Cutting Edge: T Cell Migration Regulated by CXCR4 Chemokine Receptor Signaling to ZAP-70 Tyrosine Kinase

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*J Immunol* 2001; 167:1857-1861; doi: 10.4049/jimmunol.167.4.1857

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T lymphocytes migrate in response to chemoattractant gradients provided by chemokines, a family of small proteins that bind to heterotrimeric serpentine G protein-coupled receptors (1). Thus, chemokines provide directional cues critical to T cell trafficking, migration into inflammatory sites, retention of hemopoietic precursors in the bone marrow, and anatomic compartmentalization of lymphocyte subpopulations in secondary lymphoid organs (1, 2). In addition, chemokines can regulate integrin adhesion receptor functional activity (3–5), as well as other cellular responses such as leukocyte differentiation, angiogenesis, and susceptibility to HIV infection (1). Biochemical analysis of chemokine receptor signaling has demonstrated that chemokines initiate a wide array of intracellular signals, including G protein- and tyrosine kinase-dependent calcium fluxes, activation of protein and lipid kinases, as well as tyrosine phosphorylation of a number of cellular substrates, including focal adhesion kinase, Pyk2, and paxillin (6–13). Although phosphatidylinositol 3-kinase (PI 3-K) has been implicated in chemokine-dependent migration (6, 8, 12), the functional relevance of tyrosine kinase-dependent signaling events to T cell migration remains unclear. In this report, we document a novel function for the ZAP-70 tyrosine kinase in the migration of human T cells in response to the chemokine CXCL12 (stromal-derived factor 1α/pre-B cell-stimulating factor).

Materials and Methods

Cells

The Jurkat E6-1 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The ZAP-70-deficient Jurkat mutant P116 was obtained from Drs. R. Abraham and B. Irvin (Mayo Clinic, Rochester, MN) (14). The SLP-76-deficient mutant J14 and Src homology 2 domain-containing leukocyte protein of 76 kDa (SLP-76)-reconstituted J14-76-11 were provided by Dr. A. Weiss (University of California, San Francisco, CA) (15). Jurkat, P116, and J14 were maintained in RPMI 1640 supplemented with 10% FCS (Atlanta Biologicals, Norcross, GA), L-glutamine, and penicillin/streptomycin (complete medium). P116 cells expressing ZAP-70 constructs and J14-76-11 cells were maintained in complete medium containing 2 mg/ml G418 (Mediatech-Cellgro, Herndon, VA).

Abs and other reagents

The SLP-76-specific Ab was provided by Dr. G. A. Koretzky (University of Pennsylvania, Philadelphia, PA). The β1 integrin-specific mAb TS2/16 was obtained from ATCC. Human CXCL12 (SDF-1α/PBSF) was purchased from PeproTech (Rocky Hill, NJ) or R&D Systems (Minneapolis, MN). The CXCR4-specific mAb was purchased from R&D Systems. The phosphotyrosine-specific mAb 4G10 was purchased from Upstate Biotechnology (Lake Placid, NY). The FLAG-specific mAb M2 was obtained from Sigma (St. Louis, MO). The anti-hemagglutinin (anti-HA) mAb 16B12 was purchased from Covance (Richmond, CA). Human fibronectin was purchased from Invitrogen/Life Technologies (Carlsbad, CA).

DNA constructs

The pRES-EGFP-HA-ZAP-70 and pRES-EGFP-HA-ZAP-70(K369R) bicistronic plasmid expression constructs encoding for HA-tagged forms of

3 Abbreviations used in this paper: PI 3-K, phosphatidylinositol 3-kinase; CXCR, CXC chemokine receptor; HA, hemagglutinin; SLP-76, Src homology 2 domain-containing leukocyte protein of 76 kDa; eGFP, enhanced green fluorescent protein.
human ZAP-70 have been previously described (16). The pIRESEGFP-HA-ZAP-70(Y292F) mutant construct was created by site-directed mutagenesis using the QuickChange Site Directed Mutagenesis kit (Stratagene, La Jolla, CA). Mutations were confirmed by sequencing at the University of Minnesota Microchemical Facility (Minneapolis, MN). The pEF-FLAG-SLP-76 construct was provided by Dr. G. A. Koretzky (17).

Transfections

P116 cells (10^6 cells) were transfected by electroporation as previously described (16) using a BTX (San Diego, CA) Square Wave electroporator with 25 μg each of the SLP-76 and ZAP-70 constructs. After electroporation, cells were incubated in complete medium for 16–20 h at 37°C. The generation of stable P116 transfectants expressing ZAP-70 or P116 was performed as previously described (18). Screening for transfectants was performed 20–24 days following the electroporation by flow cytometry. Bulk populations of transfectants expressing HA-tagged ZAP-70 constructs were collected and analyzed in migration assays (as described below).

Migration assays

Transfected Jurkat cells were serum-starved in migration medium (RPMI 1640 containing 1% BSA, 10 mM HEPEs buffer, pH 6.9) for 4 h. Migration assays were performed in transwell chambers with 5-μm polycarbonate membrane (catalog no. 3421; Costar, Cambridge, MA) precoated with 20 μg/ml fibronectin or BSA on both sides of the filter as previously described (19). Human CXCL12 was diluted to appropriate concentrations in migration medium and added to the lower chamber of the transwells. Medium alone was added to wells left unstimulated. The membranes were placed on top, and 5 × 10^5 serum-starved cells were loaded into the upper chamber in migration medium. The cells were allowed to migrate for 2.5 h at 37°C in 5% CO₂, and migrated cells were collected, pelleted, and resuspended in 200 μl of ice-cold FACS buffer (HBSS supplemented with 10% bovine calf serum and 0.2% sodium azide). A fixed number of 9-μm latex reference beads (Interfacial Dynamic, Portland, OR) and 25 μl of propidium iodide (Sigma) were added to each tube, and samples were analyzed by flow cytometry (19). An aliquot of each cell population was also analyzed by flow cytometry using standard procedures to assess the expression of CXCR4, α₅, and αβ₅ integrin. The percentage of migration of cells expressing comparable levels of HA-ZAP-70, as assessed by enhanced green fluorescent protein (eGFP) expression (16), was calculated as previously described (19).

Preparation of cell lysates

Cell lysates were prepared as previously described (20). Briefly, cells were serum starved for 4 h, washed in OPTI-MEM (Invitrogen) and incubated in OPTI-MEM with CXCL12 at the indicated concentrations at 37°C for the indicated amount of time. The cells were then lysed by adding an equivalent volume of 2× lysis buffer (2% Nonidet P-40, 0.5% sodium deoxycholate, 300 mM NaCl, 100 mM Tris-HCl, 2 mM sodium vanadate, 20 μg/ml leupeptin, 20 μg/ml aprotinin, and 2 mM PMSF). Cell lysates were clarified by centrifugation (13,000 × g, 4°C for 30 min). Unstimulated cells were incubated for 0 min with CXCL12 and immediately lysed as described above.

Immunoprecipitation and Western blotting

Immunoprecipitations from cell lysates (10 × 10^6 cells) were performed as previously described (20) with 5 μl anti-SLP-76 Ab or 4 μl anti-FLAG M2 mAb. Immunoprecipitates were separated by SDS-PAGE, and samples were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) for analysis by Western blotting.

Western blotting was performed as previously described (20). Blots were incubated with primary Ab (1/1000 dilution of anti-SLP-76, 0.5 μg/ml anti-phosphotyrosine mAb 4G10, or 0.5 μg/ml anti-FLAG M2 mAb in PBS containing 5% milk) for 2 h at room temperature, washed, and then incubated with HRP-conjugated goat anti-mouse IgG (Caltag Laboratories, Burlingame, CA) for 1 h at room temperature. The membranes were then washed and developed using ECL (Pierce, Rockford, IL). For repeated immunoblotting, membranes were stripped as previously described (20) and reprobed using the procedure described above.

Results and Discussion

Human Jurkat T cells express the CXCR4 chemokine receptor (Ref. 21, and data not shown) and migrate rapidly in response to a gradient of the CXCR4 ligand CXCL12 (Fig. 1). Although a basal level of migration through BSA-coated filters was observed in response to CXCL12 (data not shown), migration was enhanced dramatically if the filters were precoated with the extracellular matrix protein fibronectin. Migration through both BSA- and fibronectin-coated filters was maximal at 30–100 ng/ml CXCL12. We also tested the migration of the ZAP-70-deficient Jurkat T cell line P116 (14) in response to CXCL12. Although low amounts of CXCL12 (3 ng/ml) enhanced migration of P116 T cells compared with migration in the absence of chemokine, higher amounts of CXCL12 did not further enhance migration. In addition, the migration of P116 T cells in response to these higher concentrations of CXCL12 was always lower than the migration of wild-type Jurkat T cells. Although the overall percentage of P116 T cells that migrated varied between 10 and 30% from experiment to experiment, the migration of P116 T cells was always 2- to 3-fold lower than wild-type Jurkat T cells analyzed in the same experiment. This lower migratory response was not due to differences in chemokine receptor or β₅ integrin expression, as flow cytometric analysis demonstrated comparable levels of expression of CXCR4, α₅β₅, and ααβ₅ integrins on P116 T cells and wild-type Jurkat T cells (Ref. 16 and data not shown).

To determine whether re-expression of ZAP-70 in P116 T cells could enhance CXCL12-mediated migration, we produced stable transfectants of P116 T cells expressing either wild-type ZAP-70 or various mutant forms of ZAP-70. Exogenous ZAP-70 was encoded by an eGFP bicistronic vector (16), which allowed us to monitor expression of ZAP-70 in intact cells by flow cytometry. Western blotting analysis was also used to verify expression of the ZAP-70 protein in transfected P116 T cells (data not shown). A bulk population of transfected P116 T cells was analyzed for migration through fibronectin-coated filters in response to 30 ng/ml CXCL12. Because this bulk population of cells expressed varying levels of eGFP expression, we collected the cells that migrated and quantitated them by flow cytometry for eGFP expression (19). Postacquisition gating was then used to determine the migration of eGFP⁺ P116 T cell transfectants. The results in Fig. 2 demonstrate that although P116 T cell transfectants expressing the eGFP bicistronic vector alone retained a low level of migration through fibronectin-coated filters in response to CXCL12, expression of wild-type ZAP-70 dramatically enhanced the migration of P116 T cells.
Similar effects of wild-type ZAP-70 were observed when migration through BSA-coated filters was assessed, although the overall level of migration was diminished when compared with migration through fibronectin-coated filters (Fig. 2). In contrast, expression of a kinase-inactive form of ZAP-70 (K369R) at levels similar to wild-type ZAP-70 exhibited minimal effects on CXCL12-dependent migration of P116 T cells. Expression of eGFP or eGFP and ZAP-70 did not alter the expression of CXCR4, α4β1 integrin, or α5β1 integrin (data not shown).

The tyrosine residue at position 292 in the interdomain B region of ZAP-70 negatively regulates TCR-mediated activation of ZAP-70 by mediating the association of the negative regulatory adapter protein p120Cbl with ZAP-70 (22). Thus, mutation of the tyrosine at position 292 to phenylalanine (Y292F) enhances ZAP-70 activity following TCR stimulation (22). We examined the effect of expression of the Y292F ZAP-70 mutant in P116 T cells stably expressing the Y292F ZAP-70 mutant exhibited higher levels of migration in response to CXCL12 than transfectants expressing comparable levels of wild-type ZAP-70 (Fig. 2). This suggests that similar to its role in TCR signaling, tyrosine 292 negatively regulates ZAP-70-dependent effects on T cell migration.

Activation of ZAP-70 during TCR stimulation leads to the tyrosine phosphorylation of the adapter protein SLP-76, which plays a critical role in T cell activation and development (23). We analyzed the ability of CXCL12 stimulation to regulate tyrosine phosphorylation of SLP-76 in wild-type and P116 Jurkat T cells. Treatment of wild-type Jurkat T cells with CXCL12 resulted in a 3- to 4-fold increase in tyrosine phosphorylation of SLP-76 that was dependent on ZAP-70 tyrosine kinase activity. A, Jurkat and P116 cells were stimulated for the indicated periods of time with 100 ng/ml CXCL12 at 37°C, lysed, and subjected to immunoprecipitation with an anti-SLP-76 Ab. Anti-SLP-76 immunoprecipitates were separated by SDS-PAGE, and membranes were immunoblotted with anti-phosphotyrosine mAb 4G10 (top panel). The membrane was subsequently stripped and reprobed with the anti-SLP-76 Ab (bottom panel). B, P116 cells were transiently transfected with FLAG epitope-tagged wild-type SLP-76 (FLAG-SLP76) and the indicated ZAP-70 constructs. Equivalent numbers of eGFP cells were serum-starved and stimulated with 100 ng/ml CXCL12 for either 0 or 2 min at 37°C. Cells were lysed, subjected to anti-FLAG immunoprecipitation, separated on a 7.5% SDS-polyacrylamide gel, transferred to membrane, and immunoblotted with anti-phosphotyrosine (ANTI-PTYR) mAb (top panel) to detect tyrosine-phosphorylated SLP-76 as described in Materials and Methods. The membrane was subsequently stripped and reprobed with the anti-FLAG mAb (bottom panel).
detectable within 2 min of CXCL12 stimulation (Fig. 3A). In contrast, treatment of ZAP-70-deficient P116 T cells with CXCL12 did not result in any detectable tyrosine phosphorylation of SLP-76 at any of the time points tested (Fig. 3A), even though comparable amounts of SLP-76 were immunoprecipitated from wild-type Jurkat T cells and P116 T cells. Doses of CXCL12 ranging from 3 ng/ml to 1 μg/ml also did not result in tyrosine phosphorylation of SLP-76 in P116 T cells (data not shown). CXCL12-mediated tyrosine phosphorylation of SLP-76 was dependent on the kinase activity of ZAP-70, because expression of wild-type ZAP-70, but not kinase-inactive ZAP-70, in P116 T cells restored CXCL12-mediated tyrosine phosphorylation of SLP-76 (Fig. 3B). Furthermore, expression of the Y292F ZAP-70 mutant in P116 T cells resulted in enhanced CXCL12-mediated tyrosine phosphorylation of SLP-76 when compared with P116 T cells expressing wild-type ZAP-70. These results suggest that CXCR4 receptor stimulation activates ZAP-70 tyrosine kinase activity.

Because CXCL12 stimulation of T cells resulted in ZAP-70-dependent tyrosine phosphorylation of SLP-76, we also examined the migration of the SLP-76-deficient Jurkat T cell line J14 (15) in response to CXCL12. Similar to the results obtained with P116 T cells, the migration of J14 cells in response to CXCL12 was reduced compared with wild-type Jurkat T cells (Fig. 4). However, expression of wild-type SLP-76 in J14 T cells did not enhance the migration of J14 T cells in response to CXCL12, as the J14-76-11 stable transfected expressing wild-type SLP-76 (15) migrated to CXCL12 at levels comparable to untransfected J14 T cells. Both the SLP-76-deficient J14 and SLP-76-positive J14-76-11 cell lines exhibited comparable levels of CXCR4, αβ integrin, and αβ integrin (data not shown).

Our results demonstrating a role for ZAP-70 in CXCR4 signaling are similar to previous studies demonstrating activation of ZAP-70 upon stimulation of T cells with CCL5 (RANTES) (7). ZAP-70 has also been implicated in regulating the LFA-1-dependent migration of T cell hybridomas (24). Although LFA-1 stimulation resulted in activation of ZAP-70 in these T cell hybridomas, we have not observed stimulation of ZAP-70 activity upon ligation of β1 integrins expressed on Jurkat T cells (data not shown). Furthermore, the ability of ZAP-70 to enhance the migration of P116 T cells was also observed when using BSA-coated filters, although the overall level of migration in response to CXCL12 was dramatically reduced when compared with migration through fibronectin-coated filters (Fig. 2). These findings suggest that β1 integrin signaling to ZAP-70 does not play a major role in the effects of ZAP-70 on T cell migration in this system.

These results demonstrate a novel function for ZAP-70 in regulating T lymphocyte migration in response to the CXCR4 chemokine receptor ligand CXCL12. Although CXCR4 signaling resulted in ZAP-70-dependent tyrosine phosphorylation of SLP-76, we did not observe any effect of SLP-76 expression on the low level of migration of SLP-76-deficient Jurkat T cells. Thus, our results suggest that ZAP-70-dependent regulation of T cell migration in response to CXCL12 does not involve ZAP-70-dependent tyrosine phosphorylation of SLP-76. This was somewhat surprising, given reports that SLP-76 may be involved in TCR-mediated modulation of the actin cytoskeleton (25). Previous studies have suggested a role for PI 3-K, the GTPase cdc42, and NO in regulating CXCR4-mediated cell migration (6, 8, 12, 26, 27). The relationship between ZAP-70 and these other signaling proteins in coordinating the biochemical events required for T cell migration remains unclear. However, it is interesting to note that B cell receptor-activated cytoskeleton of PI 3-K requires Syk tyrosine kinase activity (28) and that PI 3-K has been implicated in the regulation of various GTPases (29). Thus, ZAP-70 might mediate CXCR4 signaling to PI 3-K and other downstream effectors. Although CXCR4 ligation also activates the mitogen-activated protein kinase pathway (6), mitogen-activated protein/extracellular signal-related kinase kinase inhibitors do not block CXCL12-dependent migration (8, 26).

Although it is currently unclear whether other chemokine receptors might also regulate lymphocyte migration via ZAP-70, several aspects of CXCR4 chemokine receptor signaling suggest the possibility of unique components to biochemical signaling initiated by CXCR4. First, in contrast to other chemokine receptors, CXCR4 is capable of initiating prolonged activation of protein kinase B and extracellular signal-related kinase 2 (10). Second, costimulatory effects of CXCL12 on T cell activation have been noted (30). Thus, ZAP-70 may represent an important point of convergence between chemokine receptors and the TCR that may possibly be involved in mediating the costimulatory effects of CXCL12 on T cell activation (30). It will be important in future studies to determine the role of ZAP-70 in regulating these other responses of T cells to CXCR4 stimulation.

Acknowledgments We thank Drs. R. Abraham, B. Irvin, G. A. Koretzky, and A. Weiss for generously providing cell lines and reagents, and Drs. J. McCarthy and R. Robinson-Lawler for helpful comments and discussion.

References


