, 4°C), followed by anti-mouse IgM-agarose or by protein A-Sepharose if the first antibody was derived from rabbit serum. Immunoprecipitates or protein extracts were separated in 12% SDS-PAGE gels and transferred to Immobilon membranes. Membranes were probed with a specific mAb, incubated in 5% nonfat dry milk, and washed in TBS/0.1% Tween. The blot was visualized by horseradish peroxidase-labeled second antibody (Santa Cruz Biotechnology) and chemiluminescence (ECL; Amersham). The membranes were reprobed with a different mAb, washed, and re-probed as above. A representative result is shown in Figure 2A. The technical details are found in Materials and Methods.

1. Abbreviations used in this paper: DSS, disuccinimidyl suberate; FAK, focal adhesion kinase; FN, fibronectin; GPCR, G protein–coupled receptors; JAK, Janus associated kinase; PTX, pertussis toxin.
of other chemokine receptors to Gαi in transfected HEK-293 cells (Aragay et al., 1998; Mellado et al., 1998), indicating the utility of this cell line in the study of signaling through GPCR.

We have described the ability of RANTES to polarize peripheral blood T cells, such that the CCR5 receptor migrates to the cell's leading edge while the ICAM-1, ICAM-3/CD43, CD44 molecules localize to the uropod (Nieto et al., 1997). Therefore, we tested the ability of both of these ligands to induce polarization in peripheral T cell blasts. Time-course studies of ICAM-3 redistribution to the cell uropod of FN-adhered T lymphoblasts showed that RANTES induced ICAM-3 redistribution in a significant proportion of the cells as early as 15 min after stimulation. Maximum redistribution, seen at 30 min, persisted until at least 90 min of incubation with the chemokine. In contrast, (AOP)-RANTES–induced ICAM-3 polarization showed a much slower, weaker response, with a peak at 30 min that vanished thereafter (Fig. 3 A).

We next studied the effects of these ligands on the polarization of CCR5 to the advancing front of migrating T lymphoblasts, a phenomenon that may be involved in the chemotaxis mechanism. We found that whereas RANTES triggered redistribution of CCR5 molecules to the leading edge of the cells, membrane CCR5 expression was not detectable in (AOP)-RANTES–stimulated polarized T lymphoblasts bearing ICAM-3 redistributed to the uropod (Fig. 3 B). The downregulation of the (AOP)-RANTES–triggered chemokine receptor results in a net inhibition of CCR5 redistribution to the leading edge of T lymphoblasts.

**Results**

**RANTES and (AOP)-RANTES Induce Functional Responses in CCR5-transfected HEK-293 Cells**

We developed anti–human CCR5-specific mAb using synthetic peptides corresponding to the NH₂-terminal domain of this receptor (amino acids 13–28), a CCR5-specific sequence not present in other chemokine receptors. These mAbs recognize the CCR5 in human PBMC (not shown) and in CCR5 stably transfected HEK-293 cells, in flow cytometry (Fig. 1 A), as well as in Western blot, and immunoprecipitation analysis (Fig. 1 B). No binding is observed in mock- or CCR2-transfected HEK-293 cells, or in any other cell tested that does not express CCR5.

In response to RANTES or (AOP)-RANTES, these CCR5-transfected HEK-293 cells mobilize calcium (Fig. 2 A) and are desensitized to a second stimulation. However, only RANTES triggers migration in these cells (Fig. 2 B). PTX treatment abrogates both calcium release and cell migration in response to RANTES and (AOP)-RANTES (Fig. 2 A), whereas no effect was observed following incubation with cholera toxin (not shown). This is consistent with other studies showing that RANTES downstream signals in other cell lines and T cells are coupled to PTX-sensitive G proteins (Bacon et al., 1995b), and with coupling
stimulated with this chemokine derivative, compared to those treated with RANTES (Fig. 3 C). This concurs with previous observations showing that (AOP)-RANTES induces CCR5 downregulation by blocking receptor recycling (Mack et al., 1998).

**RANTES and (AOP)-RANTES Induce Tyrosine Phosphorylation of the CCR5 Receptor and Association of the JAK1/STAT5 Complex**

When CCR5-transfected HEK-293 cells were stimulated with RANTES or (AOP)-RANTES, a 38-kD protein phosphorylated in tyrosine residues was initially identified as the CCR5 receptor by immunoblot (not shown). Thereafter, lysates from transfected HEK-293 cells were immunoprecipitated with anti-CCR5, and Western blots developed with anti-PTyr antibodies (Fig. 4). An increase in CCR5 receptor phosphorylation in tyrosines is seen as early as 60 s after stimulation. At this time no differences were observed following treatment with either stimulus, indicating that (AOP)-RANTES not only binds to CCR5, but also signals through this receptor. The early, weak tyrosine phosphorylation level may occur because only one tyrosine (Tyr 126 in the DRY motif) was being phosphorylated, analogous to the case of Tyr 139 in the CCR2 receptor (Mellado et al., 1998). Similar results are observed in the inverse experiment, in which anti-PTyr antibody immunoprecipitates are analyzed in Western blot with anti-CCR5 receptor antibody (not shown). Again, tyrosine phosphorylation is detected in CCR5 after stimulation with either RANTES or (AOP)-RANTES. Nevertheless, significant differences are observed in tyrosine phosphorylated CCR5 after longer stimulation periods. Whereas CCR5 phosphorylation persists in RANTES-treated cells after 15 min of treatment, this is not the case after (AOP)-RANTES stimulation, reinforcing the observation that there are differences in the signaling pathways activated by these chemokines.

To ascertain which kinase is responsible for the rapid CCR5 chemokine receptor phosphorylation, CCR5-transfected HEK-293 cells were stimulated with RANTES or (AOP)-RANTES, and cell lysates immunoprecipitated with anti-CCR5, or with anti–β2-microglobulin as an isotype-matched antibody control. The use of a specific anti-JAK1 antibody identified a 130-kD protein in the anti-CCR5 immunoprecipitates (Fig. 5 A). Furthermore, the JAK1 tyrosine kinase is phosphorylated on tyrosine residues after stimulation with either ligand, since anti-PTyr immunoprecipitates can be developed with anti-JAK1 antibody in Western blot (Fig. 5 B). JAK1 association to the CCR5 receptor takes place as early as 30 s after RANTES or (AOP)-RANTES stimulation (Fig. 5 A). Small amounts of JAK1 were also found associated to the CCR5 receptor in the absence of added ligand, consistent with receptor phosphorylation in the absence of exogenous ligand in CCR5-transfected HEK-293 cells (Fig. 4, A and B).

Neither immunoprecipitation of cell lysates with isotype-matched control antibodies to other cell proteins such as β2-microglobulin nor immunoprecipitation in mock-transfected HEK-293 cells (not shown) revealed the presence of JAK1 (Fig. 5 A). This rules out nonspecific protein association to membrane components under these experimental conditions. The rapid association of JAK1 to the CCR5 receptor triggered by these two chemokines, but not by others tested (MCP-1 and SDF-1α), suggests a role for this tyrosine kinase in early receptor phosphorylation following ligand stimulation. Interestingly, while MCP-1 promotes JAK2 association to the receptor in CCR2-transfected HEK-293 cells, neither JAK2 nor JAK3 associates to CCR5 after RANTES or (AOP)-RANTES activation of CCR5-transfected HEK-293 cells (data not shown).

To identify the downstream signaling pathway activated by the JAK1 kinase, we tested its association to members of the STAT transcription factor family. We analyzed JAK1 kinase-activated STAT transcription factors in anti-CCR5 immunoprecipitates, and found that STAT5 associ-
Rodríguez-Frade et al.
Chemokine Receptor Signaling

RANTES and (AOP)-RANTES Induce CCR5 Dimerization

Similar to growth factor–induced dimerization of tyrosine kinase receptors, some GPCR, including chemokine receptors like CCR2 and CXCR4, undergo ligand-induced dimerization (Hebert et al., 1996; Rodríguez-Frade et al., 1999; Vila-Coro, A.J., J.M. Rodríguez-Frade, A. Martín de Ana, M.C. Moreno-Ortiz, C. Martínez-A., and M. Mellado, manuscript submitted for publication). We have now tested whether RANTES and (AOP)-RANTES trigger CCR5 dimerization. DSS-mediated cross-linking in CCR5-transfected HEK-293 cells was carried out after RANTES or (AOP)-RANTES stimulation. In CCR5-transfected HEK-293 cells, but not in mock-transfected cells, a high molecular mass receptor species (76 kD) was observed following both stimuli. This band corresponds to the expected molecular mass of two CCR5 molecules, as assessed by immunoprecipitation with anti-CCR5 antibo-
and when immunoprecipitation and Western blot are performed using anti-CCR5 or anti-CXCR4 antibodies, respectively. Both RANTES and (AOP)-RANTES, like other chemokines and similar to members of the large cytokine family, can trigger early signaling events such as receptor dimerization, followed by recruitment of tyrosine kinases that phosphorylate both the receptor and the STAT transcription factors.

**Goi and p<sup>125</sup>FAK Association to CCR5 Differ after RANTES or (AOP)-RANTES Stimulation**

The PTX dependence of RANTES- and (AOP)-RANTES–triggered Ca<sup>2+</sup> mobilization indicates Go<sub>i</sub> involvement in this process. It has been demonstrated recently that chemokine receptor–mediated chemotaxis is triggered by the βγ subunit of Go<sub>i</sub> (Neptune and Bourne, 1997). In MCP-1–mediated chemotaxis, we have shown that Go<sub>i</sub> associates rapidly to the CCR2 receptor, releasing βγ, which is responsible for activating migration and cell polarization. Thus, we investigated whether the differences in chemotaxis triggering reflect differences in RANTES- and (AOP)-RANTES–mediated Go<sub>i</sub>-CCR5 receptor association.

CCR5- or mock-transfected HEK-293 cells were stimulated with RANTES or (AOP)-RANTES, immunoprecipitated with anti-CCR5 or isotype-matched control antibodies, followed by a Western blot with anti-G<sub>i</sub> antibodies. After both RANTES and (AOP)-RANTES stimulation, Go<sub>i</sub> associated to CCR5 (Fig. 7 A). Whereas association persisted for longer than 15 min when cells were RANTES-stimulated, Go<sub>i</sub> was dissociated from the receptor after 5 min of (AOP)-RANTES treatment (Fig. 7 A). The CCR5 expression level was controlled in both immunoprecipitates by stripping and reblotting membranes with anti-CCR5 antibody (Fig. 7 A). This result corroborates data showing the importance of βγ release from G<sub>i</sub> in chemotaxis (Neptune and Bourne, 1997).

The activation of p<sup>125</sup>FAK and the kinase ZAP-70 has been described in T cells after RANTES activation (Bacon et al., 1996). Therefore, we tested whether p<sup>125</sup>FAK activation coincides with its association to the CCR5. CCR5-transfected HEK-293 cells were stimulated with 10 nM RANTES or (AOP)-RANTES, cell lysates were immunoprecipitated with anti-p<sup>125</sup>FAK or an isotype-matched control antibody and analyzed in Western blot for the presence of CCR5. RANTES, but not (AOP)-RANTES, promotes the association of this kinase to the receptor, reaching maximum binding after 15 min (Fig. 7 B). Even in the absence of ligand, residual association between these two proteins was observed. The lack of p<sup>125</sup>FAK association and the rapid Go<sub>i</sub> dissociation from the CCR5 receptor in the case of (AOP)-RANTES suggest that association of these proteins with the receptor is crucial in triggering the signaling pathways that lead to the different effects promoted by these two chemokines.
Discussion

Interest is rapidly growing in a broad view of the chemokine function, not only because of significance in HIV infection, but also because chemokines and their receptors are expressed by a wide variety of nonhematopoietic cells (Horuk et al., 1997). Because of the relevance of chemokines in numerous pathologies, it appears of the utmost importance to devise therapies based on the administration of chemokines that lack chemotactic and leukocyte-activating properties, but are able to act as receptor antagonists. These are the basic characteristics of (AOP)-RANTES, a RANTES analogue that has been chemically modified at its NH₂ terminus, although very little is known of the mechanism underlying its action.

We have generated anti-CCR5 mAbs and used them to characterize the signaling pathways activated by RANTES and (AOP)-RANTES. The results show that ligand-induced CCR5 dimerization occurs when these chemokines signal target cells for activation. In the response to cytokines, receptor dimerization triggers a pathway involving tyrosine kinases and transcriptional factors. Only the JAK1 tyrosine kinase responds to RANTES and to

Figure 5. Both RANTES and (AOP)-RANTES activate the JAK/STAT pathway in CCR5-transfected HEK-293 cells. (A) CCR5-transfected HEK-293 cells were stimulated with RANTES or (AOP)-RANTES (10 nM). Lysates were immunoprecipitated with mAb CCR5-03 and analyzed in Western blot with anti-JAK1 antibodies. The figure shows control RANTES-stimulated (10 nM) CCR5-transfected HEK-293 cell lysates immunoprecipitated with anti-β₂-microglobulin mAb and analyzed with anti-JAK1 antibodies. As a control, CCR5-transfected HEK-293 cell lysates were tested in Western blot with the same anti-JAK1 antibodies. CCR5 protein loading was controlled by stripping and reprobing membranes with mAb CCR5-02 (bottom). (B) Cells as in A were immunoprecipitated with anti-PTyr antibody and tested in Western blot with anti-JAK1 antibodies. As a positive control, EGF-stimulated A431 cell lysates were tested in Western blot with anti-JAK1 antibodies as above. (C) Cells as in A were immunoprecipitated with CCR5-03 mAb and analyzed in Western blot with anti-STAT5 antibody. CCR5 protein loading was controlled as before. As a positive control, CCR5-transfected HEK-293 cell lysates were tested in Western blot with the same anti-STAT5 antibody. (D) Cells as in C were immunoprecipitated with anti-PTyr antibody and analyzed in Western blot with anti-STAT5 antibody. Protein loading was carefully controlled using a protein detection kit as above. The figure shows control RANTES-stimulated (10 nM) CCR5-transfected HEK-293 cell lysates immunoprecipitated with anti–MHC class I mAb and analyzed with anti-STAT5 antibodies. Arrow indicates the position of STAT5. In all cases, one experiment of five is shown.
(AOP)-RANTES by rapidly associating to CCR5 and is phosphorylated as soon as 30 s after binding, indicating that JAK1 activation is virtually simultaneous with its association to CCR5. In the cytokine receptors, activation and association of JAK kinase to the receptor creates docking sites for SH2-containing proteins such as STAT, leading to their phosphorylation, followed by activation of gene transcription (Ihle, 1996). After binding of either of the chemokine ligands studied here, STAT5 is also associated to the CCR5, in accordance with the role of the JAK kinases in transducing signals from hematopoietic growth factor receptors (O’Shea, 1997). Our data concur with a recent report showing rapid activation of certain STAT transcription factors in T cells after RANTES and MIP-1α stimulation, although in that case association to the CCR5 receptor was not analyzed (Wong and Fish, 1998). To-
gether, these results indicate that chemokine-mediated activation of GPCR leads to signal transduction, which invokes intracellular phosphorylation intermediates used by other cytokine receptors. These earlier events are equally activated by both RANTES and (AOP)-RANTES.

Both RANTES and (AOP)-RANTES induce CCR5 dimerization, a phenomenon that requires specific interaction between a given chemokine and its respective receptor. When experiments were performed using SDF-1α as a ligand neither CCR5 dimers nor CCR5-CXCR4 heterodimers were observed, whereas CXCR4 dimers are present in anti-CXCR4 immune precipitates. Direct interaction between two CCR5 molecules has also been demonstrated by Benkirane et al. (1997), even in the absence of ligand stimulation. The functional significance of dimerization was suggested by Hebert et al. (1996) using the epitope tagging approach, showing that agonist stimulation of the β2-adrenergic receptor stabilized the dimeric state of the receptor. The present data indicate that although dimerization is a primary event following ligand interaction with the chemokine receptor, it does not necessarily initiate identical transduction events. (AOP)-RANTES thus triggers Ca2+ mobilization as does RANTES, but promotes neither cell migration nor CCR5 redistribution to the leading edge in cell polarization. The rapid Goi association to CCR5 induced by both ligands explains the similar Ca2+ mobilization response and lack of response in PTX-pretreated cells.

It has been shown for a number of GPCR that different ligands of a given receptor can induce different responses. This is the case for CXCR2, from which IL-8 and NAP-2 elicit different responses, although they bind with equivalent affinity to this receptor (Ben-Baruch et al., 1997). It is feasible that RANTES and (AOP)-RANTES induce distinct conformational changes in the CCR5. In fact, it has been shown that different structural requirements are needed in GPCR to activate different pathways (Hungyady et al., 1995; Arai et al., 1997a). In the case of angiotensin II receptor changes have been described in internalization kinetics that do not alter other functional responses (Hungyady et al., 1995).

Whereas Goi association to CCR5 persists as long as 15 min after RANTES treatment, it dissociates from CCR5 by 5 min after (AOP)-RANTES stimulation. This variance must give rise to a clear difference in the availability of free, active βγ subunits by affecting some ligand-induced responses. In fact, Gi βγ subunit release is known to be important in chemokine receptor–mediated chemotaxis (Arai et al., 1997b; Neptune and Bourne, 1997). Another conspicuous difference is the lack of late tyrosine phosphorylation in CCR5 after (AOP)-RANTES treatment, in contrast to the continuous tyrosine phosphorylation of CCR5 observed following RANTES induction. This may occur because of tyrosine phosphorylation mediated by other tyrosine kinases. The activation of both p125FAK and ZAP-70 has been described after RANTES stimulation (Bacon et al., 1996). Here we demonstrate association of p125FAK, which is related to cytoskeletal proteins and migration phenomena (Sieg et al., 1998), to the receptor following RANTES but not (AOP)-RANTES stimulation. The failure of (AOP)-RANTES to maintain Gi association for long (15 min) periods concurs with the lack of association and activation of p125FAK and the inactivation of subsequent events. Both activation of Gi and p125FAK, as well as chemotaxis, occur after RANTES activation, but are absent when cells were treated with (AOP)-RANTES, indicating a possible relationship between these events.

Chemotaxis involves several phenomena including: changes in cell shape, integrin affinity, and integrin recycling at the cell’s leading edge (Condeelis, 1993; Lawson and Maxfield, 1995). These events appear to be mediated by G protein activation, as well as phosphorylation signals through chemokine receptors (Bokoch, 1995). In fibroblasts and smooth muscle cells, the regulation of intracellular FAK and its interaction with the cytoskeletal proteins α-actinin, talin, and vinculin have also been given much attention with respect to cell polarization and migration (Clark and Brugge, 1995; Miyamoto et al., 1995). As a consequence of RANTES stimulation, T cells undergo a polarization process in which the CCR5 receptor redistributes to the leading cell edge and p125FAK associates to the receptor. Both events, implicated in the late response to chemokines, are required for chemotaxis and are triggered by RANTES. Early (AOP)-RANTES–induced signaling events, such as Ca2+ mobilization, result in weak, transient cell polarization, and redistribution of the ICAM-3 adhesion receptor to the T lymphocyte uropod. This antagonist does not mediate cellular chemotaxis. Recycling of CCR5 molecules to the cell membrane, which probably occurs at the advancing front of the cell and is inhibited by (AOP)-RANTES (Mack et al., 1998), appears to be critical for the mechanism of directional cell migration towards a chemokine gradient. This is further supported by our data showing the failure of CCR5 to cluster at the leading edge of (AOP)-RANTES–stimulated cells. Therefore, these results suggest that during the chemotaxis process early signals induced by chemokines through receptor dimerization may not be sufficient to induce cell migration. Signals triggered by sustained association of the G protein to the chemokine receptor may also regulate other events necessary for the chemotactic response, such as integrin affinity or actin polymerization at the leading cell edge. Our data also support the idea that p125FAK kinase association to the receptor is not involved in ligand-induced CCR5 internalization, since (AOP)-RANTES is as efficient as RANTES in promoting internalization (Mack et al., 1998), without inducing p125FAK association.

The data presented here can be incorporated in a model in which signaling through chemokine receptors directly involves early signals that occur in the first few minutes after ligand binding, including receptor dimerization; association and activation of JAK tyrosine kinases, and activation of STAT transcriptional factors, as well as late signals such as cell polarization, activation, and association of p125FAK kinase to the chemokine receptor (Fig. 8). The fact that chemokine receptors activate the JAK/STAT pathway and that dimerization may be a general mechanism for chemokine activity adds a new perspective to understanding how the multiplicity of chemokine functions are achieved. Furthermore, it suggests an interesting new objective for therapeutic intervention in chemokine-associated pathologies, including inflammation and AIDS.

Internalization of chemokine receptors is a phenomenon that can be dissociated from their role as HIV-1 core-
which antibodies to the CCR2 NH2-terminal domain block of chemokine receptors is an alternative mechanism for Here we correlate the known effects of (AOP)-RANTES activated by these two ligands have not been reported. Although the ability of the coreceptor to internalize is not required for HIV entry, it contributes to the HIV suppressive effect of CC and CXC chemokines (Amara et al., 1997). (AOP)-RANTES is a potent inhibitor of HIV-1 infectivity, an effect that is suggested because it enhanced CCR5 internalization and inhibition of recycling (Mack et al., 1998). As internalization is an agonist-driven event, (AOP)-RANTES as well as other NH2-terminal modified chemokines should be able to activate their receptors. The differences between RANTES- and (AOP)-RANTES–induced internalization may be related to increased affinity of (AOP)-RANTES for the CCR5 receptor (Mack et al., 1998), although differences in the signaling pathways activated by these two ligands have not been reported. Here we correlate the known effects of (AOP)-RANTES with a signaling cascade and suggest that the dimerization of chemokine receptors is an alternative mechanism for chemokine-induced HIV-1 inhibition. Receptor dimerization is also the mechanism through which antibodies to the CCR2 NH2-terminal domain block HIV-1 infection. In fact, the anti-CCR2 mAb CCR2-01 that blocks HIV-1 infection (Frade et al., 1997b), but not MCP-1–induced responses (Frade et al., 1997a), triggers CCR2 dimerization (Mellado, M., manuscript in preparation). The concept that receptor dimerization is required for prevention of HIV-1 infection, besides including the nonsignaling mechanism indicated by (AOP)-RANTES, incorporates the model based on receptor internalization, which does not take place in the absence of dimerization. For both receptor desensitization and internalization, we have shown the requirement for GRK2 association and phosphorylation in serine/threonine residues of the COOH-terminal receptor domain. This in turn associates with arrestin and clathrin, and undergoes internalization (Aragay et al., 1998). Dimerization is a necessary event for the formation of the receptor/GRK-2/arrestin/clathrin complex. This model consolidates all available experimental evidence, and offers novel prospects for screening new drugs that may prevent HIV-1 infection and/or inflammatory processes.

We thank Drs. R. Geha (Harvard Medical School, Boston, MA) and J.C. Gutierrez-Ramos (Millennium Pharmaceuticals, Boston, MA) for critical reading of the manuscript, Dr. J. Gutiérrez for CCR5 transfectants, L. Gómez for help with animals, M.C. Moreno and I. López for FACS® analysis, and C. Bastos and C. Mark for secretarial and editorial assistance, respectively.

This work was supported by grants from the Spanish Dirección General de Investigaciones Científicas, European Union, the Spanish CICyT and the Regional Government of Madrid (CAM), and Pharmacia & Upjohn. The Department of Immunology and Oncology was founded and is supported by the Spanish Research Council (CISIC) and Pharmacia & Upjohn. Received for publication 25 August 1998 and in revised form 18 December 1998.

**References**


