The chemokine RANTES (regulated on activation normal T cell expressed and secreted) and its cognate receptor CC chemokine receptor 5 (CCR5) have been implicated in regulating immune cell function. Previously we reported that in T cells, RANTES activation of CCR5 results in Stat1 and Stat3 phosphorylation-activation, leading to Stat1:1 and Stat1:3 dimers that exhibit DNA binding activity and the transcriptional induction of a Stat-inducible gene, c-fos. Given that RANTES and CCR5 have been implicated in T cell activation, we have studied RANTES-induced signaling events in a CCR5-expressing T cell line, PM1. RANTES treatment of PM1 T cells results in the rapid phosphorylation-activation of CCR5, Jak2, and Jak3. RANTES-inducible Jak phosphorylation is insensitive to pertussis toxin inhibition, indicating that RANTES-CCR5-mediated tyrosine phosphorylation events are not coupled directly to Gq protein-mediated events. In addition to Jak2, several other proteins are rapidly phosphorylated on tyrosine residues in a RANTES-dependent manner, including the Src kinase p56Lck, which associates with Jak3. Additionally we have shown that the amino-terminally modified RANTES proteins, aminooxypentane-RANTES and Met-RANTES, are agonists for CCR5 and induce early tyrosine phosphorylation events that are indistinguishable from those inducible by RANTES with similar kinetics. Our data also demonstrate that RANTES activates the p38 mitogen-activated protein (MAP) kinase pathway. This is evidenced by the rapid RANTES-dependent phosphorylation and activation of p38 MAP kinase as well as the activation of the downstream effector of p38, MAP kinase-activated protein (MAPKAP) kinase-2. Pharmacological inhibition of RANTES-dependent p38 MAP kinase activation blocks MAPKAP kinase-2 activity. Thus, activation of Jak kinases and p38 MAP kinase by RANTES regulates the engagement of multiple signaling pathways.

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this study we report that RANTES treatment of human PM1 T cells that express cell surface CCR5 results in the rapid and transient phosphorylation of CCR5 on tyrosine residues and the activation of CCR5-associated Jak kinases. Furthermore, we provide evidence for the RANTES-dependent association of the Src family kinase, p56\textsuperscript{\textcircled{\textit{lck}}}, with Jak3. Our data reveal that these RANTES-CCR5-mediated tyrosine phosphorylation events are pertussis toxin-insensitive and therefore are not coupled to G\textsubscript{\textcircled{\textit{o}}}, protein-mediated events. In comparative experiments we show that the amino-terminally modified RANTES proteins, Met-RANTES and aminooxypentane-RANTES (AOP-RANTES),\footnote{The abbreviations used are: AOP-RANTES, aminooxypentane-RANTES; MAP, mitogen-activated protein; MAPKAP, MAP kinase-activated protein; PAGE, polyacrylamide gel electrophoresis.} exhibit agonist activity on CCR5 in PM1 cells in the context of tyrosine phosphorylation events with similar kinetics to RANTES.

The p38 mitogen-activated protein (MAP) kinases are serine-threonine protein kinases that are activated by diverse stimuli including physical and chemical stresses and by various hemoipoetic and pro-inflammatory cytokines (reviewed in Refs. 26–30). Signal transduction mediated via the p38 MAP kinase pathway seems to play an important role in regulating inflammatory responses including cytokine secretion and apoptosis in a number of different biological systems. p38 MAP kinase activation is regulated by its phosphorylation on threonine and tyrosine residues. Focal adhesion kinase and Pyk2 kinase are nonreceptor protein-tyrosine kinases that are phosphorylation-activated upon T cell activation and after stimulation of G protein-linked receptors (31–33). As indicated above RANTES will stimulate the phosphorylation of focal adhesion kinase, the tyrosine kinase ζ-associated protein 70, and the focal adhesion protein paxillin in human T cells (22). Moreover, there is evidence that the focal adhesion kinase-related tyrosine kinase, Pyk2, will activate p38 MAP kinase (34). Indeed, the activation of p38 MAP kinase has been implicated in chemokine-induced responses (35). Viewed together, these observations raise the possibility that RANTES activation of CCR5 in T cells may invoke p38 MAP kinase activation. In this report we provide the first evidence that RANTES-CCR5 interactions result in the rapid phosphorylation of p38 and activation of its catalytic activity. Overall, our data establish that RANTES activation of CCR5 leads to the rapid phosphorylation of distinct signaling intermediates on tyrosine residues that invoke discrete signal-transducing pathways.

EXPERIMENTAL PROCEDURES

Cells and Reagents—Human PM1 T cells expressing CCR5 (36) were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program and maintained in RPMI 1640 medium with 10% fetal calf serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. Recombinant RANTES, Met-RANTES, and AOP-RANTES were provided by Serono Pharmaceutical Research Institute. Polyclonal antibodies against Jak2, Jak3, p38, and CCR5 and a monoclonal antibody against p56\textsuperscript{\textcircled{\textit{lck}}} were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies recognizing p38, the phosphorylation-activated form of p38, and the phosphorylation-activated form of ATP-2 were obtained from New England Biolabs. Antibodies against MAP kinase-activated protein (MAPKAP)-kinase-2 and phosphorylated tyrosine (4G10) were obtained from Upstate Biotechnology, Inc. The SB203580 inhibitor and pertussis toxin were obtained from Calbiochem.

Cell Lysis and Immunoblotting—Actively growing cells at a concentration of 1 × 10\textsuperscript{6} cells/ml were incubated with 1 M KOH at 70° C to select for tyrosine-phosphorylated proteins (37).

Flow Cytometric Analysis of Antibody Binding to Native CCR5 on PM1 Cells—FACScan analyses of CCR1 and CCR5 expression on PM1 cells was performed using monoclonal antibodies against CCR1 (provided by R. Horuk, Berlex Laboratories, Inc.) and CCR5 (National Institutes of Health AIDS Research and Reference Reagent Program) as previously described (40).

RESULTS AND DISCUSSION

The CD4+ clonal PM1 cells used in these studies were derived from the human T cell line Hut78 (36). At the onset we determined that PM1 cells express cell surface CCR5 (Fig. 1). To determine whether RANTES induced the phosphorylation of CCR5 in PM1 cells, CCR5 was immunoprecipitated from lysates from RANTES-treated cells, and the solubilized immunoprecipitate was resolved by SDS-PAGE and then immunoblotted for tyrosine phosphorylation and CCR5. The data in Fig. 2A reveal that CCR5 is phosphorylated on tyrosine residues after RANTES treatment of PM1 cells. It is known that the chemokine monocyte chemotactic protein-1 triggers Jak2 activation and tyrosine phosphorylation of CCR2 in the human monocytic cell line Mono Mac 1 (41) and that RANTES induces tyrosine phosphorylation of CCR5 in CCR5-transfected human embryonic kidney HEK-293 cells and association with Jak1 (42). We therefore examined PM1 cells for RANTES-dependent CCR5 and Jak phosphorylation. In time-course studies when cell lysates from RANTES-treated cells were immunoprecipitated with anti-CCR5 antibodies, resolved by SDS-PAGE, and immunoblotted with anti-Tyr(P) antibodies, phosphorylated bands that were identified subsequently as CCR5 and Jak2 appeared within 1 min of RANTES stimulation (Fig. 2B). Apparently phosphorylated Jak2 rapidly associates with CCR5 in a RANTES-dependent manner.

To determine whether RANTES-CCR5 interactions result in the activation of other Jak-kinases, we immunoprecipitated RANTES-induced cell extracts with different anti-Jak antibodies, resolved the immunoprecipitates by SDS-PAGE, and immunoblotted with anti-Tyr(P) antibodies. Although we were unable to detect Jak1 activation, the results in Fig. 3 show RANTES-inducible Jak-2 and Jak3 phosphorylation. Interestingly, the inclusion of pertussis toxin did not affect RANTES-inducible Jak phosphorylation, suggesting that G\textsubscript{\textcircled{\textit{o}}}, protein signaling events are not coupled to Jak activation. In subsequent experiments we examined whether the kinase activity of Jak2 and Jak3 is induced by RANTES. PM1 cells were treated with RANTES, cell lysates were immunoprecipitated with anti-Jak2 or anti-Jak3 antibodies, and in vitro kinase assays were performed on the immunoprecipitates. The data in Fig. 4 indicate that RANTES-inducible phosphorylation of Jak2 and Jak3 results in activation of their catalytic domains. The rapid phosphorylation-activation of Jak2 and Jak3 and the rapid association of Jak2 with CCR5 suggests that these kinases may effect early receptor tyrosine phosphorylation.

When cell lysates from RANTES-treated cells were immunoprecipitated with anti-Jak3 antibodies and Western blots were developed with anti-Tyr(P) antibodies, we observed a 56-kDa phosphorylated protein that associated with Jak3 in a RANTES-dependent manner (Fig. 5). Stripping and reprobing the membrane identified this phosphorylated protein as the Src kinase p56\textsuperscript{\textcircled{\textit{lck}}}.

Moreover, treatment of PM1 cells with the amino-terminally modified RANTES proteins, AOP-RANTES and Met-RANTES, resulted in similar Jak3 tyrosine phosphorylation and p56\textsuperscript{\textcircled{\textit{lck}}} association (Fig. 5). Clearly, despite the fact that both Met-RANTES and AOP-RANTES inhibit RANTES-induced activities both in vitro (43) and in vivo (44–47), both exert agonist activities on CCR5 in the context of early tyrosine phosphorylation activation.
phosphorylation events. Notably, RANTES-, Met-RANTES-, and AOP-RANTES-dependent association of phosphorylated p56\textsuperscript{lck} with Jak3 follows ligand-stimulated Jak3 activation.

Because we observe that Jak3s are rapidly associated and activated with ligand-stimulated CCR5, followed by the sequential recruitment of p56\textsuperscript{lck}, the implications are that p56\textsuperscript{lck} is re-

Fig. 1. PM1 cells express cell surface CCR5. Cell surface expression of CCR1 (A) and CCR5 (B) was determined by flow cytometric analysis of unstained, (gray fill) or antibody-stained, (black fill) PM1 cells. A log shift in CCR5 fluorescence intensity was observed (B).

Fig. 2. RANTES-dependent CCR5 phosphorylation and association with phosphorylated Jak2. PM1 cells were either left untreated (−) or treated (+) with RANTES for the times indicated. Cell lysates were immunoprecipitated (IP) with anti-CCR5 antibody. A and B, immunoprecipitated proteins were resolved by SDS-PAGE, then immunoblotted (WB) with an antibody against Tyr(P) (4G10). The blot shown in A was stripped and reprobed with anti-CCR5 antibody. In B, the arrows indicate the positions of CCR5 and Jak2. The blot shown in B was stripped and reprobed with anti-Jak2 antibody. Molecular mass markers (in kDa) are identified.

Fig. 3. RANTES induction of Jak2 and Jak3 phosphorylation. PM1 cells were either left untreated (−) or treated (+) with RANTES for the times indicated in the presence or absence of pertussis toxin (PTx, 100 ng/ml for 4 h). Cell lysates were immunoprecipitated (IP) with anti-Jak2 (A) or anti-Jak3 (B) antibodies. The immunoprecipitated proteins were resolved by SDS-PAGE, immunoblotted (WB) with an antibody against Tyr(P) (4G10), and then stripped and reprobed with either anti-Jak2 or anti-Jak3 antibodies. R IgG, rabbit immunoglobulin.
was stripped and reprobed with antibodies against Jak3 and p56
(4G10). The blot
immunoblotted (WB) immunoprecipitated proteins were analyzed by SDS-PAGE and then
immunoprecipitated with anti-Jak3 antibody, and
Cell lysates were immunoprecipitated (IP) with RANTES or Met-RANTES for the times indicated.

The preceding provides compelling evidence for protein-tyrosine
kinase association with the
interferon-receptor complex influences the antiproliferative action
of interferon in T cells (51). It has been reported that Src
kinases have been reported. Specifically, both Lyn and p59
associate via SH2 domains with interferon-α-activated Tyk2 in
hemopoietic cells (49, 50). Furthermore, there is some evidence to suggest that association of p56
with the activated interferon-receptor complex influences the antiproliferative action of interferon in T cells (51). It has been reported that Src kinases can associate with the α subunits of heterotrimeric G protein complexes (reviewed in Refs. 52 and 53), and a recent report describes a functional interaction between the folate receptor and the associated signaling molecules Lyn and Grb2 (54). Thus, RANTES-dependent recruitment of p56 in CCR5 may result in complex patterns of interactions among signaling molecules that may allow for cross-talk between G protein-coupled signaling cascades and non-G protein-linked cascades.

RANTES-inducible activation of T cells influences their proliferation and differentiation, adhesion molecule expression, and cytokine release (reviewed in Ref. 49). The precise biochemical pathways that determine specific biological consequences are unknown. The preceding provides compelling evidence for protein-tyrosine kinase involvement in mediating RANTES-CCR5 signal transduction. Apparently, non-CCR5-associated protein-tyrosine kinases such as Jaks and p56 can be recruited to the activated receptor.

The implications are that membrane-localized protein-tyrosine kinases are recruited to the ligand-stimulated receptor where they are activated and act in concert to initiate intracellular signaling cascades. MAP kinases are included among the signaling kinases regulated by chemokines (50, 51) and are known to be activated by phosphorylation on tyrosine and threonine residues. Recently, we reported that the p38 MAP kinase pathway regulates interferon-dependent gene transcription without affecting DNA binding of Stat proteins, suggesting a cooperation between the p38 MAP kinase pathway and the Jak-Stat pathway in transcriptional regulation (30). Therefore, we examined whether RANTES activation of CCR5 in PM1 T cells leads to p38 MAP kinase activation. In time-course studies, whole-cell lysates from untreated and RANTES-treated PM1 cells were analyzed by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of p38 MAP kinase (ppp38). The blot was then stripped and reprobed with an antibody against p38 MAP kinase (p38). Cells were either left untreated (−) or treated (+) with RANTES for the times indicated. Cell lysates were immunoprecipitated with an antibody against p38 MAP kinase, and immunoprecipitates were subjected to an in vitro kinase assay using glutathione S-transferase-AKT-2 as substrate. Proteins were resolved by SDS-PAGE, and phosphorylated AKT-2 was detected by immunoblotting with an anti-phospho-AKT-2 antibody. C, cells were either left untreated (−) or treated (+) with RANTES for the times indicated in the presence (+) or absence (−) of SB203580 (30 min at 10 μM). Cell lysates were immunoprecipitated with an antibody against MAPKAP kinase-2 and then an in vitro kinase assay was performed using Hsp25 as substrate. Proteins were analyzed by SDS-PAGE, and phosphorylated Hsp25 was detected by autoradiography.
nase-2, and in vitro kinase assays were performed on the immunoprecipitates using Hsp25 as the exogenous substrate. The results in Fig. 6C indicate that MAPKAP kinase-2 is activated by RANTES treatment. The data reveal a concordance between the kinetics of maximal inducible kinase activity of p38 and MAPKAP kinase-2 at 30 min post-RANTES stimulation. Moreover, using a p38-specific inhibitor, the pyridinyl imidazole compound SB203580, we provide evidence that MAPKAP kinase-2 is indeed a downstream effector of the p38 MAP kinase pathway in RANTES-stimulated T cells (Fig. 6C).

The specific role(s) of p38 MAP kinase in RANTES-inducible biological responses remains unknown. A recent report describes a role for p38 MAP kinase in the serine phosphorylation of paxillin and the consequent disassembly of focal adhesion complexes (56). The implications are that p38 MAP kinase signaling may function to negatively regulate RANTES-inducible T cell activation in the context of disassembling T cell focal adhesions (22). Certainly, the kinetics of RANTES activation of p38 kinase activity are consistent with a role for this MAP kinase in negative feedback inhibition.

In conclusion, our findings provide direct evidence for the RANTES-CCR5-dependent recruitment and activation of distinct protein kinases in T cells: the Jak5, Jak2 and Jak3; the Src kinase p56lck; and the MAP kinases p38 and MAPKAP kinase 2. Whereas activation of Jak2, Jak3, and p56lck requires both threonine and tyrosine phosphorylations, activation of p38 requires their phosphorylation on tyrosines, activation of p38 requires both threonine and tyrosine phosphorylations. Furthermore the hierarchical p38 signaling pathway invokes serine phosphorylation of target substrates. Clearly the RANTES-dependent sequestering of different signaling molecules to CCR5 provides for signal integration or reciprocal modulation of interacting signaling pathways. The specific roles of these interacting pathways during RANTES activation of CCR5 remain to be elucidated and are the subject of our ongoing investigations.

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RANTES Activates Jak2 and Jak3 to Regulate Engagement of Multiple Signaling Pathways in T Cells
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