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Adhesion Regulation of Stromal Cell-derived Factor-1 Activation of ERK in Lymphocytes by Phosphatases*

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We have investigated whether chemokine signaling to the extracellular-signal-regulated kinase (ERK) was regulated by β_1-integrin-mediated adhesion in B- and T-cell lines. Activation of ERK by the chemokine SDF-1 can be regulated by adhesion to β_1-integrin substrates in the T-cell lines MOLT-3, Jurkat, and H9 and in the Daudi B-cell line. In Jurkat T-cells, adhesion to the immobilized $\alpha_4\beta_1$-integrin ligand VCAM-1 or to the $\alpha_5\beta_1$-integrin ligand fibronectin regulated stromal-cell derived factor-1 (SDF-1) activation of ERK. Adhesion control of SDF-1 signaling was a rapid event, occurring as early as 10 min after adhesion, and loss of signaling occurred within 10 min of deadhesion. In contrast, SDF-1 activation of the ERK kinase MEK was independent of adhesion. Partial restoration of signaling to ERK in suspension was accomplished by pretreatment with pharmacological inhibitors of serine/threonine or protein-tyrosine phosphatases. In addition, we used a nonradioactive phosphatase assay using phosphorylated ERK as the substrate to determine relative ERK dephosphorylation in whole cell extracts. These results showed greater relative ERK dephosphorylation in extracts from Jurkat cells treated in suspension, as compared with adherent cells. Therefore, these data suggest that adhesion influences SDF-1 activation of ERK by regulating the activity of ERK phosphatases. This identifies a novel locus of adhesion regulation of the ERK cascade.

It is clear that adhesion to extracellular matrix components via β_1-integrins regulates a variety of cellular responses such as survival, proliferation, growth, and development. Our laboratory and others have shown that integrin-mediated adhesion can induce intracellular signaling cascades (1–3). Adhesion via integrins can also regulate signals generated from other surface receptors, such as growth factor receptor signaling to the mitogen-activated protein kinase (MAPK)[1] cascade (4, 5). More recently, activation of MAPK cascades via G protein-coupled receptors has also been shown to be regulated by β_1-integrin-mediated adhesion (6).

Most of the research on adhesion regulation of signaling to date has been performed using cell lines that are normally stably adherent. Although the work done in these systems has been valuable, depriving these cells of anchorage is rather nonphysiological. By contrast, immune system cells, such as lymphocytes, normally traffic between a nonadherent state in the blood and an adherent state in tissues. Thus, if cell adhesion modulates signaling in these cells, it could be considered to be a normal physiological event. Both $\alpha_5\beta_1$- and $\alpha_4\beta_1$-integrins are adhesion molecules involved in lymphocyte development and function (7, 8). T and B lymphocytes are key players in immunity, and their proper function is required for host defense against infection. Dysregulation of this cell type can play a role in many diseases such as lymphomas, leukemias, AIDS, and autoimmune disorders. Thus, the role of cell adhesion in regulating lymphocyte signaling has important implications for the understanding of both normal immune function and immune-related diseases.

One important molecule involved in eliciting signaling events in lymphocytes is the chemokine stromal-cell derived factor-1 (SDF-1, PBSF, CXCL12). Chemokines are small cytokine-like molecules that can elicit chemotactic responses involved in inflammation, immune cell development, and homing to secondary immune organs (9). SDF-1 was originally identified as a factor secreted by stromal cells that supports the proliferation and development of B lymphocytes (10). This chemokine elicits its effect by binding to a $G\alpha_i$ protein-coupled receptor, CXCR-4, which is expressed on B and T lymphocytes and many other cell types. CXCR-4 has also been shown to function as a co-receptor for human immunodeficiency virus infection and has recently been implicated in metastasis of several types of cancer (11–13). Signaling through CXCR-4 can affect T and B lymphocyte development, survival, and chemotactic responses (14–17). However, little is known as to whether lymphocytes respond differently to SDF-1 while in suspension as compared with adhered to other cells or extracellular matrix. The experiments presented herein will show that in B- and T-cell lines, adhesion to β_1-integrin substrates results in a dramatic increase in activation of ERK, but interestingly MEK phosphorylation can occur independent of adhesion. The ERK MAPK can promote proliferation, growth, and survival. Thus, understanding the mechanism of regulation of this kinase could have important biological implications.

In the ERK MAPK signaling cascade, in general, phosphorylation of proteins by kinases leads to activation, whereas dephosphorylation by phosphatases results in inactivation. Phosphatase regulation of signaling cascades is a relatively new area of investigation as compared with the study of kinases. Three major types of phosphatases are involved in regulating signaling cascades. Protein-tyrosine phosphatases

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; SDF-1, stromal-cell derived factor-1; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; PTP, protein-tyrosine phosphatase; PP, protein phosphatase; DSP, dual specificity phosphatase; FBS, fetal bovine serum; Fn, fibronectin; PBS, phosphate-buffered saline; RIPA, radioimmunoprecipitation assay.

(PTP) remove phosphates from tyrosine residues, protein phosphatases (PP) dephosphorylate serine and threonine residues, and dual specificity phosphatases (DSP) dephosphorylate tyrosine, threonine, and serine residues (18–20). DSPs are classified as PTP type phosphatases, because they have conserved catalytic sites and use the same mechanism for dephosphorylation. Recent research in the area has demonstrated that the phosphatases hematopoietic PTP, PP2A, and the DSP MAPK phosphatase-3 physiologically dephosphorylate ERK and thus reduce signaling via this kinase (21–24). Other DSPs, such as vaccinia H1 related (VHR) and MAPK phosphatase-1 have also been implicated in ERK dephosphorylation (25–27). Considering that active ERK is dually phosphorylated on tyrosine 204 and threonine 202 and that dysregulation of ERK signaling can result in aberrant cell growth and proliferation, it is not surprising that multiple phosphatases might be involved in removal of these phosphate groups. Surprisingly, little is known about whether adhesion can modulate the activity of these phosphatases and thus regulate ERK dephosphorylation. Adhesion can clearly modulate the activity of certain phosphatases; conversely, phosphatases are also involved in regulating adhesion (28–31). In fact, adhesion to fibronectin has been shown to increase MAPK phosphatase-1 activity in endothelial cells, and adhesion via $\alpha_2\beta_1$-integrin has been shown to increase PP2A activity in fibroblasts (28, 29). In our studies, using pharmacological inhibitors of phosphatases and an *in vitro* ERK phosphatase assay, we have shown that adhesion regulates ERK phosphatase activity in Jurkat T-cells, thus modulating the ERK MAPK signaling pathway.

EXPERIMENTAL PROCEDURES

Cell Culture, Cell Adherence, and Culture Additions—The human T-cell lines Jurkat, MOLT-3, and H9 and the B-cell line Daudi were used. Cell lines were grown in RPMI medium containing 10% FBS (HyClone, Logan, UT) or 20% FBS in the case of MOLT-3 and grown at densities between 1×10^5 and 1×10^6 cells/ml. The cells were serum-starved overnight in RPMI containing 0.5% FBS prior to adhesion and/or treatment with recombinant human SDF-1α (R&D Systems, Minneapolis, MN). SDF-1α was added at 20 ng/ml for 5 min, unless otherwise indicated.

For cell adhesion, tissue culture dishes were coated overnight with either 20 μg/ml Fn (BD Biosciences, Bedford, MD), 1 μg/ml recombinant human VCAM-1 (R&D Systems), or 10 μg/ml mouse anti-human β_1-integrin activating antibody, TS2/16 (purified from hybridoma supernatant using protein G). Coated dishes were then blocked by incubation with 2% bovine serum albumin for one h followed by washing with phosphate-buffered saline (PBS). Dishes used for suspension cultures were also incubated with 2% bovine serum albumin for 1 h. Because only a fraction of Jurkat cells adhere to Fn, the cells were adhered to Fn-coated dishes by either preselecting the adherent population and replating on Fn-coated dishes or by the addition of 10 μg/ml TS2/16 to promote adhesion. The cells were plated at $1–3 \times 10^6$ cells/ml of RPMI containing 0.5% FBS. The cells were adhered for 1 h prior to the addition of SDF-1, unless otherwise indicated.

Pharmacological inhibitors of G protein signaling, actin polymerization, MEK signaling, and phosphatase activity were added to Jurkat cell cultures prior to SDF-1 treatment. Inhibition of $G\alpha_i$ protein signaling was performed by pretreatment of Jurkat cells overnight with 100 ng/ml pertussis toxin. The actin polymerization destabilizer, cytochalasin D, was added to Jurkat cell cultures at 0.2, 2.0, or 20 μM for 30 min prior to SDF-1 treatment. UO126 (Promega, Madison, WI) was added to Jurkat cell cultures for 15 min at 25 μM to inhibit the activity of MEK. Phosphatase inhibition was performed by either the addition of 0.1 or 1.0 μM okadaic acid (Santa Cruz, San Diego, CA) for 30 min or the addition of 400 μM sodium orthovanadate for 45 min prior to SDF-1 treatment.

Preparation of Cell Lysate and Western Analysis—The cells were lysed in 0.1% Triton X-100, 0.3 M sucrose, 50 mM Tris, pH 7.5, 100 mM KCl, 1 mM $CaCl_2$, 2.5 mM $MgCl_2$ with phosphatase and protease inhibitors added fresh at 1 mM sodium orthovanadate, 1 mM nitrophenyl phosphate, 20 nM calyculin A, 100 μM 4-(2-aminoethyl)benzene-sulfonyl fluoride (AEBSF), and 0.1% aprotinin. Adherent cell cultures were lysed directly on plates. For suspension cultures, ice-cold PBS was added, the cells were centrifuged at 4 °C at 1500 RPM for 3 min, and the pellet was resuspended in lysis buffer. The cells were lysed for 40 min on ice and centrifuged at 4 °C at 14,000 RPM for 10 min, and the supernatants were collected. In some cases, where indicated, modified radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 5 mM EDTA, and protease and phosphatase inhibitors as above) was used to lyse cells. The protein concentration of cell lysates was determined using a bicinchonic acid assay (Pierce).

For Western blot analysis, 5–10 μg of cell extract were mixed with the appropriate volume of 6× Laemmli sample buffer, boiled for 3–5 min, separated by SDS-PAGE in a 10% acrylamide gel, and transferred to polyvinylidene fluoride membranes (Immobilon P, Millipore Corp., Bedford, MD). The membranes were blocked in 2% bovine serum albumin with 0.1% Tween in PBS for 1 h and incubated with primary antibody. The antibodies purchased from Cell Signaling Technology (Beverly, MA) are as follows: mouse anti-dually phosphorylated (Thr^{202}/Tyr^{204}), active, ERK-1/ERK-2, rabbit anti-dually phosphorylated ($Ser^{217/221}$), active, MEK-1/MEK-2, rabbit anti-total ERK-1/ERK-2, rabbit anti-total MEK-1/MEK-2, or mouse anti-phospho-Elk. Additionally, mouse anti-MEK-1 (K-23) or mouse anti-ERK-2 (D-2) (Santa Cruz) were used as indicated under "Results" for some Western blot analyses. Following labeling with primary antibodies, the membranes were washed in 0.1% Tween 20 in PBS and labeled with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG for 1 h. The immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Biosciences).

Immunoprecipitation and Immune Complex Kinase Assays—Endogenous ERK was immunoprecipitated from 300 μg of cell extract obtained by lysis in RIPA lysis buffer. The extracts were first precleared with protein G-Sepharose for 30 min at 4 °C, and then 1 μg of rabbit anti-ERK 2 (C-14) directly conjugated to agarose (Santa Cruz) was added to extracts for a total of 2 h at 4 °C. Negative controls were performed by incubation with rabbit nonspecific IgG. Immune complex beads were washed one time in modified RIPA containing protease and phosphatase inhibitors; three times in 500 mM lithium chloride, 100 mM Tris, pH 8.6; one time in 100 mM lithium chloride, 25 mM Tris, pH 8.6; and one final time in 100 mM sodium chloride, Tris, pH 7.5. The samples were resuspended in 30 μl of kinase assay buffer (10 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM ATP). For the ERK kinase reaction, 1 μg of recombinant GST-Elk protein (Cell Signaling) was added to the reaction mixture for 30 min at room temperature. Laemmli sample buffer was added at the appropriate concentration, and the samples were boiled for 3 min to stop the kinase reaction. Western blot analysis for P-Elk was performed to determine the relative kinase activity.

ERK Phosphatase Assays—To determine ERK phosphatase activity in whole cell extracts, 150 μg/sample of cell extract (without phosphatase inhibitors) was diluted 1:4 in phosphatase assay buffer (10 mM $MgCl_2$, 10 mM Hepes, pH 7.4, and 10 μM MEK inhibitor UO126). Recombinant phosphorylated His_6-ERK-2 (Biomol, Plymouth Meeting, PA) was added at 30 ng/sample and incubated for various lengths of time at room temperature. Urea (8 M, pH 8.6) containing 10 mM imidizole (to reduce nonspecific binding to Ni^{2+}-agarose) was added to the mixture to stop the reaction, and the samples were placed on ice. To precipitate the His-ERK, nickel-conjugated agarose (30 μl) was added to the reaction and incubated at 4 °C for 1 h. The samples were then washed three times in 8 M urea, pH 6.8, 10 mM imidizole and two times in 300 mM $NaCl_2$, 25 mM Tris, pH 7.5. The amount of phosphorylated ERK remaining was then determined by Western analysis using antibodies against dually phosphorylated ERK and total ERK to control for protein loading. The protein levels were quantitated using a Fluor-S scanner and Quantity One software for analysis (Bio-Rad). The data were analyzed and statistics were performed using Microsoft Excel software.

RESULTS

Adhesion to β_1-Integrin Substrates Regulates SDF-1 Activation of ERK in Jurkat T-cells—Integrin-mediated adhesion has been shown to regulate a number of important signal transduction events in normally adherent cell lines (3). Surprisingly, little is known about whether integrin-mediated adhesion can regulate signaling processes in lymphocytes, such as chemokine activation of the ERK MAPK. Therefore, experiments were performed to determine whether adhesion to β_1-integrin substrates affected SDF-1 signaling to ERK in the Jurkat T-cell

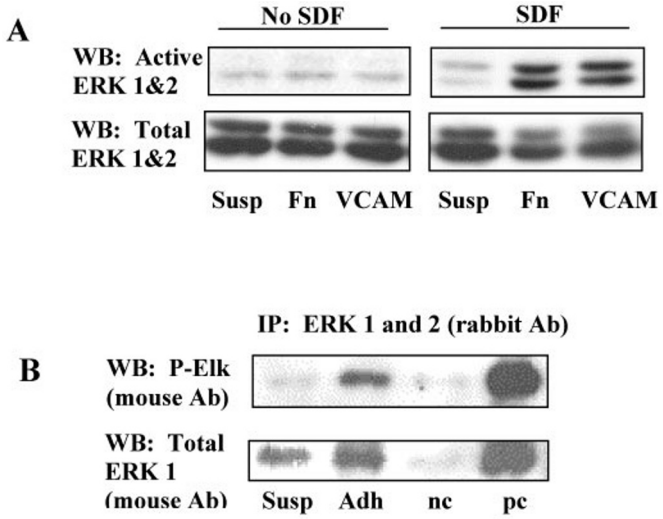

FIG. 1. **Adhesion to β_1-integrin substrates regulates SDF-1 activation of ERK in Jurkat T-cells.** *A*, Jurkat cells were starved overnight and preselected for adherence by plating on Fn for 1 h, removing suspended cells, deadhering by briskly tapping the cultures, and replating in suspension or on either Fn or VCAM-coated dishes for 1 h. The cells were then either treated with 20 ng/ml SDF-1 for 5 min or not and lysed in modified RIPA buffer and subjected to Western blot (*WB*) analysis. Active ERK was determined using a monoclonal antibody against dually phosphorylated ERK-1/ERK-2, and the membranes were stripped with 2 M NaOH and reprobed with rabbit anti-ERK-1/ERK-2 to determine the amount of total protein loaded. *B*, immune complex kinase assays were performed using ERK immunoprecipitates (*IP*) from extracts obtained from Jurkat cells treated with SDF-1 either in suspension (*Susp*) or adhered to Fn in the presence of TS2/16 to promote adhesion. Negative controls (*nc*) were cell extracts immunoprecipitated with nonspecific rabbit Ig, and positive controls (*pc*) were from whole cell extracts. Kinase activity was determined by Western analysis of P-Elk, and the total ERK-1 levels were determined to control for equal loading and ERK immunoprecipitation.

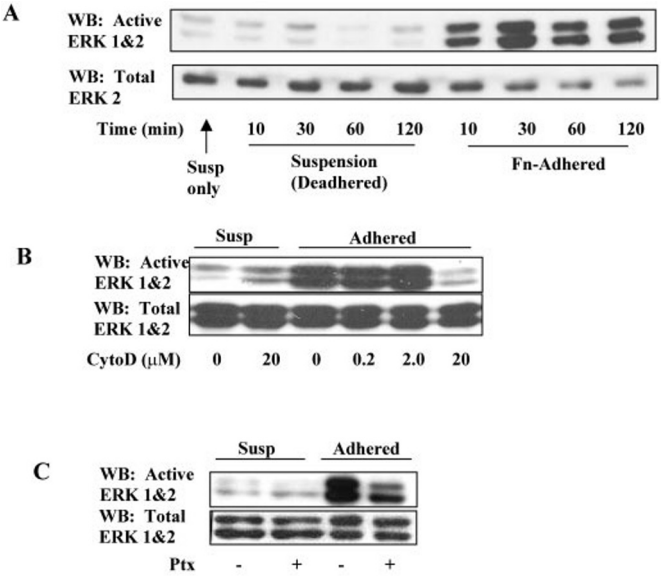

FIG. 2. **Adhesion regulation of SDF-1 activation of ERK is a rapid event that is both cytochalasin- and pertussis toxin-sensitive.** *A*, Jurkat cells were starved overnight and either held in suspension (*Susp*), allowed to adhere to Fn for 10, 30, 60, or 120 min (plus TS2/16 to promote adhesion), or adhered to Fn (plus TS2/16) for 45 min followed by resuspension for 10, 30, 60, or 120 min. The cells were stimulated for 5 min with 20 ng/ml SDF-1. Following culture, the cells were lysed, and the cell extracts subjected to Western blot (*WB*) analysis for the detection of active ERK, stripped, and reprobed for total ERK-2 protein to control for loading. *B*, Jurkat cells were starved, preselected for adherence, and plated on Fn-coated plates as previously described. The actin polymerization destabilizer cytochalasin D was added 30 min prior to SDF-1 treatment at the indicated concentrations. Following treatment, the cells were lysed and analyzed by Western blot for active and total ERK1 and 2 protein levels. *C*, Jurkat cells were treated with 100 ng/ml pertussis toxin (*Ptx*) overnight, preselected, and replated in suspension or adhered to Fn-coated tissue culture dishes. Following treatment with SDF-1 for 5 min, the cells were lysed and subjected to Western analysis as described above.

line. Thus, Jurkat cells that were adhered to immobilized Fn or VCAM displayed high levels of active, dually phosphorylated, ERK in response to treatment with SDF-1 for 5 min (Fig. 1*A*). Incubation of Jurkat cells in suspension with the β_1-activating antibody TS2/16 with or without soluble VCAM-1 did not result in increased ERK phosphorylation, indicating that ligand binding to integrins *per se* was not sufficient (data not shown). Additionally, adhesion alone did not induce ERK phosphorylation, because cells not treated with SDF-1 did not display levels of phospho-ERK comparable with those treated with SDF-1 while adhered. This demonstrates that integrin-mediated adhesion enhances SDF-1 activation of ERK in this lymphocyte cell line.

In vitro kinase assays were also performed to verify the results obtained from Western blot analysis. Fig. 1*B* shows the kinase activity of immunoprecipitated endogenous ERK protein from SDF-1-treated Jurkat cells in suspension or adhered to Fn. ERK kinase activity was apparent only in immunoprecipitates obtained from adherent cells. Therefore, these results also demonstrate that adhesion promotes SDF-1 activation of ERK.

Adhesion Regulation of SDF-1 Activation of ERK Is a Rapid Event That Is Cytochalasin- and Pertussis Toxin-sensitive—To determine whether adhesion regulation of ERK occurred rapidly or gradually over time, adhesion and deadhesion time course experiments were performed. As little as 10 min of adhesion to Fn allowed SDF-1 activation of ERK and prolonged adhesion through 90 min also allowed for SDF-1 activation of ERK (Fig. 2*A*). Further, deadhesion of Jurkat cells from Fn for as little as 10 min resulted in a complete loss of the ability of SDF-1 to activate ERK. These data suggest that adhesion enhancement of SDF-1 activation of ERK is a rapid process and that this effect is rapidly lost after deadhesion.

Because adhesion, not integrin-activation alone, appeared to regulate SDF-1 signaling to ERK, there could be a role for the cytoskeleton in mediating this adhesion control. Cytochalasin D is a pharmacological inhibitor of actin polymerization and can cause disruption of the actin cytoskeleton. The addition of relatively high concentrations of cytochalasin D to adherent Jurkat T-cells resulted in disruption of adhesion-mediated control of SDF-1 activation of ERK (Fig. 2*B*). Therefore, adhesion regulation of SDF-1 activation of ERK in lymphocytes can be inhibited by cytochalasin and thus might, either directly or indirectly, depend on cytoskeletal integrity.

The SDF-1 receptor, CXCR-4, has been reported to be a $G\alpha_i$ protein-coupled receptor (12). To determine whether adhesion-regulated SDF-1 activation of ERK is mediated by $G\alpha_i$, rather than by other receptor-associated $G\alpha$ subunits, Jurkat cells were treated with the $G\alpha_i$ inhibitor, pertussis toxin. Treatment with pertussis toxin overnight dramatically reduced SDF-1 activation of ERK in adherent Jurkat cells (Fig. 2*C*). These results suggest that adhesion-dependent SDF-1 activation of ERK is mediated, at least in part, by a pertussis toxin-sensitive mechanism.

SDF-1 Activation of MEK Occurs Independent of Adhesion: Adhesion-regulated ERK Activation Occurs in Other Lymphoid Cell Lines—The locus of adhesion control of signal transduction events has been demonstrated at various steps in signaling cascades. For example, our lab has shown that there is a locus of adhesion regulation of G protein signaling at the level of Raf

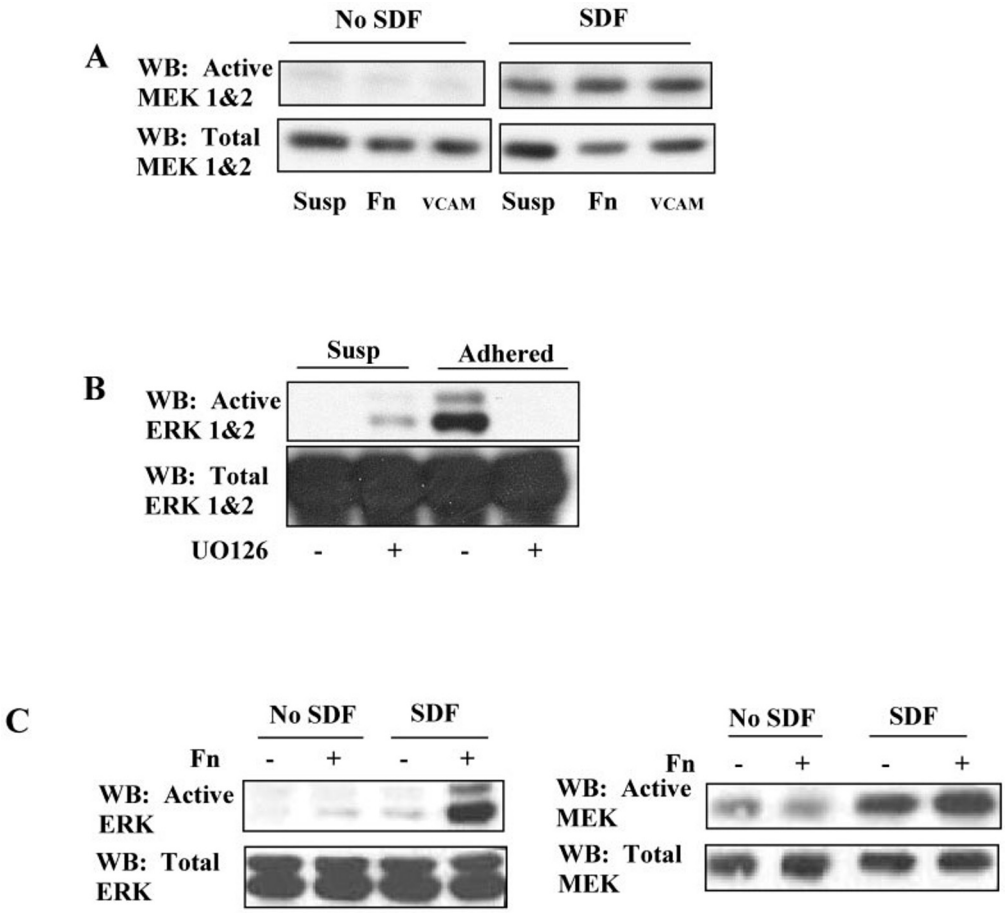

FIG. 3. **SDF-1 activation of MEK is independent of adhesion.** *A*, Jurkat cells were starved overnight and preselected for adherence by plating on Fn for 1 h, removing suspended (*Susp*) cells, deadhering by briskly tapping the cultures, and replating in suspension or on either Fn or VCAM-coated dishes for 1 h. The cells were then either treated with 20 ng/ml SDF-1 for 5 min or not and lysed in modified RIPA buffer and subjected to Western blot (*WB*) analysis. Active MEK was determined using a polyclonal antibody against dually phosphorylated MEK-1/MEK-2, and the membranes were stripped with 2 M NaOH and reprobed with mouse anti-MEK-1 to determine the amount of total protein loaded. *B*, Jurkat cells were starved, preselected, and replated on Fn-coated plates as described above. The MEK inhibitor, UO126, was added at 25 μM for 15 min prior to treatment with SDF-1. Active and total ERK protein levels were determined by Western analysis as described in the legend to Fig. 1. *C*, the B-cell line Daudi was starved overnight and either kept in suspension or plated on Fn-coated tissue culture dishes for 1 h. The cells were then treated with 20 ng/ml SDF-1 for 5 min or not and lysed in 0.1% Triton X-100 lysis buffer. As previously described, the cell extracts were subjected to Western blot analysis for active ERK or active MEK, stripped, and reprobed for total ERK or total MEK, respectively.

activation in endothelial cells (6). The upstream kinase of ERK is MEK, and thus experiments were performed to determine whether adhesion to β_1-integrin substrates could also modulate SDF-1 activation of this kinase. Interestingly, SDF-1 activation of MEK was comparable in Jurkat cells maintained in suspension or adhered to Fn or VCAM (Fig. 3*A*). Nonadherent cells treated with soluble TS2/16 also displayed activation of MEK upon treatment of SDF-1 but not in untreated cells (data not shown). In summary, SDF-1 activation of MEK can occur independent of adhesion in the Jurkat T-cell line, thus suggesting a locus of adhesion control at the level of ERK activation.

Because it was determined that SDF-1 activation of ERK was dependent on adhesion, whereas the activation of MEK was shown to be independent of adhesion, it was important to determine whether ERK activation was dependent on MEK in SDF-1-treated adherent cells. To determine whether MEK was responsible for ERK activation in adherent cells, the MEK inhibitor UO126 was added to cells either cultured in suspension (negative control) or adhered to Fn-coated plates (Fig. 3*B*). UO126 treatment resulted in complete inhibition of SDF-1 activation of ERK in adherent Jurkat cells. Therefore, adhesion-regulated SDF-1 activation of ERK is dependent on MEK activity.

A variety of lymphocyte cell lines were tested to determine whether, as in Jurkat cells, SDF-1 activation of ERK was dependent on adhesion, whereas activation of MEK was largely independent of adhesion. Daudi is a B-cell line derived from a patient with Burkitt's lymphoma. Treatment of this cell line with SDF-1 for 5 min resulted in much higher activation of ERK in adherent cells as opposed to suspension cells, whereas MEK activation was similar in either situation (Fig. 3*C*). Additionally, the T-cell lines MOLT-3 and H9 displayed similar results (data not shown). These results demonstrate that adhesion regulates SDF-1 activation of ERK in a number of lymphocyte cell lines.

Pharmacological Inhibitors of Phosphatases Can Partially Restore SDF-1 Activation of ERK in Suspension—We wished to explore the mechanism underlying adhesion regulated SDF-1 activation of ERK. Our results demonstrating that the activation of MEK by SDF-1 occurred in suspension and that under the same circumstances ERK was not activated suggested that the locus of adhesion regulation was at the level of ERK itself. We explored mechanisms that addressed the spatial regulation of MEK and ERK, such as adhesion-regulated MEK/ERK association or adhesion-regulated endocytosis, but no evidence was found to support these hypotheses (data not shown). Another potential mechanism for adhesion regulation of SDF-1 activation of ERK could be control of ERK dephosphorylation

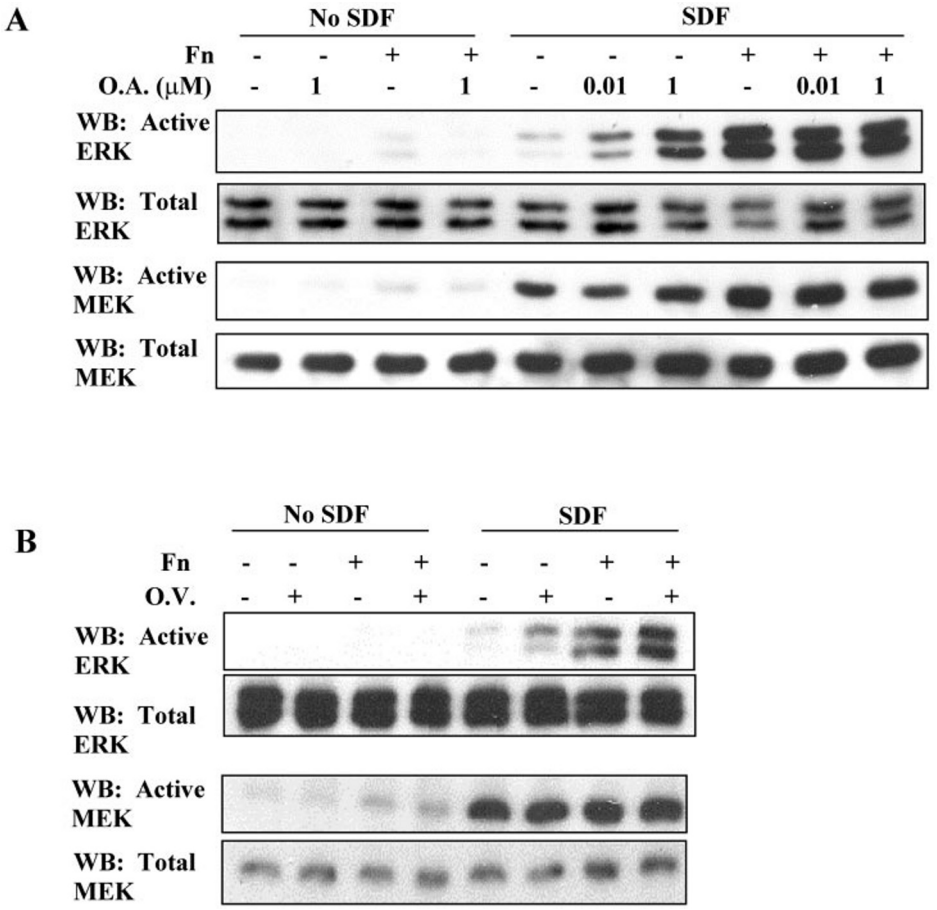

FIG. 4. **Okadaic acid and sodium orthovanadate can partially restore SDF-1 activation of ERK in suspended Jurkat T-cells.** Jurkat cells were starved overnight and either maintained in suspension or plated on Fn-coated tissue culture plates (with the addition of TS2/16 antibody to promote adhesion) for 1 h prior to SDF-treatment. Prior to SDF-1 treatment cells were either pretreated with 1.0 or 0.01 μM okadaic acid (*OA*) for 30 min (*A*) or 400 nM sodium orthovanadate (*OV*) for 45 min (*B*). Following treatment, as previously described, the cells were lysed in 0.1% Triton X-100 lysis buffer and subjected to Western blot (*WB*) analysis for active ERK and active MEK, stripped, and reprobed for total ERK and total MEK.

by the regulation of phosphatase activity. To determine whether phosphatase activity played a role, initial experiments utilizing pharmacological inhibitors of phosphatases were performed. Okadaic acid at low concentrations selectively inhibits the serine/threonine phosphatase PP2A, PP4, and PP5, and at 10-fold higher concentration PP1 is also inhibited (32, 33). Fig. 4*A* shows that the addition of low concentrations of okadaic acid to Jurkat T-cells cultured in suspension resulted in partial restoration of SDF-1 activation of ERK as compared with adherent cells. Higher concentrations resulted in even more SDF-1 activation of ERK in suspension. It is also important to note that treatment with okadaic acid did not result in ERK activation in cells not treated with SDF-1, nor did inhibition of serine/threonine phosphatases modulate SDF-1 activation of MEK. Sodium orthovanadate is an inhibitor of tyrosine phosphatase activity (34). Pretreatment with this inhibitor also resulted in partial restoration of SDF-1 activation of ERK in suspended lymphocytes (Fig. 4*B*). As seen with okadaic acid treatment, sodium orthovanadate treatment did not result in ERK activation in cells not treated with SDF-1, nor did it modulate the SDF-1 induced activation of MEK in these cells. In summary, pharmacological inhibition of serine/threonine or tyrosine phosphatases results in partial restoration of SDF-1 activation of ERK in suspension, whereas activation of MEK is unaffected.

Lymphocyte Adhesion to Fn Reduces ERK Phosphatase Activity—The above results suggest that ERK dephosphorylation is greater in suspended Jurkat T-cells as compared with adherent cells but does not directly address the level of phosphatase activity. To evaluate whether adhesion regulates ERK dephosphorylation by regulating phosphatase activity, we developed an assay to determine the ERK phosphatase activity in whole cell extracts. In fact, recombinant His-conjugated phosphorylated ERK-2 was more rapidly dephosphorylated when added to extracts obtained from Jurkat cells incubated in suspension than when added to extracts from adherent cells (Fig. 5*A*). These results were not influenced by ERK rephosphorylation, because MEK activity was inhibited by the addition of UO126 to the phosphatase assay buffer. Significant differences in phosphatase activity were established by quantitating and averaging multiple Western blots in Fig. 5*B*. These results, therefore, show that ERK phosphatase activity is higher in Jurkat cells incubated in suspension as compared with cells adhered to a Fn substrate.

Phosphatase Activity Can Be Inhibited in Vitro by High Concentrations of Okadaic Acid and Orthovanadate—Phosphatase inhibition by okadaic acid and sodium orthovanadate was additionally performed using the *in vitro* phosphatase assay (Fig. 6). The previous inhibition studies were performed on whole cells in which inhibitor accumulation can be influenced by cell permeability and cellular export. Inhibition of Ser/Thr phosphatases with relatively high concentrations of okadaic acid resulted in effective inhibition of ERK dephosphorylation. The addition of the tyrosine phosphatase inhibitor, sodium orthovanadate, also significantly inhibited ERK dephosphorylation, and an additive inhibition was observed with both sodium orthovanadate and moderate concentrations of okadaic acid. The greatest protection from ERK dephosphorylation was achieved by a combination of high concentrations (200 nM) of okadaic acid and sodium orthovanadate. Therefore, ERK dephosphorylation in SDF-1-treated suspended Jurkat cells is likely mediated by both okadaic acid- and orthovanadate-sensitive phosphatases.

DISCUSSION

Adhesion regulation of signaling pathways in lymphocytes has been little studied but could provide important insight into how lymphocytes respond to stimuli in various physical envi-

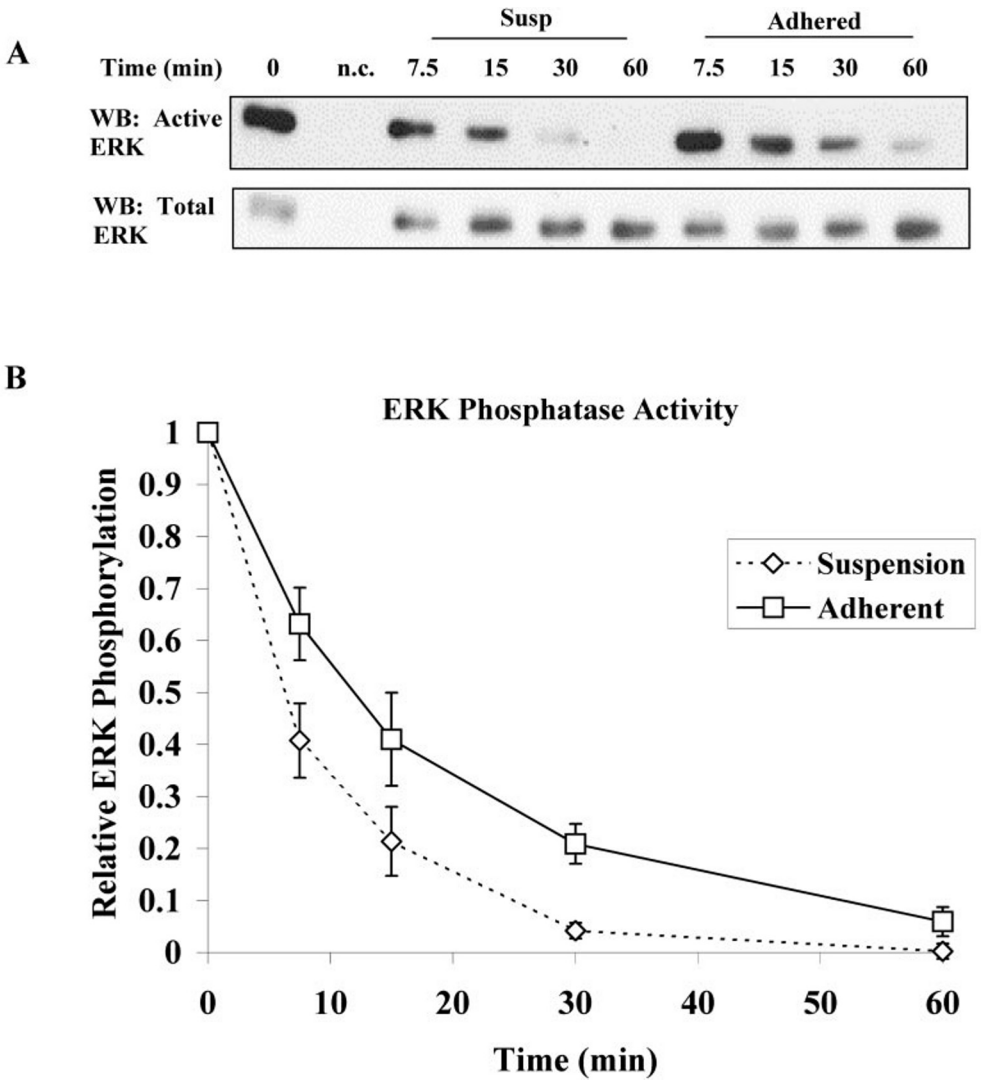

FIG. 5. **Extracts from Jurkat cells incubated in suspension display greater ERK dephosphorylation.** *A*, Jurkat cells were starved overnight and either maintained in suspension (*Susp*) or adhered to Fn-coated dishes for 1 h and treated with SDF-1 for 5 min. The cells were then lysed in 0.1% Triton X-100 lysis buffer. Recombinant active His-ERK-2 was added to cell extracts for various lengths of time, and the reaction was stopped in urea. His-ERK was precipitated from extracts using Ni^{2+}-conjugated agarose. The precipitates were washed several times as described under "Experimental Procedures" and analyzed by Western blot (*WB*) for phosphorylated ERK and total ERK. *B*, phosphorylation was quantitated using a fluorescent scanner, and the level of P-ERK was controlled for loading variations and normalized to time 0. The averages and standard error ($n = 3$) were plotted using Microsoft Excel software.

ronments, for example adhered in tissue as compared with suspended in blood. Clearly, the chemokine SDF-1 is an important signaling molecule in normal immune function and in disease. Therefore, understanding how adhesion might regulate its signal transduction pathways has been explored here. The studies herein clearly show that adhesion to β_1-integrin substrates can enhance SDF-1 activation of the ERK MAPK in B and T lymphocyte cell lines. This activation is dependent on MEK activity, because a MEK inhibitor ablates SDF-1 activation of ERK. Additionally, we verified that this process is at least partially pertussis toxin-sensitive and is dependent on actin polymerization.

A previous study had shown that SDF-1 could activate ERK in suspended Jurkat cells, but comparisons to adherent cells were not made (12). Additionally, the experiments were performed at a relatively high cell density that could potentially cause cell aggregation, perhaps creating adherent interactions. In some cases we in fact did observe slight activation of ERK with SDF-1 treatment in suspension, but compared with the ERK activation in adherent cells this was negligible. In another study SDF-1 induced somewhat weak activation of ERK in interleukin-2-stimulated primary human T-cells (35). Although the state of adhesion was not commented on, one would assume a suspended phenotype for these cells, but a direct comparison with our experiments cannot be made because our cells were not co-stimulated with interleukin-2 upon SDF-1 treatment.

Interestingly, in further analysis of the MAPK signaling cascade, the upstream kinase to ERK, MEK-1, was shown to be activated by SDF-1 independent of adhesion. This suggested a novel locus of adhesion control of SDF-1 signaling at the level of ERK. Several loci of adhesion control of the MAPK pathway have been described previously, including receptor tyrosine kinase activation, Raf activation, and trafficking of ERK to the nucleus, but direct adhesion regulation of ERK activation has not been previously described (6, 36, 37). It is noteworthy that the studies cited above were all performed in stably adherent cell lines and that adhesion regulation of signaling events in cells that are normally transiently adherent might utilize different mechanisms of control. It was therefore important to further investigate how adhesion might regulate SDF-1 activation of ERK in lymphocytic cell types.

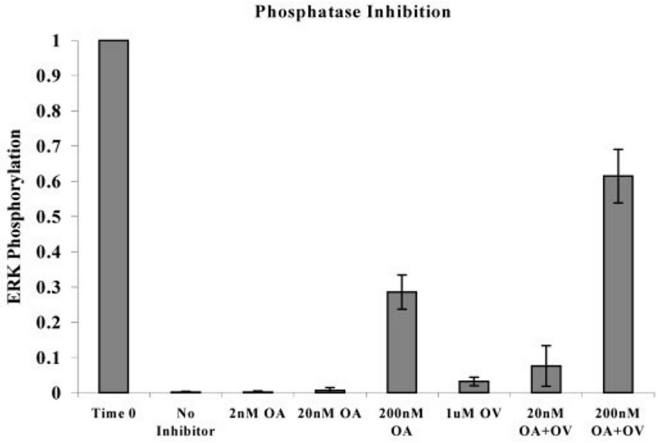

FIG. 6. **Adhesion regulation of phosphatase activity is a rapid event, and phosphatase activity can be inhibited *in vitro* by high concentrations of okadaic acid and orthovanadate.** The extracts were obtained from SDF-1 treated Jurkat cells in suspension and were pretreated for 30 min with either 2, 20, or 200 nM okadaic acid (*OA*) or 1 μM sodium orthovanadate (*OV*) or a combination of 20 or 200 nM okadaic acid with 1 μM sodium orthovanadate. The *in vitro* phosphatase assay was performed as previously described with the addition of active His-ERK to cell extracts for 45 min. Phosphorylation was determined by Western analysis, and the data were controlled for loading based on total ERK levels and normalized to phosphorylation at time 0. The data obtained from Western blots were averaged, and the standard error *bars* are shown ($n = 4$).

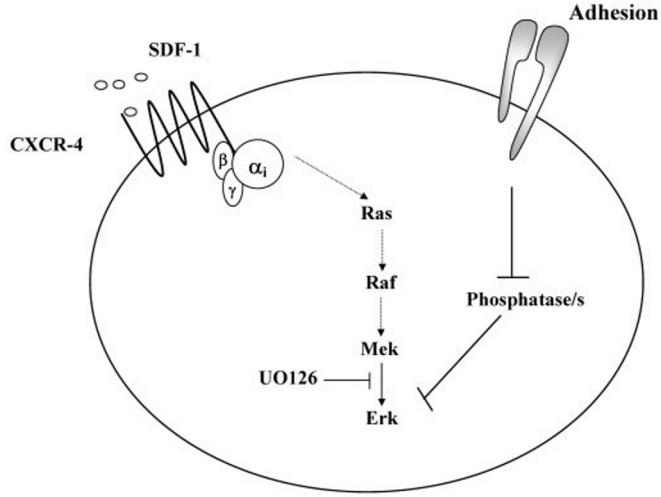

FIG. 7. **A model for adhesion regulation of SDF-1 activation of ERK in lymphocytes.**

One potential mechanism of regulation of ERK activation is by controlling ERK dephosphorylation. Pharmacological inhibitors of Ser/Thr phosphatases and Tyr phosphatases were utilized to determine whether inhibition of these phosphatases could restore SDF-1 activation of ERK in suspended lymphocytes. Results from experiments performed *in situ* showed that inhibition of either Ser/Thr or Tyr phosphatases could partially restore ERK activation in suspended cells. The activation status of MEK was unaffected by inhibitor addition, suggesting that the effect was specific to ERK under these conditions. Therefore, adhesion regulation of ERK phosphatase activity was indicated as a potential mechanism of adhesion control of SDF-1 signaling to ERK.

To more directly determine whether adhesion controls ERK dephosphorylation by regulating phosphatase activity, a nonradioactive phosphatase assay utilizing active ERK as the substrate was developed. These experiments clearly demonstrated that ERK dephosphorylation was more rapid in extracts obtained from suspended Jurkat T-cells as compared from extracts obtained from adherent cells. Thus, adhesion appears to down-regulate the activity of phosphatase(s) that dephosphorylate ERK. The exact phosphatases responsible were not determined here, but contributions of both Ser/Thr and Tyr phosphatases were indicated by *in vitro* experiments using okadaic acid and sodium orthovanadate similar to the *in situ* experiments. Previous studies have indicated that ERK dephosphorylation rates by PP2A or hematopoietic PTP on monophosphorylated as compared with dually phosphorylated ERK can differ (23, 38). Because the anti-active ERK antibody used here detects only dually phosphorylated ERK, it might also be interesting to use anti-phosphothreonine or phosphotyrosine antibodies to determine whether there is a sequential regulation of ERK dephosphorylation that is regulated by adhesion.

Time course experiments were performed to determine when adhesion regulation of SDF-1 activation of ERK occurs. Although not directly implicating any particular regulatory mechanism, the kinetics of the adhesion response could indicate whether regulation is likely due to regulation of protein synthesis or post-translational modifications. In the Jurkat cells studied here the increase in SDF-1 signaling to ERK took place almost immediately (10 min) upon adhesion. Conversely, deadhering cells from Fn results in a rapid loss of SDF-1 activation of ERK. This indicates a rapid mechanism of regulation that occurs in minutes rather than hours. Perhaps adhesion results in modulation of the phosphorylation status or some other post-translational modification of phosphatases, thus rapidly regulating their activity. This is somewhat surprising, because transcriptional regulation of protein phosphatase expression is a widely reported mechanism of control (18, 25, 28). However, post-translational modifications such as phosphorylation or regulation by subcellular localization have also been reported to modulate phosphatase activity (32, 39, 40). These events could result in differential association between the phosphatases and ERK.

In summary, adhesion can regulate activation of ERK by the chemokine, SDF-1 in lymphocyte cell lines. One mechanism for this regulation appears to be by adhesion-mediated down-regulation of the activity of phosphatases that can specifically dephosphorylate ERK. A model of how adhesion might regulate SDF-1 activation of ERK in lymphocytes is presented in Fig. 7. In the future it will be interesting to determine what the specific adhesion-controlled phosphatases are and how the activity of these phosphatases might be controlled. Additionally, whether adhesion control of ERK dephosphorylation is specific to chemokine signaling in lymphocytes or whether it is a more universal mechanism could have important implications for understanding complex signal transduction pathways and how they are modulated.

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