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TCR Activation Inhibits Chemotaxis Toward Stromal Cell-Derived Factor-1: Evidence for Reciprocal Regulation Between CXCR4 and the TCR

James W. Peacock* and Frank R. Jirik2*†

Stromal cell-derived factor-1 (SDF-1), a C-X-C family chemokine, is a potent T lymphocyte chemoattractant. We investigated the effects of T cell activation on the chemotactic response to SDF-1. Anti-CD3 Ab stimulation of either Jurkat T cells or murine peripheral CD4+ T lymphocytes produced a dramatic inhibition of SDF-1-induced chemotaxis. In contrast, the SDF-1 responses of Jurkat clones with deficiencies in key TCR signaling components (Lck, CD45, and TCR-β), were only marginally reduced by anti-CD3 stimulation. Similar to PMA treatment, which abolished both CXCR4 receptor expression and the chemotactic response of Jurkat cells to SDF-1, anti-CD3 Ab treatment reduced cell surface expression of CXCR4 to 65% of the control value, an effect that was blocked by protein kinase C inhibitors. Our data suggest that initial T cell activation events inhibit the response of Jurkat T cells to CXCR4 stimulation. In contrast, SDF-1 treatment resulted in a reduction of tyrrosine phosphorylation of the TCR downstream effectors, ZAP-70, SLP-76, and LAT (linker for activation of T cells), suggesting that this chemokine potentially regulates the threshold for T cell activation. The Journal of Immunology, 1999, 162: 215–223.

The trafficking of lymphocytes to sites of inflammation is thought to be mediated by the local production of chemotactic cytokines (1). In vitro, T lymphocytes migrate in response to concentration gradients of both α (or C-X-C) chemokines (IL-8, IFN-γ-inducible protein-10, and stromal cell-derived factor-1 (SDF-1α)) (2–7) and β (or C-C) chemokines (RANTES, macrophage inhibitory protein (MIP-1α), and MIP-1β) (8, 9). Although stimulation of T cells with anti-CD3 Ab enhances cell migration toward MIP-1α, MIP-1β (9), and IFN-γ-inducible protein-10 (3), anti-CD3 Ab treatment of T cells can also inhibit chemotactic responses. For example, stimulation of CD45RO+ memory T cells induced a rapid down-regulation of CCR1 and CCR2 receptor expression, associated with an inhibition of cell migration in response to RANTES or monocyte chemotactic protein-1 (10, 11). Also, vasoactive intestinal peptide, a T lymphocyte chemoattractant, induced migration of unstimulated T lymphocytes, and the response to vasoactive intestinal peptide was decreased by anti-CD3 activation (12). Pretreatment with MIP-1α, was shown to inhibit the anti-CD3 Ab-induced proliferation of murine splenic T lymphocytes (13) Therefore, the response to a specific chemokine appears to be determined by both the differentiation and the activation state of the responding T cell population.

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Abbreviations used in this paper: SDF-1, stromal cell-derived factor-1; MIP-1α, macrophage inhibitory protein-1α; LAT, linker for activation of T cells; MAPK, mitogen-activated protein kinase; R-PE, R-phycocerythrin; PKC, protein kinase C; PLCβ3, phospholipase Cβ3.

SDF-1 mRNA is expressed constitutively in a wide variety of tissues (14, 15), and this chemokine has been identified as a lymphocyte chemoattractant (16). In keeping with this activity, SDF-1 induces actin polymerization in lymphocytes, a process thought to be a prerequisite for cell motility (16). CXCR4, a seven-transmembrane domain, G protein-coupled receptor predominantly expressed on the unactivated naive subset of T cells (17) is the only known receptor for SDF-1 (16, 18) and has also been shown to serve as an accessory factor for cell entry by T cell-tropic HIV isolates (19–22). Interestingly, there is evidence that the HIV-1 envelope protein, gp120, makes contact with both the CD4 molecule and the CXCR4 receptor (23), and soluble gp120 has been shown to alter anti-CD3-driven proliferation of CD4+ T cells (24, 25). These results suggest that TCR and CXCR4 signaling may be regulated by T cell contact with gp120, and that this association potentially regulates the responses of these cells to SDF-1.

To gain a greater understanding of the biological responses of T cells to SDF-1, we investigated the effect of SDF-1 pretreatment on T cell activation as well as the effect of TCR activation on SDF-1-induced cellular migration. We demonstrate that anti-CD3 Ab stimulation markedly inhibits the migration of T cells toward SDF-1. This inhibitory effect is much weaker in cell lines lacking essential components of the TCR signaling complex, namely, Lck, CD45, and the TCR β-chain. TCR activation also led to a down-regulation of CXCR4 surface expression. The preincubation of T cells with SDF-1 reduced the anti-CD3-stimulated phosphorylation of critical downstream effectors of TCR signaling, including ZAP-70, SLP-76, and pp36, providing evidence for cross-talk between CXCR4 and the TCR.

Materials and Methods

Chemokines and Abs and chemicals

SDF-1 (residues 1–67), generated using an ABI peptide synthesizer (Applied Biosystems, Foster City, CA), was provided by I. Clark-Lewis (16, 18). The following Abs were used: OKT3 (American Type Culture Collection, Manassas, VA), the ascites containing the C305 monoclonal with specificity against the Jurkat T β-chain (a gift from A. Weiss), 2C11 purified hamster anti-mouse CD3ε mAb (PharMingen Canada, Mississauga, Canada), anti-phosphotyrosine monoclonal 4G10 and anti-LAT
TCR ACTIVATION INHIBITS SDF-1-MEDIATED CHEMOTAXIS

Assessment of cellular phosphotyrosine content

Cells were washed in PBS and pelleted by centrifugation. Cells (1.0 × 10^7/100 μl) were incubated in serum-free RPMI 1640 medium containing 20 mM HEPES (pH 7.2) for 30 min (at 5% CO2) to restore basal levels of protein phosphorylation. Cells were treated with SDF-1 at 50 μg/ml as indicated. In addition, cells were stimulated with Abs at a 1/100 dilution of ascites (C305) or 10 μg/ml OKT3. At the indicated times, stimulated cells were rapidly lysed in 1.0% Nonidet P-40 lysis buffer and flash-frozen in liquid nitrogen.

Immunoprecipitation and immunoblotting

Cells (1.0 × 10^7/100 μl) were lysed in Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris (pH 7.5), and 10% glycerol) in the presence of protease inhibitors (100 μg/ml PMSF, 1.0 μg/ml aprotinin, 0.7 μg/ml pepstatin, 0.5 μg/ml leupeptin, 40 μg/ml bestatin, 10 μg/ml soybean trypsin inhibitor (Boehringer Mannheim, Indianapolis, IN), 10 mM sodium fluoride, 1 mM sodium vanadate, and 1 mM sodium molybdate (BDH, Toronto, Canada)). After 30 min on ice, lysates were centrifuged for 30 min at 13,000 rpm, then precleared with 30 μg of protein A-Sepharose 4 Fast Flow, (Pharmacia Canada) for 1 h. Lysates were then centrifuged at 8000 rpm for 1 min. Total protein (300–500 μg) was incubated with 1.0 μg/ml of the indicated immunoprecipitating Ab for 2 h. Immunoprecipitates were collected by centrifugation as described above and were washed three times in 1% Nonidet P-40 buffer containing PMSF and sodium vanadate. Washed immunoprecipitates were resuspended in 1x diluted Laemmli buffer (4% SDS, 5.7 M 2-ME, 20% glycerol, and 20% bromophenol blue). Alternatively, lystate volume corresponding to 15 μg of total protein was diluted 3/1 with Laemmli sample buffer. Samples were boiled for 10 min before electrophoresis. Immune complexes or total protein lysates were separated by SDS-PAGE at 150 V and transferred to nitrocellulose paper by electroblotting at 100 V for 1 h (stirred) at 4.0°C in a solution containing 192 mM glycine, 25 mM Tris, and 20% methanol. Filters were incubated overnight in TBST (10 mM Tris (pH 8.0), 150 mM NaCl, and 0.05% Tween-20) containing 5% BSA. Filters were subsequently incubated for 60 min at room temperature in TBST containing 1.0% BSA supplemented with primary Ab at a 1/10,000 (4G10), 1/250 (anti-ZAP-70), 1/500 (SLP-76), or 1/1000 (anti-LAT) dilution. After three additional washes in TBST, filters were incubated for 1 h in the appropriate horseradish peroxidase-conjugated secondary Ab and washed again as described, and proteins were detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL) and exposure to Eastman Kodak Biomax MR film (Eastman Kodak, Rochester, NY).

Densitometry

Densitometry was performed using a GS 300 densitometer (Hoefer Scientific Instruments, San Francisco, CA), and results were analyzed using the GS370 1-D Data System, version 2.0 for Macintosh.

Statistical methods

We applied Student’s t test (Microsoft Excel) when analyzing the results. p < 0.05 was considered significant.

Results

OKT3 stimulation of Jurkat cells blocks SDF-1-induced chemotaxis and requires an intact TCR signaling mechanism

To assess the effect of T cell activation on migration in response to SDF-1, chemotaxis assays were performed on Jurkat T cell leukemia cells and a Jurkat clone (JE6.1) that were competent for TCR signaling and on Jurkat mutant clones (J.CaM1.6, J45.01, and J.RT3.T3.5) that were defective in TCR signaling. Conditions for chemotaxis in the transwell system were established by resuspending cells (5 × 10^5) in serum-free HEPES (20 mM)-buffered RPMI 1640 medium and incubating them in transwells containing SDF-1. The maximum transmigration response to synthetic SDF-1 (residues 1–67) was observed at a concentration of 1.5 μg/ml (187.5 nM) and was essentially complete within 3 h, as established by prior dose-response and time-course experiments (data not shown). As previously shown (16), we also observed a bell-curve chemotactic response with SDF-1 (0.5–3.0 μg/ml). In addition, Jurkat cells demonstrated a chemotactic, as opposed to chemokinetic, response to SDF-1. Maximal transmigration was retained when 0.15...
μg/ml SDF-1 was introduced to the upper well with a concentration of 1.5 μg/ml in the bottom well.

Table I shows the migratory responses of Jurkat cells (Jurkat, JE6.1) and Jurkat mutant clones (J.CaM1.6, J45.01, and J.RT3.T3.5) either treated with OKT3 (10 μg/ml) or untreated. The number of Jurkat and JE6.1 cells that migrated after treatment with OKT3 was greatly reduced compared with that of untreated controls. Thus, incubation of Jurkat or JE6.1 cells with OKT3 before addition of cells to the upper chamber reduced transmigration to 63% (J.CaM1.6), 64% (J45.01), and 78% (J.RT3.T3.5) of that of untreated cells (Table I). Although the lack of OKT3-mediated inhibition was much less apparent in the Lck−, CD45−, and TCR β-chain− clones (Table I), the decreased response to SDF-1 was most evident in clones expressing the highest levels of CD3. This inhibition was much less apparent in the Lck−, CD45−, and TCR β-chain− clones (Table I). Although the lack of OKT3-mediated inhibition in these clones may in part have been due to lower CD3 levels, it most likely reflected the loss of key signaling molecules, such as Lck and CD45. In summary, the data demonstrate that the SDF-1-induced chemotactic response of Jurkat cells with an intact TCR signaling complex was inhibited (5- to 10-fold) compared with that of control cells, and that cells deficient in TCR activation were considerably less sensitive to OKT3-mediated inhibition (1- to 1.6-fold).

The contribution of spontaneous migration of control and OKT3-treated Jurkat or JE6.1 cells represented <10% of the response to SDF-1 (Table I). Interestingly, OKT3 treatment reduced the level of spontaneous transmigration occurring in the absence of an SDF-1 gradient, consistent with OKT3 treatment exerting an inhibitory effect on T cell motility. Background transmigration of clones with limited capacity for TCR signaling, as expected, was not significantly reduced by OKT3 treatment. Differences in transmigration might simply be due to variation in CXCR4 levels, however, as shown in Fig. 1, similar levels of CXCR4 were detected in all clones. We also examined cell surface expression of CD3 by flow cytometry. Whereas Jurkat and JE6.1 expressed similar levels of CD3 on the cell surface, CD3 expression of J.CaM1.6 and J45.01 was three- to eightfold lower, and the TCR-defective clone J.RT3.T3.5 showed only minimal staining (Fig. 1). Although the transmigration response of all clones was inhibited to varying degrees by OKT3 treatment, the decreased response to SDF-1 was most evident in clones expressing the highest levels of CD3. This inhibition was much less apparent in the Lck−, CD45−, and TCR β-chain− clones (Table I). Although the lack of OKT3-mediated inhibition in these clones may in part have been due to lower CD3 levels, it most likely reflected the loss of key signaling molecules, such as Lck and CD45. In summary, the data demonstrate that the inhibition of SDF-1 activity by anti-CD3 Ab treatment requires a functional TCR signaling complex.

Anti-CD3 stimulation of mouse peripheral T cells blocks SDF-1-induced chemotaxis

To establish whether anti-CD3 stimulation would inhibit the migration of normal cells toward SDF-1, the response of murine splenocytes and CD4+ cells was evaluated. As shown in Fig. 2, the
SDF-1-induced migratory responses of both unfractionated splenocytes and purified CD4+ T lymphocytes were inhibited by treatment of the cells with the anti-CD3 Ab, 2C11. The response of unfractionated splenocytes pretreated with anti-CD3 was 15% of the migration of cells in response to SDF-1 alone (p < 0.00005), and the migration of anti-CD3-treated CD4+ cells was 30% that of untreated controls (p < 0.00003; Fig. 2). These results indicate that anti-CD3-mediated TCR activation of normal murine T cells inhibits transmigration in response to SDF-1.

**PMA blocks chemotaxis of Jurkat cells and down-regulates expression of CXCR4**

Phorbol esters are known to mimic many of the effects of CD3 stimulation of the TCR complex, involving the activation of protein kinase C (PKC) (32, 33). To investigate whether PKC activation would mimic the OKT3-mediated inhibition of chemotaxis to SDF-1, we stimulated JE6.1 cells with PMA (100 ng/ml) and measured cell transmigration as described above. Treatment with PMA inhibited SDF-1-induced transmigration >300-fold compared with that in untreated controls, whereas we observed a 13-fold inhibition of transmigration in OKT3-treated cells. In addition, the level of spontaneous migration of JE6.1 cells following PMA treatment was reduced 30-fold compared with 5.6-fold in OKT3-treated cells (data not shown). These data demonstrated that OKT3-mediated inhibition of T cell migration could be simulated by PMA treatment. However, PMA was a much more effective inhibitor of chemotaxis.

We next investigated whether PMA stimulation would down-regulate CXCR4 surface receptor expression in Jurkat cells, perhaps accounting for the observed inhibition of chemotaxis. Jurkat cells were treated with 100 ng/ml PMA and subsequently stained at various points over a 24-h time course with anti-CXCR4-PE-conjugated Ab. We observed a marked down-regulation of CXCR4 surface staining within 4 h of PMA treatment. Cell surface expression was reduced to 22 and 14% of the control value after 1 and 2 h of incubation, respectively. After 4 h of PMA treatment, most (94%) CXCR4 cell surface staining was abolished (Fig. 3 and data not shown). Our observations agree with those of Signoret et al., who reported similar results with phorbol ester–treated SupT1 T cells (34).

**Treatment of Jurkat cells with OKT3 down-regulates CXCR4 surface expression**

One potential mechanism that could account for the decreased migration of OKT3-activated Jurkat cells would be the down-modulation of CXCR4. We thus treated JE6.1 cells with OKT3 (10 μg/ml) and assessed cell surface staining of CXCR4. A representative experiment is shown in Fig. 4. Total fluorescence staining of CXCR4 was reduced to 65% of the control value by 1 h (p < 0.0002), with no further change observed over a 2-h time course (data not shown). Our data demonstrate an OKT3-induced down-regulation of CXCR4.

**OKT3-mediated down-regulation of CXCR4 requires PKC**

To establish whether the decreased cell surface expression of CXCR4 in OKT3-treated cells was dependent on PKC activation, we pretreated JE6.1 cells with 0.50 μM staurosporine or 1.0 μM RO 31-8220 (a staurosporine analogue) to block PKC activation (35). Both agents were able to inhibit CXCR4 down-regulation induced by OKT3 (p > 0.10), indicating that the effect of this Ab was at least in part a consequence of PKC activation (Fig. 4 and data not shown). Interestingly, an increase in cell surface expression of CXCR4 was seen in JE6.1 cells treated with RO 31-8220, with peak fluorescence increasing twofold (Fig. 4), suggesting that PKC may regulate basal levels of CXCR4 in Jurkat cells. Staurosporine, a nonspecific kinase inhibitor, failed to modulate basal CXCR4 surface staining (data not shown), indicating that the two agents probably inhibit overlapping, but nonidentical, molecules. In summary, our results strongly suggest that the OKT3-induced down-regulation of CXCR4 is in part mediated via PKC activation.
SDF-1 inhibits the OKT3-stimulated phosphorylation of TCR signaling molecules ZAP-70, SLP-76, and pp36

We demonstrated that OKT3 treatment blocked the chemotactic response of Jurkat cells. It was therefore of interest to determine whether SDF-1 was capable of regulating TCR signaling. Thus, we examined early TCR signaling events triggered by treatment with OKT3 or C305 in the presence or the absence of SDF-1. Fig. 5 shows the effects of SDF-1 treatment on Jurkat cells stimulated in combination with C305 Ab over 15–120 s. Results obtained in separate experiments using OKT3 stimulation were identical with those using C305 (data not shown). Cells were lysed, processed, and blotted with anti- phosphotyrosine as described above. Interestingly, in the presence of SDF-1, tyrosine phosphorylation of a 70-kDa protein was attenuated at time points up to 30 s. Even at 1–2 min, when phosphorylation due to C305 stimulation was maximal, the level of tyrosine phosphorylation of the pp70 band was reduced compared to that of control C305-stimulated cells (Fig. 5a). We did not detect any significant change in C305-induced p70 phosphorylation when SDF-1 preincubations of 15 and 30 min were compared (compare Fig. 5, b and d).

A prominent phosphoprotein of 36 kDa was also observed to be markedly underphosphorylated in C305-stimulated cells preincubated with SDF-1 compared with that in cells stimulated with C305 alone. In the absence of SDF-1, there was a time-dependent increase in tyrosine phosphorylation of the 36-kDa protein, which reached a maximum at 60 s poststimulation. In a parallel time course, this protein remained at least twofold underphosphorylated as described above. Interestingly, in the presence of SDF-1, tyrosine phosphorylation of the 36-kDa protein was attenuated at time points up to 30 s. Even at 1–2 min, when phosphorylation due to C305 stimulation was maximal, the level of tyrosine phosphorylation of the pp36 band was reduced compared to that of control C305-stimulated cells (Fig. 5a). We did not detect any significant change in C305-induced pp36 phosphorylation when SDF-1 preincubations of 15 and 30 min were compared (compare Fig. 5, b and d).

There was a concomitant increase in the tyrosine phosphorylation of the MAP kinases ERK-1 and ERK-2, which reached a maximum at 120 s, as demonstrated by reprobing the blot in Fig. 5b with a phospho-specific MAPK Ab (Fig. 5c). Preincubation of these cells with SDF-1 resulted in immediate phosphorylation of p42 and p44 MAP kinases at time points as early as 15 s, which remained constant for up to 1 min and further increased at 2 min. As the 36-kDa protein remained underphosphorylated at a time when MAPK phosphorylation was increasing, it suggested that the latter was primarily the result of SDF-1-stimulated CXCR4 signaling (compare Fig. 5, b to c). Thus, SDF-1 exposure attenuated the C305- and OKT3-induced tyrosine phosphorylation of proteins of 70 and 36 kDa, which appeared to represent downstream targets of TCR activation. These results were consistent with the temporal kinetics of C305-mediated activation of ZAP-70 and pp36 (38).

SLP-76, which is phosphorylated on tyrosine as a result of TCR activation, appears to be a substrate of ZAP-70 (37). Equal amounts of total protein were immunoprecipitated with 4G10 Ab, resolved by SDS-PAGE, and blotted with either ZAP-70 or SLP-76 Abs. Fig. 6, a and b, shows a temporally dependent inhibition of ZAP-70 and SLP-76 tyrosine phosphorylation as a result of SDF-1 treatment. Densitometric analysis was performed on the autoradiograph represented in Fig. 6, a and b. The results represent the ratio of the peak band intensities of SLP-76 and ZAP-70 (normalized to the peak band intensity of the lower band shown in Fig. 6, a and b). There was up to a 50% reduction in the SLP-76 phosphotyrosine signal by 60 s. While there was no significant difference in ZAP-70 at early time points, there was a 30% reduction in phosphotyrosine signal by 60 s following stimulation with anti-CD3 (data not shown). These observations suggest that the 4G10-detectable phosphotyrosine levels of a number of different TCR signaling molecules were reduced by SDF-1 treatment. To further characterize the 36-kDa protein that we observed to be underphosphorylated in C305-stimulated Jurkat cells treated with SDF-1, total protein lysates from the 2 min point in Fig. 5d were blotted with anti-LAT Ab (Fig. 7b), then stripped and reprobed with anti-phosphotyrosine Ab (4G10; Fig. 7a). As shown in Fig. 7 there was a significant reduction (60% of the control value) in the phosphotyrosine content of the 36-kDa protein in SDF-1-treated cells. The attenuation of pp36 phosphorylation was much greater than that of ZAP-70 and SLP-76, suggesting that the effects of SDF-1 on TCR signaling have a greater impact on events downstream of ZAP-70 and SLP-76 activation.

To determine whether the 70-kDa protein observed to be underphosphorylated as a result of SDF-1 treatment was ZAP-70, equal amounts of total protein (Fig. 8, lanes 1 and 2) from precleared lysates were incubated (lanes 3 and 4) with polyclonal anti-ZAP-70 Ab (Fig. 8). Total lysates or immunoprecipitations were resolved by SDS-PAGE followed by immunoblotting with 4G10 Ab (Fig. 8, upper panel). The filter was then stripped and reprobed with rabbit polyclonal ZAP-70 Ab (Fig. 8, lower panel). Our results suggest that the pp70 protein that is underphosphorylated as a result of SDF-1 treatment is ZAP-70.
were stimulated in serum-free RPMI containing 20 mM HEPES with 10 nM OKT3 in SDF-1-treated cells, total lysates were immunoprecipitated with anti-LAT Ab (4G10) and resolved by SDS-PAGE, and filters were probed with anti-ZAP-70 and SLP-76 kinase.

The 36-kDa protein that was underphosphorylated in C305-stimulated Jurkat cells following SDF-1 pretreatment.

Given that p70 phosphorylation was reduced in OKT3-treated Jurkat cells, we investigated whether stimulation with SDF-1 alone would have an effect on the basal phosphorylation level of p70. SDF-1-treated and untreated Jurkat cells were thus compared in a time-course experiment (Fig. 9a, b, and c). Interestingly, SDF-1 treatment was associated with a progressive loss in the phosphotyrosine content of p70 in a time-dependent manner in the absence of TCR activation (Fig. 9a). In contrast, in the absence of SDF-1, p70 phosphotyrosine content remained unchanged (Fig. 9b).

Discussion

Stimulation of lymphocytes with SDF-1 or PMA results in mobilization of calcium (33, 39, 40), internalization, and subsequent desensitization of the CXCR4 receptor (7, 16, 18, 39, 41). PMA

FIGURE 5. Intracellular protein tyrosine phosphorylation in Jurkat cells in response to treatment with SDF-1 and C305. a, Jurkat cells (1.0 × 10⁶) were stimulated in serum-free RPMI containing 20 mM HEPES with 10 µg/ml C305 (left panel) or were costimulated with 50 µg/ml SDF-1 and 10 µg/ml C305 (right panel) for the indicated times (15–120 s) before lysis. b and d, Cells were preincubated in serum-free medium (control panel) or in medium containing 50 µg/ml SDF-1 for either 15 min (b) or 30 min (d), then stimulated with C305 for the indicated times. Equal concentrations of total protein from each sample were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine Ab (4G10; a, b, and d). Arrows indicate phosphoproteins of 70 and 36 kDa, respectively. Molecular mass markers are indicated on the left. c, The blot indicated in b was stripped and reprobed with anti-LAT Ab. Arrows indicate ERK-1 and ERK-2.

FIGURE 6. Effect of SDF-1 treatment on the tyrosine phosphorylation of ZAP-70 and SLP-76 in C305-stimulated Jurkat cells. As indicated in b, c, and d, cells that were untreated or were preincubated with SDF-1 were stimulated with C305 for 15–60 s. Total lysates were immunoprecipitated with 4G10 Ab and resolved by SDS-PAGE, and filters were probed with anti-ZAP-70 and SLP-76 kinase. By densitometry the ratios of the peak band intensity of ZAP-70 (normalized to the lower band in a) of control and SDF-1-treated panels were 15 s, 1.0; 30 s, 1.0; and 60 s, 0.70. a, The blot was then stripped and reprobed with anti-SLP-76. By densitometry the ratios of the peak band intensity of SLP-76 (normalized to the lower band in b) of control and SDF-1-treated panels were: 15 s, 1.04; 30 s, 0.66; and 60 s, 0.55. b, Overlay of the immunoblots confirmed two distinct proteins of different mobilities. Arrows indicate phosphoproteins ZAP-70 and SLP-76, respectively.

FIGURE 7. The 36-kDa protein observed to be underphosphorylated as a result of SDF-1 treatment in C305-stimulated Jurkat cells is probably LAT. Cells were pretreated with SDF-1 (+) or with medium alone (−) for 30 min as in Fig. 5. a, Fifteen micrograms of total protein lysates (TL) of unstimulated (un) or C305-stimulated (120 s) Jurkat cells (remaining two lanes) were blotted with anti-phosphotyrosine (4G10). b, The blot in a was stripped and reprobed with anti-LAT Ab. Densitometry ratios of the peak band intensity of the anti-phosphotyrosine band (a) and the peak band intensity of the anti-LAT Ab (b) were: un, 0.061; +, 0.133; and −, 0.704. c, Lysates (88 µg), as described above, were immunoprecipitated (IP) with 4 µg of anti-LAT Ab and resolved in parallel with 15 µg of total lysates (TL). The filter was then incubated with anti-phosphotyrosine (4G10).
literature that demonstrates that SDF-1 induces CXCR4 coupling desensitization pathway (43). Although there is evidence in the activation, which, in turn, mediates the inhibition of CXCR4 ac-
lation we observed in OKT3-stimulated Jurkat cells accounted for that the relatively modest levels of CXCR4 receptor down-modu-
or, bisindolylmaleimide (45). The former inhibited responses ini-
phosphorylation by PKC may block activation by G
3 (PLC
3) (41). In support of the former, Signoret et al. have shown that PKC activation is not essential for SDF-1-induced CXCR4 down-modulation, indicating that SDF-1 and phorbol esters probably operate through different intracellular signaling pathways to induce their effects (34).

It is possible that the TCR activation of PKC leads to PLC
3 activation, which, in turn, mediates the inhibition of CXCR4 activity, an event leading to SDF-1 unresponsiveness. There appears to be at least two mechanisms that down-regulate signal transduction through CXCR4: one at the level of receptor phosphorylation and the other, independent of receptor phosphorylation, at the level of PKC-dependent phosphorylation of phospholipase C
3 (PLC
3) (41). In support of the former, Signoret et al. have shown that PKC activation is not essential for SDF-1-induced CXCR4 down-modulation, indicating that SDF-1 and phorbol esters probably operate through different intracellular signaling pathways to induce their effects (34).

FIGURE 8. Evidence that the 70-kDa protein that is underphosphorylated by SDF-1 treatment in C305-stimulated cells is ZAP-70. Cells were stimulated with C305 alone (−) or were costimulated with C305 and SDF-1 (+) for 60 s before lysis. Total protein lysates (lanes 1 and 2) and anti-ZAP-70 immunoprecipitations (lanes 3 and 4) were resolved by SDS-PAGE. The filter was probed with 4G10, stripped, and then reprobed with rabbit polyclonal anti-ZAP-70 Ab.

activation of PKC induces phosphorylation of CXCR4 (41), followed by receptor internalization (40–42). As PKC activation follows TCR activation (33), the TCR-dependent block of SDF-1-induced migration that we observed may have been a consequence of PKC activation, an event leading to SDF-1 unresponsiveness. There appears to be at least two mechanisms that down-regulate signal transduction through CXCR4: one at the level of receptor phosphorylation and the other, independent of receptor phosphorylation, at the level of PKC-dependent phosphorylation of phospholipase C
3 (PLC
3) (41). In support of the former, Signoret et al. have shown that PKC activation is not essential for SDF-1-induced CXCR4 down-modulation, indicating that SDF-1 and phorbol esters probably operate through different intracellular signaling pathways to induce their effects (34).

It is possible that the TCR activation of PKC leads to PLC
3 activation, which, in turn, mediates the inhibition of CXCR4 activity, an event that represents an agonist nonspecific heterologous desensitization pathway (43). Although there is evidence in the literature that demonstrates that SDF-1 induces CXCR4 coupling to both G
i (pertussis toxin-sensitive) (5, 39) and G
o (pertussis toxin-insensitive) (40, 42) family members of G proteins, it is possible that a given receptor uses more than one G protein subunit and may initiate several different signaling pathways (44). The G
o subunit can directly activate PLC
3 (45). On the contrary, PLC
3 phosphorylation by PKC may block activation by G
o (45). In addition, PLC
3 phosphorylation was blocked by the PKC inhibitor, bisindolylmaleimide (45). The former inhibited responses initiated by the G protein-coupled receptor for platelet-activating factor (45). Given that PKC inhibitors blocked the OKT3-mediated down-regulation of CXCR4, it is likely that the down-regulation of receptor expression subsequent to CD3-stimulation involved the activation of PKC (Fig. 4). PMA treatment ablated transmigration of Jurkat cells, an inhibition that correlated with the complete loss of CXCR4 cell surface expression (Fig. 3). It is unlikely, however, that the relatively modest levels of CXCR4 receptor down-modulation we observed in OKT3-stimulated Jurkat cells accounted for the striking inhibition of chemotaxis. It may be hypothesized that CD3-mediated activation of PKC leads to the phosphorylation PLC
3, which inhibits the activation of G
o (46) and thereby produces heterologous desensitization of CXCR4. Our results are in agreement with such a model and also provide evidence for crosstalk between the TCR complex and CXCR4. The response of T cells to TCR activation is probably complex, first involving a migration stop signal, followed by the down-regulation of CXCR4 activity and cell surface expression.

Another possibility is that the TCR and CXCR4 signaling pathways share the same G
o subunit. TCR signaling also mediates GTP exchange within G
o/11 and leads to its association with CD3ζ in Jurkat cells (46). G protein activation was tyrosine kinase dependent and mediated activation of PLC
3. Stanners et al. demonstrated a Ga/11-dependent modulation of CD3 ζ- and ε-chain phosphorylation and that a functionally deficient Ga/11 mutant was associated with diminished tyrosine phosphorylation of CD3 ζ and ε and of ZAP-70 upon anti-CD3 Ab stimulation (46). Thus, TCR-CD3 activation could sequester G
o from CXCR4, thereby preventing SDF-1-induced effects. SDF-1, on the other hand, might produce a rapid up-regulation of CXCR4 activity (40), leading to G
o recruitment. Such a mechanism agrees with a model proposed by Dustin (47) suggesting that the TCR-induced stop signal would be dominant in resting T cells when CXCR4 levels are low, but the signal from SDF-1 may gain dominance once CXCR4 levels on the T cell blasts are up-regulated. Moreover, a further consequence of a shift in G
o usage induced by SDF-1 might be the inhibition of signaling molecules, such as ZAP-70 and pp36, that lie downstream of the TCR.

Whereas we observed an inhibition of SDF-1-induced chemotaxis and a reduction in CXCR4 cell surface expression during initial activation of lymphocytes, in Jurkat cells others have reported that CXCR4 receptor levels and chemotaxis were up-regulated after either PHA stimulation or IL-2 priming, reaching a peak at 3–6 days. These effects were associated with an increased chemotactic response (17). Moreover, Hesselgesser et al. (39) observed a marginal increase in the chemotactic response of anti-CD3-stimulated Jurkat cells. The discrepancy between these observations and those reported herein probably reflect differences in the TCR stimulation protocols. Thus, treatment of cells for 3–6 h with anti-CD3 Abs before the chemotaxis assay would probably lead to the activation of a number of genes, including those encoding cytokines (32), that might modulate the response to SDF-1. Our experiments, in contrast, examined the effect of initial T cell activation events on the migratory response to SDF-1.

To establish whether the inhibition of chemotaxis seen in C305-stimulated Jurkat cells would also be a feature of normal T cells, we assessed the migratory response of SDF-1-treated C305-stimulated, unfractionated murine splenocytes or CD4+ -enriched T cells. As with Jurkat cells, the SDF-1-induced migratory response of normal T cells was also inhibited by anti-CD3 treatment. Interestingly, there was some variation in the response of
unfractionated splenocytes compared with purified CD4+ T cells (Fig. 2), suggesting either that a non-CD4+ T cell population made up a large fraction of the migratory population in the unfractionated splenocytes or perhaps that the CD4 selection procedure altered the chemotactic response of the purified T cells. In any event, our data clearly demonstrate that the block of cell migration in response to T cell activation is probably a mechanism common to both human and murine species.

There was no significant difference in the cell surface expression of CXCR4 among the various Jurkat clones used in our study (Fig. 1). There was, however, some variability in SDF-1-induced transmigration among these clones (Table I). While the reason(s) for this is probably complex, the finding of reduced chemotaxis in the lines deficient in Lck and CD45 raises the possibility that these molecules play some facilitatory role in either SDF-1 signaling or cell motility. Taken together with the inhibitory effect of T cell activation on SDF-1-induced chemotaxis, these observations raise the possibility that reciprocal regulation of TCR activation by SDF-1 might also occur. We therefore examined the effect of SDF-1 treatment on the signaling events subsequent to TCR activation. These events have been well characterized (for review, see Refs. 48 and 49). Thus, engagement of the TCR results in the activation of Lck/Fyn, members of the Src family of protein tyrosine kinases. Phosphorylation of TCR-ζ leads to the recruitment, phosphorylation, and activation of ZAP-70 protein tyrosine kinase (50–52) and subsequent phosphorylation of other key molecules, such as SLP-76 (26, 37) and LAT (38). Events downstream and parallel with the activation of SLP-76 and LAT eventually culminate in T cell activation, proliferation, and cytokine production (49, 53, 54). The uncoupling of this cascade at initial phosphorylation events would be predicted to have a great impact on the magnitude of the downstream responses. For example, a reduction in ZAP-70 activity could greatly alter the character of the activation response. In keeping with this hypothesis, we demonstrate a reduction in the phosphotyrosine content of ZAP-70 and SLP-76 following SDF-1 treatment. This effect is, in turn, associated with a pronounced inhibition of pp36 phosphorylation. The 36- to 38-kDa protein LAT was shown to be a substrate for ZAP-70 (38). Moreover, pp36–38 has been shown to associate with Grb2 and PLCγ1 in C305-stimulated Jurkat cells (38). Our results further demonstrate that the 36-kDa protein that is underphosphorylated in Jurkat cells treated with SDF-1 represents LAT, a molecule that appears to be critical to T cell activation (38). Our results suggest that SDF-1 is capable of inhibiting critical components of the TCR signaling cascade. The precise level at which SDF-1 exerts its inhibitory effect on TCR signaling remains to be determined. Interestingly, the naive, CD26low, CD45RA+, CD45RO− T cells express high levels of CXCR4 protein and mRNA (17, 55). Given the ability of SDF-1 to suppress phosphorylation of ZAP-70, pp36, and SLP-76, there is a possibility that this chemokine may regulate the activation of naive cells in vivo.

In addition to having a role in lymphocyte chemotaxis, CXCR4 is a coreceptor for T cell tropic HIV-1 strains (56). Although CXCR4 signaling does not appear to be required for viral entry into the cell (57), it is not clear whether gp120 binding induces CXCR4 signaling. HIV-1 gp120 is known to contact both CD4 and CXCR4, probably forming a complex (23), which, in turn, may modulate both T cell activation (58) and migration (59). Several studies have indicated that gp120 is able to inhibit TCR signaling, an effect that is accompanied by a reduction in downstream and subsequent phosphorylation events (25, 58, 60, 61). Moreover, T cells obtained from early asymptomatic and symptomatic/AIDS patients demonstrated a reduction in the tyrosine kinase activity of Lck, Fyn, and ZAP-70 associated with a decrease in ζ-chain phosphorylation (62). In view of our results suggesting that CXCR4 signaling may regulate TCR signaling, it would be important to establish whether the effects of gp120 on T cells might be due at least in part to binding of this molecule to CXCR4.

In conclusion, we have demonstrated in Jurkat and normal murine T cells that anti-CD3 stimulation of the TCR complex inhibits SDF-1 function. Jurkat clones deficient in TCR signaling were much less sensitive to the effects of anti-CD3 treatment, confirming the role of TCR components in this process. Anti-CD3 Ab stimulation of Jurkat cells also reduced CXCR4 cell surface expression, a process that was inhibited by staurosporine and RO 31-8220. These results suggest that TCR complex-mediated activation of PKC is involved in CXCR4 receptor down-modulation. We have also shown that SDF-1 treatment of C305-stimulated cells is associated with reduced phosphorylation of critical TCR downstream effectors, ZAP-70, SLP-76, and pp36. Taken together, these observations are suggestive of reciprocal regulation between TCR and CXCR4. The latter finding suggests that the SDF-1 chemokine is potentially capable of regulating the threshold of T cell activation.

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References

stromal cell-derived factor 1 receptor, a murine homolog of the human immu


