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

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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 $G\alpha_i$ 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 α chemokines (in which the two cysteines nearest the NH_2 terminus are separated by a single amino acid) and the CC or β 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: lymphotactin, 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

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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)[1] (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 $G\alpha_i$ 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) (p^{125} FAK) and ZAP-70 (Bacon et al., 1996); increased association of Src family kinases with p^{21}ras (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 (Rodríguez-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 Ca^{2+} 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.

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.

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-$G\alpha_i$ and anti-STAT5 (Santa Cruz Biotechnology); monoclonal anti-PTyr (4G10; Upstate Biotechnology Inc.), anti-PTyr (PY20) (Transduction Laboratories), and anti–β_2-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 (2.5×10^5 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-3AM; Calbiochem). Cells (2.5×10^6 cells/ml), were resuspended in RPMI containing 10% FCS, 10 mM Hepes, and incubated with 10 μl/10^6 cells of Fluo-3AM (300 μM in DMSO, 15 min, 37°C) (Frade et al., 1997a). After incubation, cells were washed, resuspended in complete medium containing 2 mM $CaCl_2$, and maintained at 4°C until just before chemokine addition to minimize membrane trafficking and to eliminate spontaneous Ca^{2+} 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 similar load, as assessed by ionophore-induced Ca^{2+} mobilization, were used (5 μg/ml ionomycin; Sigma Chemical Co.).

Cell Migration

CCR5-transfected HEK-293 cells (0.5×10^6 cells/ml) were starved for 120 min at 37°C, 5% CO_2 in RPMI 1640 containing 0.1% BSA. Cells (0.25×10^6 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% CO_2) 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 aprotinin) 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 precleared 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

SDS-PAGE and transferred to nitrocellulose membranes. Western blot analysis was performed as described (Mellado et al., 1997), using 2% BSA in TBS as blocking agent for the antiphosphotyrosine analyses. When stripping was required, membranes were incubated for 60 min at 60°C with 62.5 mM Tris-HCl, pH 7.8, containing 2% SDS and 0.5% β-mercaptoethanol. After washing with 0.1% Tween 20 in TBS for 2 h, membranes were reblocked, reprobed with the appropriate antibody, and developed as above. In all cases, protein loading was carefully controlled using a protein detection kit (Pierce Chemical Co.) and, when necessary, by reprobing the membrane with the immunoprecipitating antibody.

Receptor Cross-linking Assays

Serum starved CCR5 transfected HEK-293 cells (20×10^6) were stimulated with 10 nM RANTES, 10 nM (AOP)-RANTES, or 20 nM SDF-1α for 1 min at 37°C. The reaction was terminated by addition of 1 ml of cold PBS and centrifugation (30 s, 15,000 *g*). After washing twice with cold PBS, 10 μl of 100 mM disuccinimidyl suberate (DSS; Pierce Chemical Co.) was added for 10 min at 4°C with continuous rocking. The reaction was terminated by adding 1 ml of cold PBS and washing three times. The pellet was lysed for 60 min and immunoprecipitated as above.

Immunofluorescence Microscopy: Polarization of Blast Cells

Immunofluorescence experiments were performed essentially as described (Nieto et al., 1997). Briefly, 2×10^6 T lymphoblasts in 500 μl complete medium were allowed to adhere to fibronectin (FN)-coated coverslips in 24-well plates (Costar Corp.). Cytokines and chemokines (10 ng/ml) were added, and cells were incubated (37°C, 5% CO_2). After 30 min, cells were fixed with 3.7% formaldehyde in PBS for 10 min at room temperature, and stained with specific mAb plus a FITC-labeled rabbit anti–mouse IgG $F(ab')_2$ (Pierce Chemical Co.). The proportion of CCR5 polarization was calculated by direct cell count (n = 400–500) of 10 random fields for each condition using a photomicroscope with 60× oil immersion objectives (Labophot-2; Nikon Inc.). Preparations were photographed on Ektachrome 400 film. Images were acquired with a high performance CCD camera (Cohu) coupled to the microscope and connected to a workstation (model Q550CW; Leica Imaging Systems, Ltd.). Images were visualized, processed, and stored using QFISH software (version V1.01; Leica Imaging Systems, Ltd.) and printed on a color printer (Phaser 440; Tektronix Inc.).

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 NH_2-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

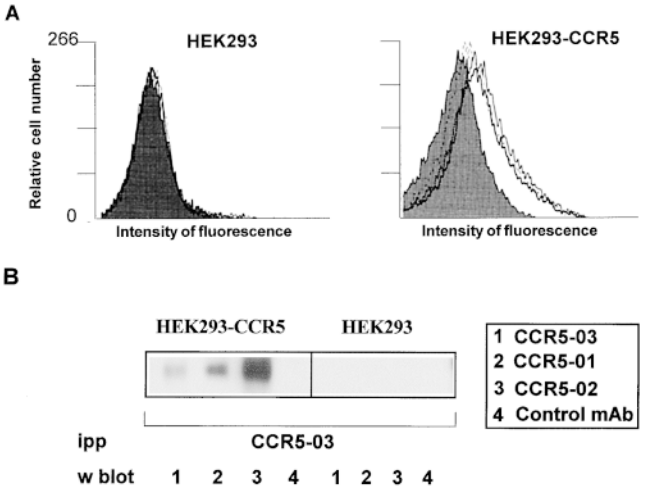

Figure 1. CCR5-01, -02, and -03 antibodies are specific for the CCR5 chemokine receptor. mAb specificity for the CCR5 receptor was assessed by flow cytometry analysis of mock- and CCR5-transfected HEK-293 cells. (A) CCR5-transfected HEK-293 cells (HEK-293-CCR5) or mock-transfected controls (HEK-293) were incubated with the biotin-labeled CCR5-01, -02, or -03 antibodies, followed by streptavidin-PE. An isotype-matched mAb was used as a control. (B) CCR5- or mock-transfected HEK-293 cells were immunoprecipitated as described in Materials and Methods using CCR5-03 mAb, then analyzed in Western blot with CCR5-01, -02, -03, or control mAb, as indicated. One representative experiment of five is shown.

of other chemokine receptors to G_i 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 at 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

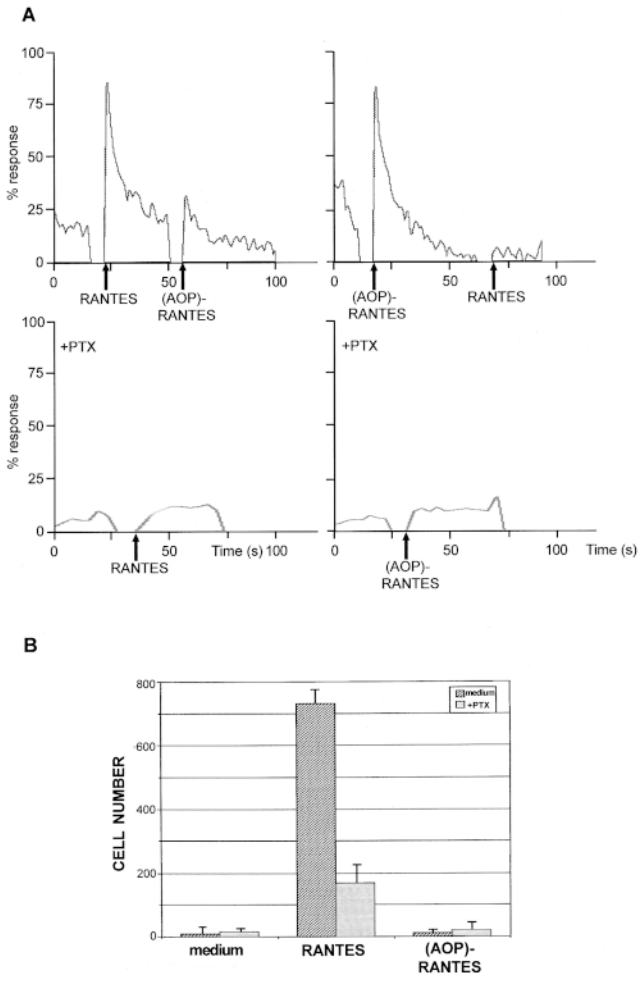

Figure 2. Functional responses induced by RANTES and (AOP)-RANTES. (A) Both RANTES and (AOP)-RANTES–induced Ca^{2+} mobilization in CCR5-transfected HEK-293 cells was desensitized by the other ligand. Pretreatment with PTX (0.1 μg/ml, 16 h) completely blocks responses in both cases. One of three experiments is shown. Results are expressed as a percentage of the maximum chemokine response. (B) RANTES, but not (AOP)-RANTES (10 nM), induces chemotaxis in CCR5-transfected HEK-293 cells. PTX pretreatment (0.1 μg/ml, 16 h) blocks the RANTES-induced response. The figure depicts one of five experiments performed, with the SD indicated.

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–β_2-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 antibodies 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 β_2-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-

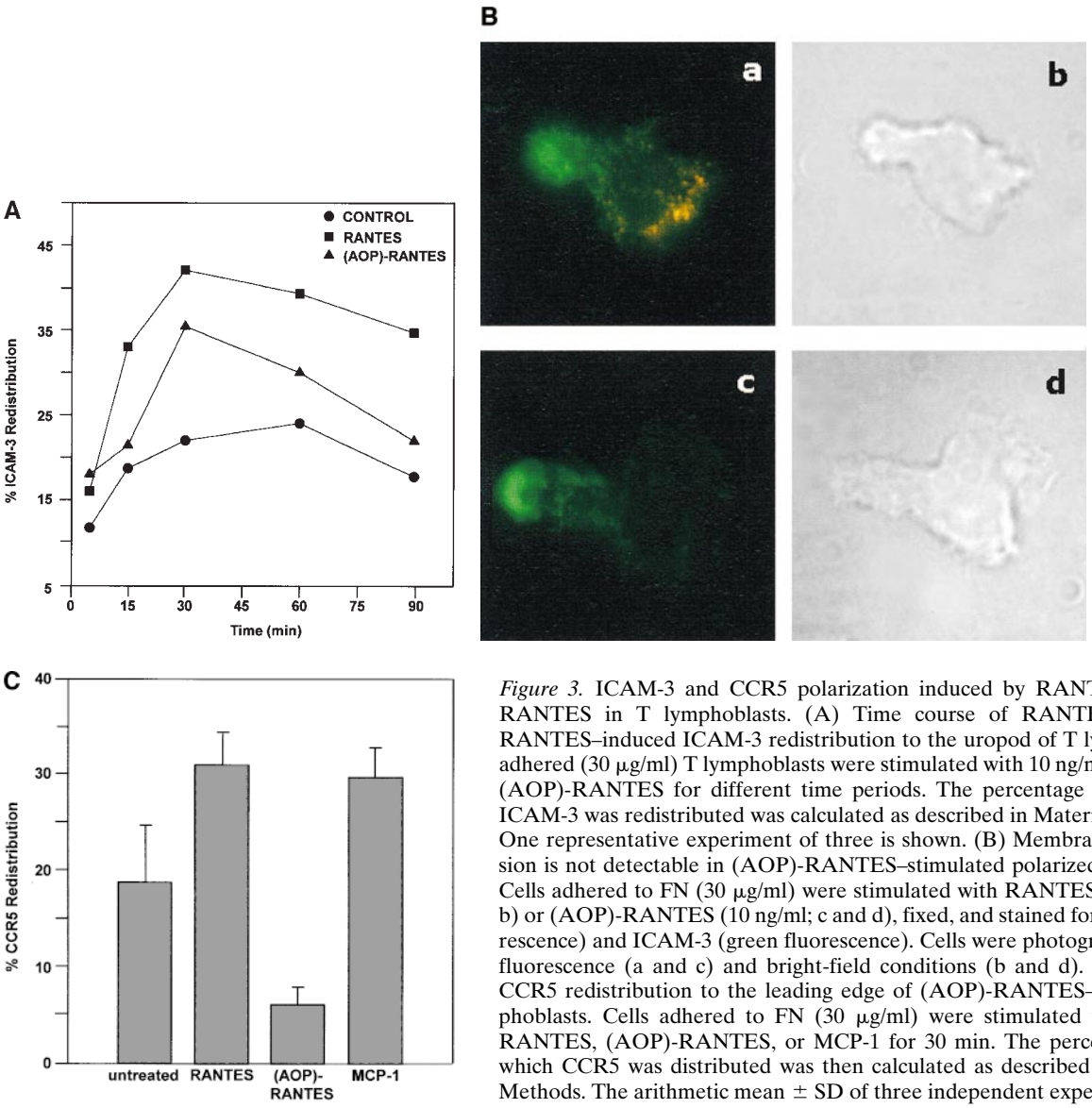

Figure 3. ICAM-3 and CCR5 polarization induced by RANTES and (AOP)-RANTES in T lymphoblasts. (A) Time course of RANTES- and (AOP)-RANTES–induced ICAM-3 redistribution to the uropod of T lymphoblasts. FN-adhered (30 μg/ml) T lymphoblasts were stimulated with 10 ng/ml of RANTES or (AOP)-RANTES for different time periods. The percentage of cells in which ICAM-3 was redistributed was calculated as described in Materials and Methods. One representative experiment of three is shown. (B) Membrane CCR5 expression is not detectable in (AOP)-RANTES–stimulated polarized T lymphoblasts. Cells adhered to FN (30 μg/ml) were stimulated with RANTES (10 ng/ml; a and b) or (AOP)-RANTES (10 ng/ml; c and d), fixed, and stained for CCR5 (red fluorescence) and ICAM-3 (green fluorescence). Cells were photographed under epifluorescence (a and c) and bright-field conditions (b and d). (C) Inhibition of CCR5 redistribution to the leading edge of (AOP)-RANTES–polarized T lymphoblasts. Cells adhered to FN (30 μg/ml) were stimulated with 10 ng/ml of RANTES, (AOP)-RANTES, or MCP-1 for 30 min. The percentage of cells in which CCR5 was distributed was then calculated as described in Materials and Methods. The arithmetic mean ± SD of three independent experiments is shown.

ates to the receptor complex in response to both ligands (Fig. 5 C). The association of STAT5 correlates in time with JAK1 phosphorylation and binding. Furthermore, the precipitated STAT5 is phosphorylated on tyrosine residues, showing it is in the activated state (Fig. 5 D). Immunoprecipitates of RANTES-stimulated cell lysates with isotype-matched control antibodies do not contain STAT5. These results were further validated by the reverse experiments, in which anti-STAT5 antibodies were used for immunoprecipitation and Western blots developed with either anti-PTyr or anti-CCR5 antibodies (not shown). Therefore, binding of both RANTES and (AOP)-RANTES to the CCR5 receptor induces receptor and JAK1 phosphorylation and STAT5 binding. Independent of the fact that other STAT transcriptional factors may be implicated in CCR5-mediated signaling, our data show that no major differences are observed in RANTES or (AOP)-RANTES activation of the JAK/STAT pathway.

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 antibod-

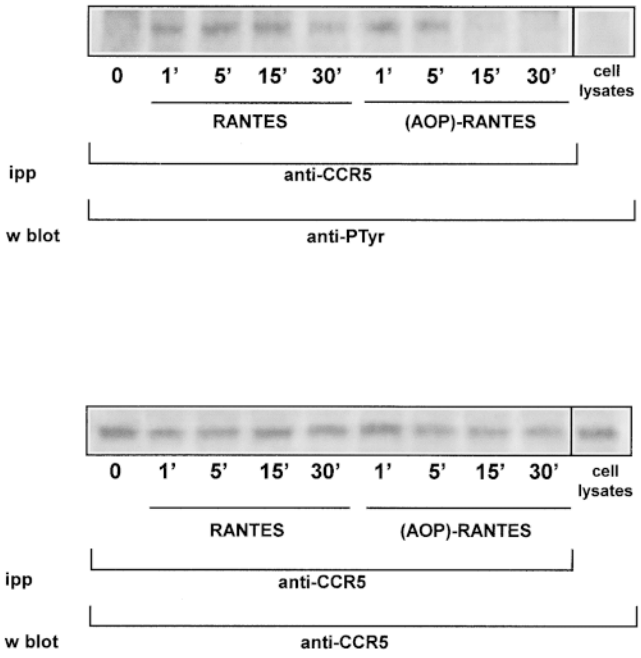

Figure 4. Both RANTES and (AOP)-RANTES induce early tyrosine phosphorylation of CCR5. RANTES- or (AOP)-RANTES (10 nM)–induced CCR5-transfected HEK-293 cells were stimulated with 10 nM RANTES or (AOP)-RANTES, lysed, immunoprecipitated with anti-CCR5 antibody, and developed in Western blot with the anti-PTyr mAb 4G10. Protein loading was controlled using a protein detection kit (Pierce Chemical Co.). To control for equivalent CCR5 loading, the blot was stripped and reprobed with the anti-CCR5 mAb CCR5-02. A control of RANTES-stimulated CCR5-transfected HEK-293 cell lysate, immunoprecipitated with anti–β_2-microglobulin antibody, was analyzed with anti-CCR5 antibody (not shown). One representative experiment is shown of four performed.

ies and Western blot developed with anti-CCR5 antibodies (Fig. 6 A). When CCR5-transfected HEK-293 cells were stimulated with SDF-1α, no CCR5 dimerization was observed under these experimental conditions (Fig. 6 A), despite the fact that HEK-293 cells express a functional CXCR4 receptor as determined by flow cytometry and SDF-1α–induced calcium mobilization assays (Fig. 6 B). When the same experiment was done using anti-CXCR4 mAb for immunoprecipitation and Western blot, a high molecular mass species (84 kD) of the CXCR4 receptor is observed only after SDF-1α, but not after RANTES or (AOP)-RANTES stimulation (Fig. 6 B).

All together, these data indicate specific ligand-induced dimerization, as SDF-1α is not able to induce CCR5 dimerization, while RANTES or (AOP)-RANTES are not able to induce CXCR4 dimers. In addition, association of CCR5 with other membrane components, including other chemokine receptors, can be discarded under these experimental conditions, as cells treated with SDF-1α render only CXCR4 dimers, and those treated with RANTES and (AOP)-RANTES render only CCR5 dimers. High molecular mass species containing CCR5 and CXCR4 simultaneously are not detected, as shown when cells are stimulated with SDF-1α or RANTES and (AOP)-RANTES, 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.

$G\alpha_i$ and p^{125}FAK Association to CCR5 Differ after RANTES or (AOP)-RANTES Stimulation

The PTX dependence of RANTES- and (AOP)-RANTES–triggered Ca^{2+} mobilization indicates $G\alpha_i$ involvement in this process. It has been demonstrated recently that chemokine receptor–mediated chemotaxis is triggered by the βγ subunit of $G\alpha_i$ (Neptune and Bourne, 1997). In MCP-1–mediated chemotaxis, we have shown that $G\alpha_i$ 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 $G\alpha_i$-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-$G\alpha_i$ antibodies. After both RANTES and (AOP)-RANTES stimulation, $G\alpha_i$ associated to CCR5 (Fig. 7 A). Whereas association persisted for longer than 15 min when cells were RANTES-stimulated, $G\alpha_i$ is 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). These data concur with the similar Ca^{2+} mobilization promoted by both of these ligands (Fig. 2 A), as $G\alpha_i$ association to CCR5 is unaltered at the times employed for these experiments (1–3 min). Rapid $G\alpha_i$ dissociation from the CCR5, induced only by (AOP)-RANTES, implies a role for G_i in later chemokine-triggered events such as chemotaxis. This result concurs with data showing the importance of βγ release from G_i in chemotaxis (Neptune and Bourne, 1997).

The activation of p^{125}FAK and the kinase ZAP-70 has been described in T cells after RANTES activation (Bacon et al., 1996). Therefore, we tested whether p^{125}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^{125}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^{125}FAK association and the rapid $G\alpha_i$ 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.

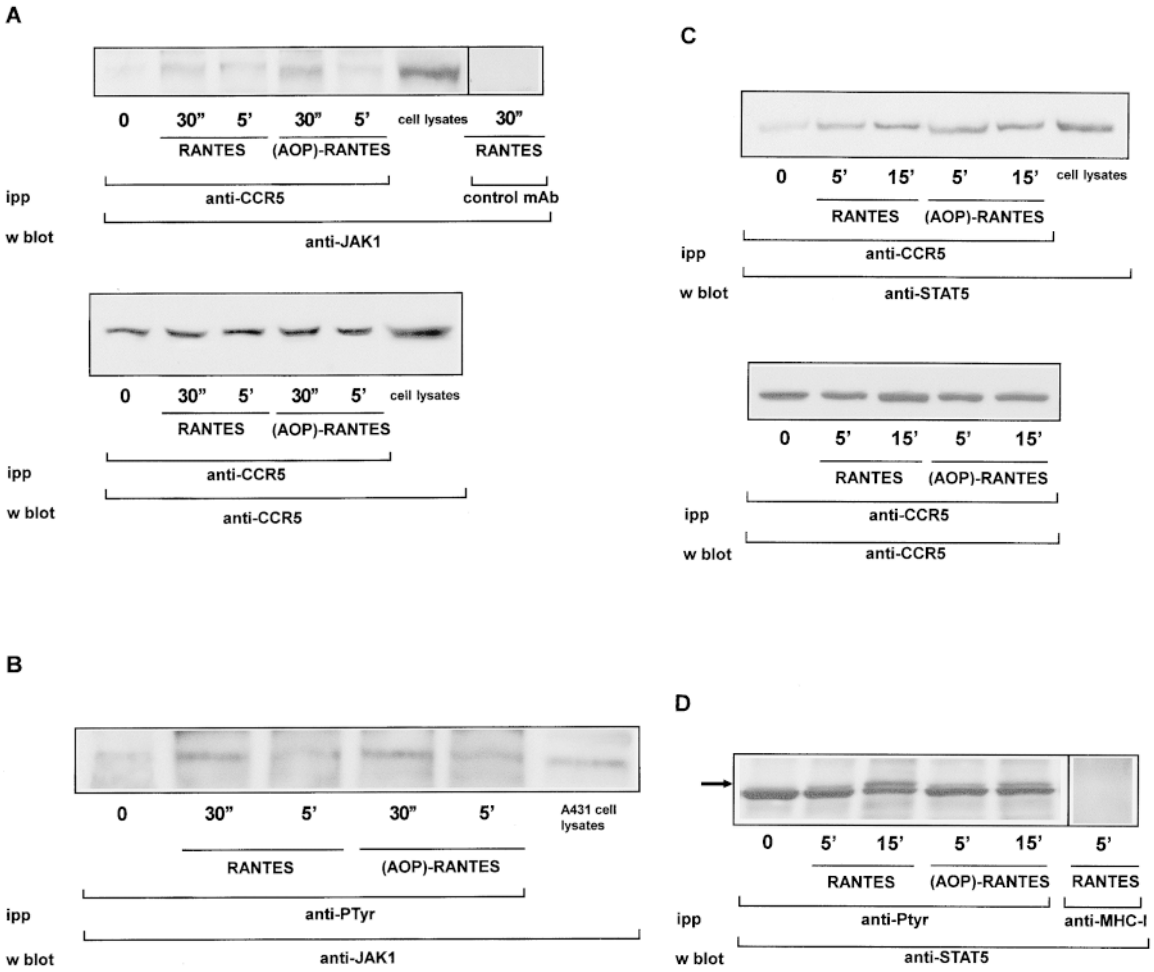

Figure 5. Both RANTES and (AOP)-RANTES activate the JAK/STAT pathway in CCR5-transfected HEK-293 cells. (A) CCR5-transfected HEK-293 cells were stimulated with RANTES or (AOP)-RANTES (10 nM). Lysates were immunoprecipitated with mAb CCR5-03 and analyzed in Western blot with anti-JAK1 antibodies. The figure shows control RANTES-stimulated (10 nM) CCR5-transfected HEK-293 cell lysates immunoprecipitated with anti–β_2-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.

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 NH_2 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

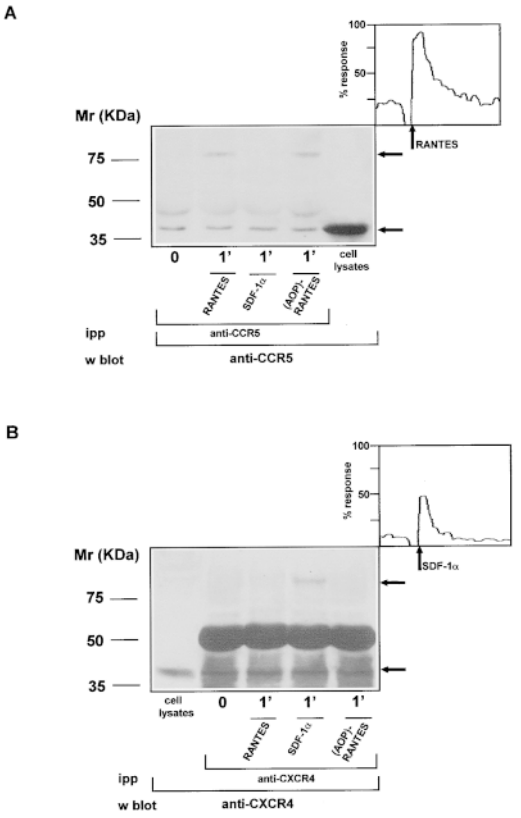

Figure 6. RANTES and (AOP)-RANTES induce dimerization of CCR5. (A) CCR5-transfected HEK-293 cells were stimulated for 60 s with 10 nM RANTES, 10 nM (AOP)-RANTES, or 20 nM SDF-1α at 37°C, and cross-linked using 1 mM DSS. Cell lysates were immunoprecipitated with mAb CCR5-03, electrophoresed, and transferred to nitrocellulose membranes. The Western blot was analyzed with mAb CCR5-02; as a positive control, CCR5-transfected HEK-293 cell lysates were immunoblotted with mAb CCR5-02. Arrows indicate the CCR5 monomer (38 kD) and dimer (76 kD). Upper corner insert shows RANTES-induced Ca^{2+} mobilization in CCR5-transfected HEK-293 cells, performed as described in Materials and Methods. Results, expressed as in Fig. 2 A, are a percentage of the maximum chemokine response. (B) CCR5-transfected HEK-293 cells as in A were immunoprecipitated and analyzed in Western blot with anti-CXCR4 mAb. Arrows indicate the CXCR4 monomer (42 kD) and dimer (84 kD). Protein loading was controlled using a protein detection kit (Pierce Chemical Co.). Upper corner insert shows SDF-1α–induced Ca^{2+} mobilization in CCR5-transfected HEK-293 cells, performed as described in Materials and Methods. Results are expressed as in A. Note that the response of CCR5-transfected HEK-293 cells to SDF-1α is weaker than RANTES, as measured by Ca^{2+} influx. This lower response correlates with the lower amounts of dimerized CXCR4 receptor, and explains the longer exposure time required to develop the Western blot for the dimerized receptor. The 52-kD protein band corresponds to the heavy chain of the immunoprecipitating antibody.

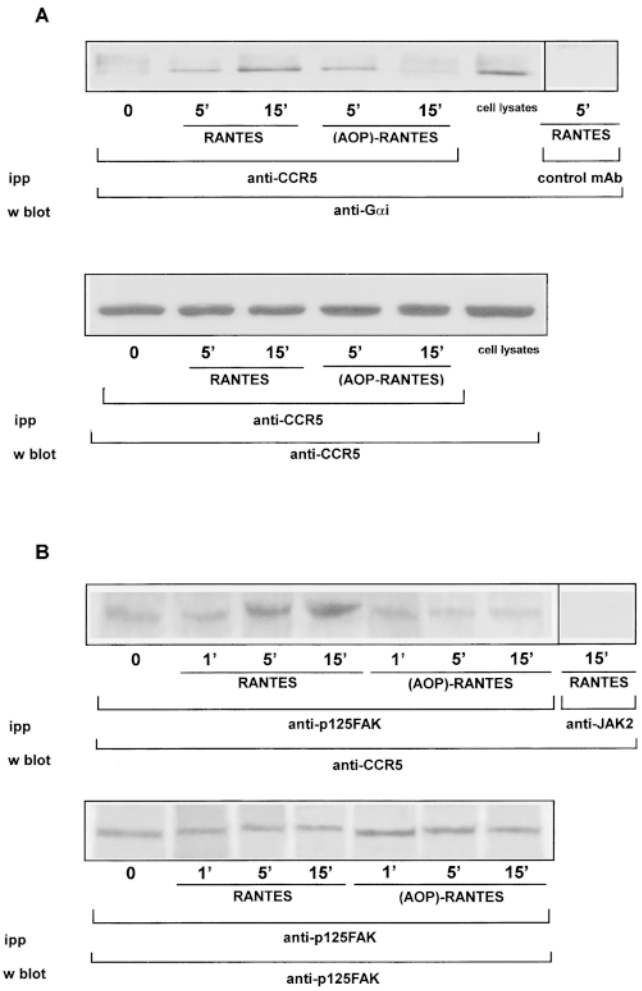

Figure 7. RANTES and (AOP)-RANTES promote $G\alpha_i$ and p^{125}FAK association to the CCR5 receptor. (A) 10 nM RANTES or (AOP)-RANTES–induced CCR5-transfected HEK-293 cell lysates were immunoprecipitated with anti-CCR5 (CCR5-03) or with an anti–β_2-microglobulin control mAb, and the Western blot developed with anti-$G\alpha_i$ antibody. As a control for CCR5 loading equivalence, the blot was stripped and reprobed with the anti-CCR5 mAb CCR5-02. As a positive control, CCR5-transfected HEK-293 cell lysates were tested in Western blot with the same anti-$G\alpha_i$ or anti-CCR5 antibodies. (B) CCR5-transfected HEK-293 cells were stimulated with RANTES or (AOP)-RANTES as in A. Cell lysates were immunoprecipitated with anti-p^{125}FAK, or anti-JAK2 antibodies as control, the Western blot developed with anti-CCR5 mAb CCR5-02. Protein loading was controlled by stripping the membrane and reprobing with anti-p^{125}FAK antibody.

(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 immunoprecipitates. 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 β_2-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 Ca^{2+} mobilization as does RANTES, but promotes neither cell migration nor CCR5 redistribution to the leading edge in cell polarization. The rapid $G\alpha_i$ association to CCR5 induced by both ligands explains the similar Ca^{2+} 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 (Hunyady 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 (Hunyady et al., 1995).

Whereas $G\alpha_i$ 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, G_i 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 p^{125}FAK and ZAP-70 has been described after RANTES stimulation (Bacon et al., 1996). Here we demonstrate association of p^{125}FAK, 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 G_i association for long (15 min) periods concurs with the lack of association and activation of p^{125}FAK and the inactivation of subsequent events. Both activation of G_i and p^{125}FAK, 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 p^{125}FAK 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 Ca^{2+} 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 p^{125}FAK 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 p^{125}FAK 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 p^{125}FAK 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-

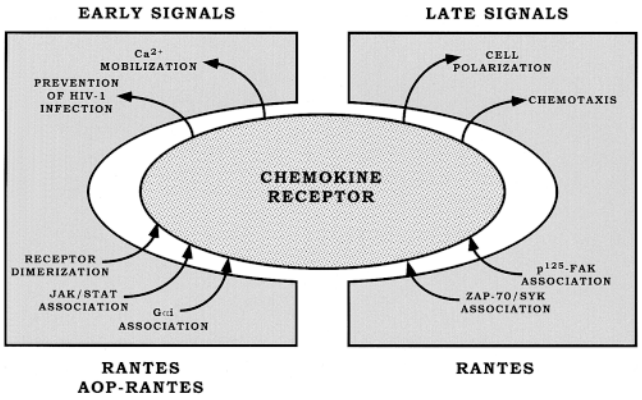

Figure 8. Diagrammatic representation of the signals delivered following binding of chemokines to their receptors. The emphasis is placed on the division between early (left) and late signals (right), indicating the biochemical signals (bottom) implicated in the function (top). The figure shows the differential ability of RANTES and (AOP)-RANTES to trigger early and late signals.

ceptors (Aramori et al., 1997; Howard et al., 1998). 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 NH_2-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 NH_2-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.

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