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# Dual Role of H-Ras in Regulation of Lymphocyte Function Antigen-1 Activity by Stromal Cell-derived Factor-1$\alpha$: Implications for Leukocyte Transmigration

**Kim S.C. Weber,* Georg Ostermann,* Alma Zernecke,* Andreas Schröder,* Lloyd B. Klickstein,‡ and Christian Weber*†§**

*Institute for Prevention of Cardiovascular Disease, Ludwig-Maximilians-University, Munich, Germany 80336; †Department of Cardiovascular Molecular Medicine, University Hospital, Aachen, Germany D-52074; and ‡Division of Rheumatology and Immunology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115

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We investigated the role of H-Ras in chemokine-induced integrin regulation in leukocytes. Stimulation of Jurkat T cells with the CXC chemokine stromal cell-derived factor-1$\alpha$ (SDF-1$\alpha$) resulted in a rapid increase in the phosphorylation, i.e., activation of extracellular signal receptor-activated kinase (ERK) but not c-Jun $NH_2$-terminal kinase or p38 kinase, and phosphorylation of Akt, reflecting phosphatidylinositol 3-kinase (PI3-K) activation. Phosphorylation of ERK in Jurkat cells was enhanced and attenuated by expression of dominant active (D12) or inactive (N17) forms of H-Ras, respectively, while N17 H-Ras abrogated SDF-1$\alpha$-induced Akt phosphorylation. SDF-1$\alpha$ triggered a transient regulation of adhesion to intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 mediated by lymphocyte function antigen-1 (LFA-1) and very late antigen-4 (VLA-4), respectively, and a rapid increase in LFA-1 binding to soluble ICAM-1.Ig, which was inhibited by D12 but not N17 H-Ras. Both D12 and N17 H-Ras abrogated the regulation of LFA-1 but not VLA-4 avidity, and impaired LFA-1–mediated transendothelial chemotaxis but not VLA-4–dependent transmigration induced by SDF-1$\alpha$. Analysis of the mutant Jurkat J19 clone revealed LFA-1 with constitutively high affinity and reduced ERK phosphorylation, which were partially restored by expression of active H-Ras. Inhibition of PI3-K blocked the up-regulation of Jurkat cell adhesion to ICAM-1 by SDF-1$\alpha$, whereas inhibition of mitogen-activated protein kinase kinase impaired the subsequent down-regulation and blocking both pathways abrogated LFA-1 regulation. Our data suggest that inhibition of initial PI3-K activation by inactive H-Ras or sustained activation of an inhibitory ERK pathway by active H-Ras prevail to abolish LFA-1 regulation and transendothelial migration induced by SDF-1$\alpha$ in leukocytes, establishing a complex and bimodal involvement of H-Ras.

## INTRODUCTION

Integrins are heterodimeric transmembrane proteins and are important for cellular adhesive functions during various processes such as cell proliferation and development (Springer, 1990; Hynes, 1992). Integrins may act as checkpoints for signal transduction pathways originating from inside the cell (inside-out signaling) or resulting from binding to its ligand (outside-in signaling) (Clark and Brugge, 1995; Dedhar and Hannigan, 1996; Humphries, 1996; Kolanus and Seed, 1997; Giancotti and Ruoslahti, 1999, van Kooyk and Figdor, 2000). As a result, integrins may be linked to important signaling processes, such as tyrosine phosphorylation, and cellular events, such as actin cytoskeletal reorganization and focal adhesion formation. Numerous studies have contributed to the understanding of the molecular mechanisms that eventuate in integrin avidity regulation and this has given rise to two predominant theories. First, extracellular conformational changes in the molecule may induce an increased affinity to ligand (Diamond and Springer 1994; Humphries, 1996; Stewart *et al.*, 1996). Second, cytoskeletal release of integrins enabling their lateral clustering may result in increases in integrin avidity (Lub *et al.*, 1997; Yauch *et al.*, 1997; Hato *et al.*, 1998; van Kooyk *et al.*, 1999).

§ Corresponding author. E-mail address: cweber@post.linikum.rwth_aachen.de

Although integrin activation may involve a combination of clustering and affinity change, the contributions of each mechanism may depend on the mode of stimulation. Integrins may be activated in vitro by nonphysiological stimuli such as divalent cations or activating monoclonal antibodies (mAbs), which induce an extracellular conformational change and thus an increase in affinity (Diamond and Springer, 1994; Humphries, 1996; Stewart *et al.*, 1996). On the other hand, cellular stimulation of protein kinase C may induce an increase in integrin avidity accompanied by cell spreading (Stewart *et al.*, 1996). During the sequential model of leukocyte emigration, regulation of integrin activity is important for the firm arrest and subsequent transendothelial migration of leukocytes (Springer, 1994). In this model, it has been suggested that integrin activation may occur as a result of selectin cross-linking or the exposure to chemokines (Springer, 1994; Simon *et al.*, 1995; Hwang *et al.*, 1996; Weber *et al.*, 1996a,b; Peled *et al.*, 1999; Simon *et al.*, 2000). Chemokines are a family of chemotactic cytokines released by various tissues during inflammation (Baggiolini, 1998; Nelson and Krensky, 1998). Although initially described as mediators of chemotaxis, chemokines have also been demonstrated to induce the firm arrest of cells and transmigration via the specific modes of avidity regulation for distinct integrins, i.e., transient for lymphocyte function antigen-1 (LFA-1) and very late antigen-4 (VLA-4) and sustained for Mac-1 or VLA-5 (Smith *et al.*, 1989; Weber *et al.*, 1996a,b, 1997a; Sadhu *et al.*, 1998; Weber and Springer, 1998b).

In recent years, it has been described that the small GTPases of the Ras family may also be involved in regulation of integrin avidity (Zhang *et al.*, 1996; Hughes *et al.*, 1997; O'Rourke *et al.*, 1998; Shibayama *et al.*, 1999; Tanaka *et al.*, 1999). The small GTPase R-ras has been found to increase the avidity of $\beta$1 integrins, which was associated with cell spreading (Zhang *et al.*, 1996). In contrast, H-Ras acts as a negative regulator of $\beta$1 and $\beta$3 integrin activation, which was mediated by the Raf-1/extracellular signal receptor-activated kinase (ERK) pathway and resulted in a decrease in integrin affinity (Hughes *et al.*, 1997). These studies were largely restricted to malignant cell types or to integrins expressed exogenously, i.e., not in their natural cellular context. More recently, however, studies have found that in leukocytes stimulated by T-cell receptor engagement or interleukin-3 (IL-3), H-Ras may signal to activate the avidity of $\beta$1 and $\beta$2 integrins independently of the Raf-1/ERK kinase pathway, thereby suggesting a more complex role of H-Ras in integrin activation (O'Rourke *et al.*, 1998; Shibayama *et al.*, 1999). It has been shown that the CXC chemokine stromal cell-derived factor-1$\alpha$ (SDF-1$\alpha$) and the chemoattractant factor formyl-methionyl-leucyl-phenylalanine can induce the activation of two downstream effectors of Ras, i.e., rapid activation of ERK and phosphatidylinositol 3-kinase (PI3-K) (Ganju *et al.*, 1998; Sotsios *et al.*, 1999; Vicente-Manzanares *et al.*, 1999; Constantin *et al.*, 2000; Tilton *et al.*, 2000). Therefore, we studied the role of H-Ras in the regulation of leukocytic integrins in response to chemokines. Here we demonstrate that H-Ras is involved in the transient avidity regulation of the $\beta$2 integrin LFA-1 and transendothelial chemotaxis of lymphocytes stimulated by SDF-1$\alpha$. Although the initial up-regulation in LFA-1 avidity is dependent on PI3-K, the subsequent down-regulation appears to be mediated by ERK.

## MATERIALS AND METHODS

### *Cell Culture, mAbs, and Reagents*

The T-lymphoma Jurkat cells were cultured as described (Weber *et al.*, 1997b). The J19 cell clone was generated by radiation mutagenesis. In brief, Jurkat cells were treated with $\gamma$-irradiation producing 50% cell death. Cells were returned to culture, and cells expressing LFA-1 in a constitutively active state were selected by immunopanning on intercellular adhesion molecule-1 (ICAM-1). Single cell colonies were screened by adhesion to ICAM-1 and the J19 cell clone was used for further experiments. Flow cytometry and comparison of purified LFA-1 in adhesion assays confirmed that the LFA-1 molecule in the J19 cells was unaltered. DNA sequencing of the $\alpha$L and $\beta$2 cytoplasmic domain cDNA generated by reverse transcription-polymerase chain reaction in the wild-type (Jn.9) and the J19 cells was the same. The active (D12) and inactive (N17) forms of H-Ras (kindly provided by Dr. A. Hall, University College, London, United Kingdom) were subcloned into pcDNA3 (Invitrogen, Carlsbad, CA). For stable transfections, cells were electroporated with cDNA and selected in RPMI-1640 medium supplemented with 0.75 mg/ml geneticin (Invitrogen). Single cell colonies were screened by immunoblotting for expression of H-Ras and phosphorylated ERK (pERK). In addition, immunoblotting with a c-myc mAb directed against a tag epitope preceeding the sequence of H-Ras mutant constructs also revealed expression in transfectants our unpublished data, thus distinguishing mutant from endogenous H-Ras. As a control, cells were also transfected with pcDNA3 vector alone. For select experiments with transient transfections, cells were cotransfected with cDNA encoding H-Ras mutants and with cDNA encoding a green fluorescence protein (GFP) by electroporation (107 cells with 30 $\mu$g of cDNA in 250 $\mu$l at 300 V and 1200 $\mu$F; Zeitlmann *et al.*, 1998). The efficiency of transfection was assessed after 12 h by analyzing GFP expression in a flow cytometer. Cell populations transfected with an equivalent efficiency of >50% were instantly used in adhesion assays. The TS1/22 mAb blocking $\alpha$L(Sanchez-Madrid *et al.*, 1982) is available from American Type Culture Collection (Rockville, MD) and blocking VLA-4 mAb HP1/2 was a kind gift from Dr. Martin Hemler (Dana Faber Cancer Institute, Boston, MA). mAbs to pERK, ERK, phosphorylated c-Jun $NH_2$-terminal kinase (JNK), JNK, phosphorylated-p38, p38, phosphorylated Akt, Akt, and H-Ras were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) or New England Biolabs (Beverly, MA). The mAb to CXCR4 was purchased from BD PharMingen (San Diego, CA). SDF-1$\alpha$ was purchased from Pepro Tech (Rocky Hill, NJ). Recombinant soluble vascular cell adhesion molecule-1 (VCAM-1) was a kind gift from Dr. R. Rothlein (Boehringer Ingelheim, Ridgefield, CT) or was purchased from R & D Systems (Wiesbaden, Germany). The 40-kDa fragment of fibronectin (FN40) containing the connecting segment 1 was purchased from Invitrogen. All other reagents were from Sigma (Deisenhofen, Germany) unless otherwise specified.

### *Immunoblotting*

Cell lysates were made in sample buffer containing protease inhibitors and proteins were separated by SDS-PAGE. Membranes were blocked in 5% milk/Tris-buffered saline (TBS) and incubated with the indicated specific mAbs for 1 h or overnight, washed, incubated with horseradish peroxidase-conjugated secondary antibody for 1 h, and washed. Proteins were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Freiburg, Germany).

### *Flow Cytometry*

Cells were incubated with specific mAbs or the isotype control (10 $\mu$g/ml) for 30 min on ice, washed, and stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse mAb for 30 min on ice. Surface expression was then analyzed in an FACScan (BD Biosciences, Heidelberg, Germany). To assess expression of GFP, cells were lysed with 0.2% Triton X-100 on ice for 2 min, washed,

and fluorescence intensity was measured by flow cytometry. For soluble ICAM-1 binding assays, cells were reacted with increasing concentrations of ICAM-1 fused with an Ig domain (ICAM-1.Ig) in TBS supplemented with 2 mM $Mg^{2+}$ and 1 mM $Ca^{2+}$ for 1 h at 37°C, stained with FITC-conjugated goat anti-human IgG mAb on ice, and analyzed by flow cytometry (Stewart *et al.*, 1996; Geiger *et al.*, 2000). In some experiments, cells were incubated in TBS supplemented with 10 mM $Mg^{2+}$/1 mM EGTA to induce high-affinity LFA-1 receptors (Stewart *et al.*, 1996). For analysis of SDF-1$\alpha$–induced binding of ICAM-1, cells and transfectants were preincubated with ICAM-1.Ig at 100 $\mu$g/ml and FITC-conjugated goat anti-human IgG mAb mAb in TBS/2 mM $Mg^{2+}$/1 mM $Ca^{2+}$ for 30 min, stimulated with SDF-1$\alpha$ (1 $\mu$g/ml) for 1 min, removed from the soluble phase by spin centrifugation at 400 $\times$ *g* for 1 min, fixed in 2% paraformaldehyde, and analyzed in an FACScan. After subtraction of unstimulated background binding, induction of ICAM-1 binding was expressed as specific median fluorescence intensity stimulated by SDF-1$\alpha$.

### *Cell Adhesion Assays*

Cell adhesion to ICAM-1 or VCAM-1 adsorbed at 10 $\mu$g/ml and 0.25–2.5 $\mu$g/ml, respectively, was performed as described (Weber *et al.*, 1996a,b). Proteins were coated onto 96-well microtiter plates (Linbro Titertek; Eschwege, Germany) and nonspecific adhesion was blocked by the addition 0.5% bovine serum albumin (BSA) in HHMC (Hanks' balanced salt solution, 10 mM HEPES pH 7.4, 1 mM $Mg^{2+}$, 1 mM $Ca^{2+}$) for 2 h at room temperature. Cells were labeled with the fluorescent dye 2,7-biscarboxyethyl-5(6)-carboxyfluorescein acetaxymethyl ester (BCECF-AM) (1 $\mu$g/ml), and resuspended in HHMC supplemented with 0.5% BSA. For inhibition of PI3-K or mitogen-activated protein kinase kinase (MEK) kinase, cells were preincubated with wortmannin (100 nM) and PD 98059 (20 $\mu$M) (both Calbiochem, San Diego, CA), respectively, for 15 min at 37°C and maintained in the assay. For mAb inhibition, cells were preincubated with the indicated mAbs (10 $\mu$g/ml) for 30 min on ice and maintained in the assay. Labeled cells ($5 \times 10^4$ in 50 $\mu$l) were added to ligand-coated wells in the presence of assay medium (control) or stimuli. Nonadherent cells were removed by multiple rapid inversions of the plate and washes in HHMC (flick wash) and fluorescence of input and adherent cells was quantified with a fluorescence plate reader (Tecan-SLT; Tecan, Crailsheim, Germany). Specific binding was expressed as percentage of input and are the mean $\pm$ SD of at least four separate experiments performed in triplicate.

### *Transendothelial and Transfilter Chemotaxis Assay*

Isolation and culture of human umbilical vein endothelial cells and transendothelial migration assays were performed as described (Weber *et al.*, 1996b). For transendothelial assays, human umbilical vein endothelial cells were grown on collagen-coated 6.5-mm-diameter Transwell inserts (5 $\mu$m pore size; Costar, Wiesbaden, Germany, MA). For chemotaxis assays, the Transwell inserts were coated overnight with the specific VLA-4 ligand FN40 or VCAM-1 (10 and 0.5 $\mu$g/ml, respectively), washed, and blocked with RPMI-1640 supplemented with 0.5% BSA or were left untreated (bare filter assay). For inhibition experiments, cells were pretreated with blocking mAbs for 30 min on ice before being added to the assay. Chemokines in assay medium (RPMI-1640/medium 199, 0.5% BSA) were added to 24-well tissue culture plates. Transwells were inserted and cells added to the top chamber. A dilution of cells served as a measure of input. Jurkat cells were allowed to transmigrate across filters coated with endothelial cells for 4 h or across bare or protein-coated filters for 3 h. Input and transmigrated cells were counted by microscopy and in an FACScan with the use of appropriate light scatter gates.

## RESULTS

### *CXC Chemokine SDF-1α Selectively Phosphorylates Mitogen-activated Protein (MAP) Kinase ERK and Akt*

To study the effect of chemokine stimulation on the H-Ras/Raf-1/MAP kinase pathway, Jurkat T lymphoma cells, which endogenously express the SDF-1$\alpha$ receptor CXCR4 (Hesselgesser *et al.*, 1998), were stimulated with the CXC chemokine SDF-1$\alpha$, and the phosphorylated forms of the MAP kinases, ERK, JNK, and p38, were detected by immunoblotting with the use of specific mAbs. Stimulation of Jurkat cells with 1 $\mu$g/ml SDF-1$\alpha$ for different periods of time resulted in a rapid and pronounced increase in the levels of phosphorylated, i.e., activated ERK, which reached a maximum at 1 and 5 min before being gradually down-regulated at later time points (Figure 1A). In contrast, the phosphorylation of JNK or p38 kinase was not significantly up-regulated in response to SDF-1$\alpha$, which is consistent with a previous report (Figure 1A; Ganju *et al.*, 1998). These results were confirmed by quantitative densitometry comparing levels of phosphorylated relative to total protein (Figure 1B). The induction of ERK phosphorylation by SDF-1$\alpha$ was dose-dependent with maximal effects occurring at 1 $\mu$g/ml, whereas the total amounts of ERK remained unaltered (Figure 1C). In addition, SDF-1$\alpha$ induced a rapid increase in the levels of phosphorylated Akt, a downstream target of PI3-K (Figure 1D). Our data reveal that SDF-1$\alpha$ triggers activation, i.e., phosphorylation of ERK, as well as phosphorylation of Akt, indicative of PI3-K activation.

### *H-Ras Selectively Regulates Phosphorylation of ERK and Akt but not JNK or p38 Kinase*

It has been reported that integrin activity may be regulated specifically via the H-Ras/Raf-1/ERK kinase pathway (Hughes *et al.*, 1997). To directly investigate the involvement of H-Ras in the regulation of integrin avidity by chemokines in the physiological context of a leukocyte, we transfected Jurkat cells with dominant active (D12 H-Ras) and inactive (N17 H-Ras) forms of H-Ras. Transfection with both H-Ras mutants resulted in a severalfold increase in the H-Ras protein levels in selected clones and expression of a tag epitope, as assessed by immunoblotting and quantitative densitometry, indicating overexpression of the mutants (Figure 2A; our unpublished data). Jurkat clones (i.e., 22 or 23) selected after transfection with D12 H-Ras revealed increased levels of phosphorylated ERK compared with vector-transfected cells (Figure 2B). Conversely, Jurkat clones (i.e., 14 or 36) expressing N17 H-Ras exhibited lower levels of phosphorylated ERK compared with vector-transfected cells. In contrast, levels of phosphorylated JNK or p38 and total expression of the MAP kinases were not significantly affected by expression of D12 or N17 H-Ras (Figure 2B). The expression of inactive N17 H-Ras almost completely abrogated Akt phosphorylation in response to SDF-1$\alpha$, whereas the increase in ERK phosphorylation was only slightly impaired in comparison with wild-type Jurkat cells, as revealed by densitometrical quantification (Figure 2C; our unpublished data). Thus, these data indicate that H-Ras is complexly and intricately involved in signaling pathways triggered by SDF-1$\alpha$.

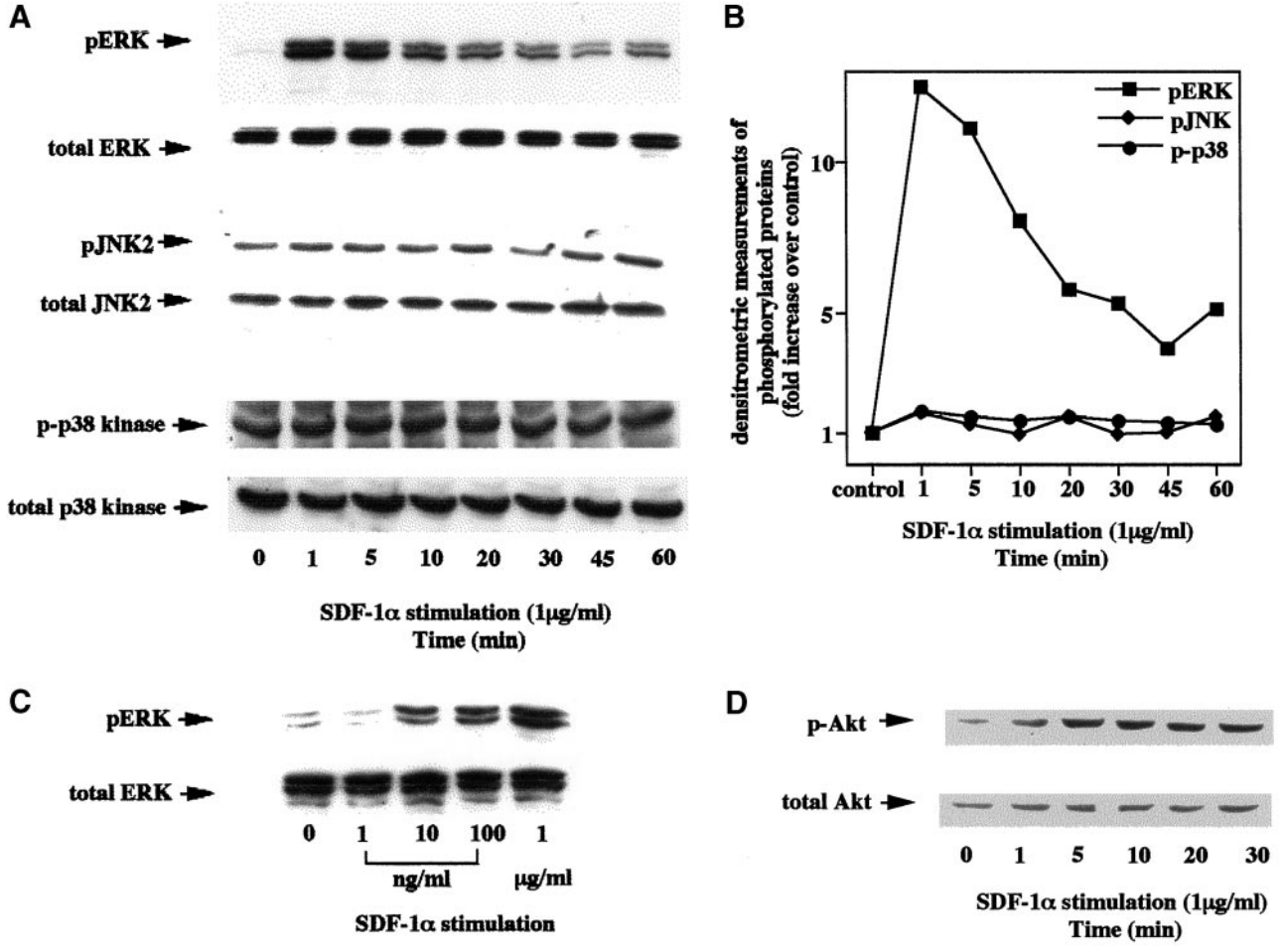


**Figure 1.** SDF-1$\alpha$ induces substantial phosphorylation of ERK and Akt but not JNK or p38. Jurkat cells were stimulated with SDF-1$\alpha$ (1 $\mu$g/ml) for indicated periods (A, B, and D) or with SDF-1$\alpha$ at indicated concentrations (C). Cell lysates were separated by 10% SDS-PAGE and specific mAbs were used to detect phosphorylated and total ERK (A–C), JNK, and p38 kinase (A and B) or Akt (D). Shown are representative blots from at least three separate experiments or quantitative densitometry (B) expressed as phosphorylated relative to total protein (percentage of control) of a representative experiment.

### *Involvement of H-Ras in Regulation of LFA-1 but not VLA-4 Avidity Induced by SDF-1α*

It has previously been demonstrated that chemokines induce the transient activation of the integrins LFA-1 and VLA-4 to their ligands ICAM-1 and VCAM-1, respectively (Campbell *et al.*, 1998; Weber *et al.*, 1996b, 1999a; Constantin *et al.*, 2000), which may be of particular relevance for cell migration and transendothelial chemotaxis. To study the potential role of H-Ras in the regulation of these integrins, we performed static adhesion assays on ICAM-1 and VCAM-1 with the use of the Jurkat D12 and N17 H-Ras transfectants stimulated with SDF-1$\alpha$. The surface expression of LFA-1, VLA-4, and the SDF-1$\alpha$ receptor CXCR4 on vector-transfected Jurkat cells, Jurkat/D12 H-Ras clone 22, and Jurkat N17/H-Ras clone 36 was compared by flow cytometry, confirming equivalent levels of expression and indicating that transfection of mutant forms of H-Ras did not interfere with the transcriptional or translational regulation of these molecules (Figure 3). Moreover, the total expression levels of the signaling kinases ERK, JNK, p38, and Akt were unaffected by stable transfection with the H-Ras mutants (Figure 2), providing further evidence that the regulation of genes involved in the processes studied was unaltered. Adhesion assays demonstrated that SDF-1$\alpha$ triggered the transient adhesion of wild-type Jurkat cells to immobilized ICAM-1 or VCAM-1 (Figure 4, A and C). The binding was mediated by LFA-1 and VLA-4, respectively, as confirmed by blocking with specific mAbs (Figure 4, A and C). Both D12 and N17 H-Ras impaired the transient regulation of LFA-1 avidity induced by SDF-1$\alpha$ (Figure 4A). In contrast, neither the active nor inactive form of H-Ras affected the regulation of VLA-4 avidity at different substrate densities of VCAM-1 (Figure 4C; our unpublished data). Moreover, both forms of H-Ras partially impaired Jurkat cell adhesion to ICAM-1 induced by the phorbol ester phorbol-12-myristate-13-acetate (PMA) (Figure 4B), whereas only the inactive form of H-Ras appeared to slightly inhibit PMA-induced adhesion to VCAM-1 (Figure 4D). These data sug-

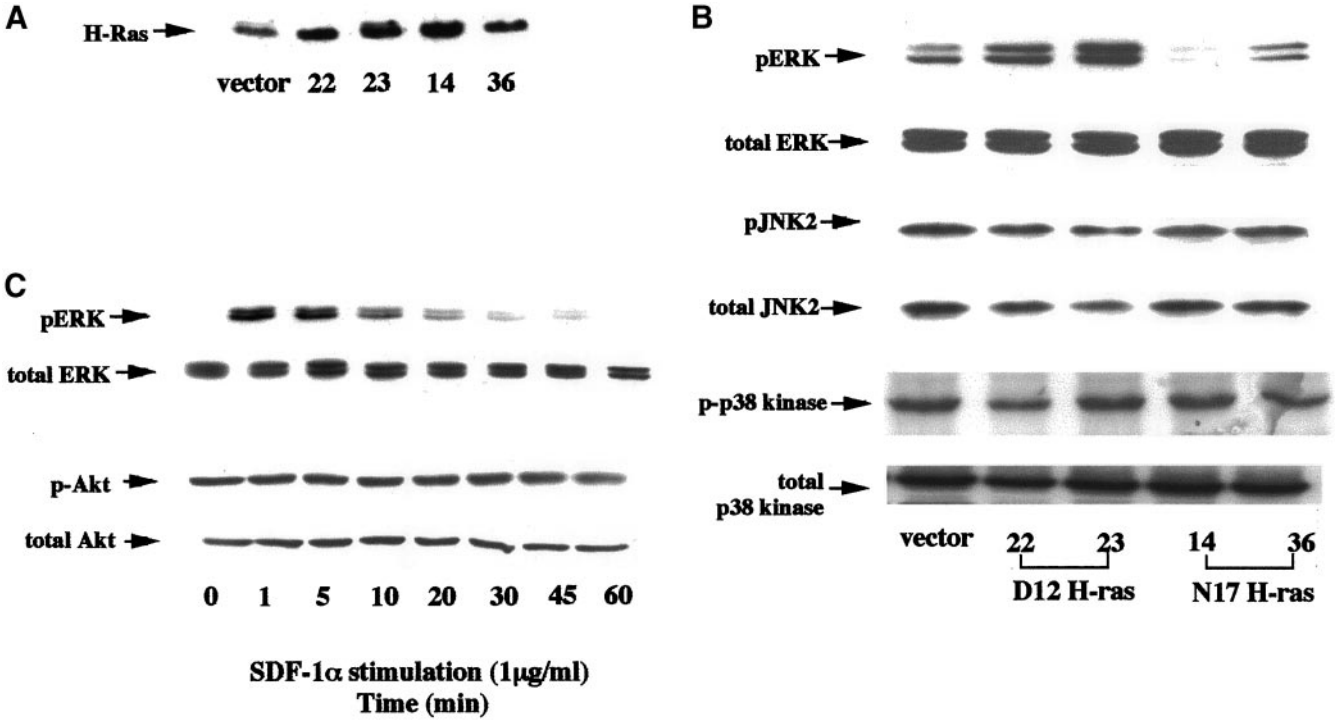


**Figure 2.** Expression of dominant active (D12) or inactive (N17) forms of H-Ras results in a selective regulation of phosphorylated ERK. Jurkat cells were stably transfected with vector alone, active (D12) or inactive (N17) H-Ras. Cell lysates from selected clones were separated by 10% SDS-PAGE and specific mAbs were used to detect total H-Ras (A), and phosphorylated and total ERK, JNK, and p38 kinase (B). Jurkat/N17 H-Ras transfectants were stimulated with SDF-1$\alpha$ (1 $\mu$g/ml) for indicated times, cell lysates were separated by 10% SDS-PAGE, and specific mAbs were used to detect phosphorylated and total ERK and Akt (C). Shown are representative blots from at least three separate experiments.

gest that H-Ras is involved in chemokine-induced regulation of LFA-1 but not VLA-4 avidity. To exclude that these observations were complicated by the use of stably transfected cell lines derived from single clones resulting in secondary adaptions, e.g., selection for altered regulation of other genes, or effects induced by the H-Ras mutants on the mRNA transcription of other signaling elements, we also performed select experiments with cells transiently transfected with the same constructs. Expression of a GFP protein confirmed that the transfection efficiency was equivalent among the cell populations used (Figure 5A). Adhesion assays on immobilized ICAM-1 indeed confirmed that the transient expression of either D12 or N17 H-Ras impaired the regulation of LFA-1 avidity by SDF-1$\alpha$ (Figure 5B).

### *Role of H-Ras in LFA-1-mediated Transendothelial Chemotaxis Stimulated by SDF-1$\alpha$*

Because both $\beta$1 and $\beta$2 can contribute to transendothelial chemotaxis of leukocytes, we performed chemotaxis assays toward an SDF-1$\alpha$ gradient with the use of the D12 and N17 H-Ras transfectants. Across bare filters, wild-type Jurkat cells, or D12 or N17 H-Ras transfectants revealed comparable levels of integrin-independent migration induced by SDF-1$\alpha$ (Figure 6A), confirming that the intrinsic motility of these cell types was equivalent (Weber *et al.*, 1997a). In contrast, both D12 H-Ras and N17 H-Ras significantly impaired transmigration across filters coated with endothelial cells, toward an SDF-1$\alpha$ gradient (Figure 6B). Inhibition with a blocking mAb to LFA-1 demonstrated that transendothelial chemotaxis of wild-type Jurkat cells was largely LFA-1-dependent, whereas blocking LFA-1 expressed on D12 or N17 H-Ras transfectants did not affect transmigration (Figure 6B). Thus, these data confirm a requirement for dynamic regulation of LFA-1 during transendothelial chemotaxis and implicate an important role for H-Ras in controlling this process. In transmigration experiments across filters coated with the specific ligands of VLA-4, the 40-kDa fragment of fibronectin containing connecting segment 1 or VCAM-1, we did not observe a significant difference in the chemotaxis of wild-type Jurkat cells, or D12 or N17 H-Ras transfectants induced by SDF-1$\alpha$ (Figure 6C; our unpublished data). Inhibition of VLA-4 equivalently increased transmigration across FN40-coated filters (Figure 6C). This is most likely due to the constitutively active state of VLA-4 expressed on Jurkat cells, and confirms previous observations that the inhibition of this VLA-4 activity facilitates transmigration of Jurkat cells (Weber et al., 1996b). These data indicate that H-Ras mutants did not affect VLA-4–dependent transmigration, and thus parallel and further support our findings that H-Ras specifically participates in LFA-1 but not VLA-4 avidity regulation by chemokines.

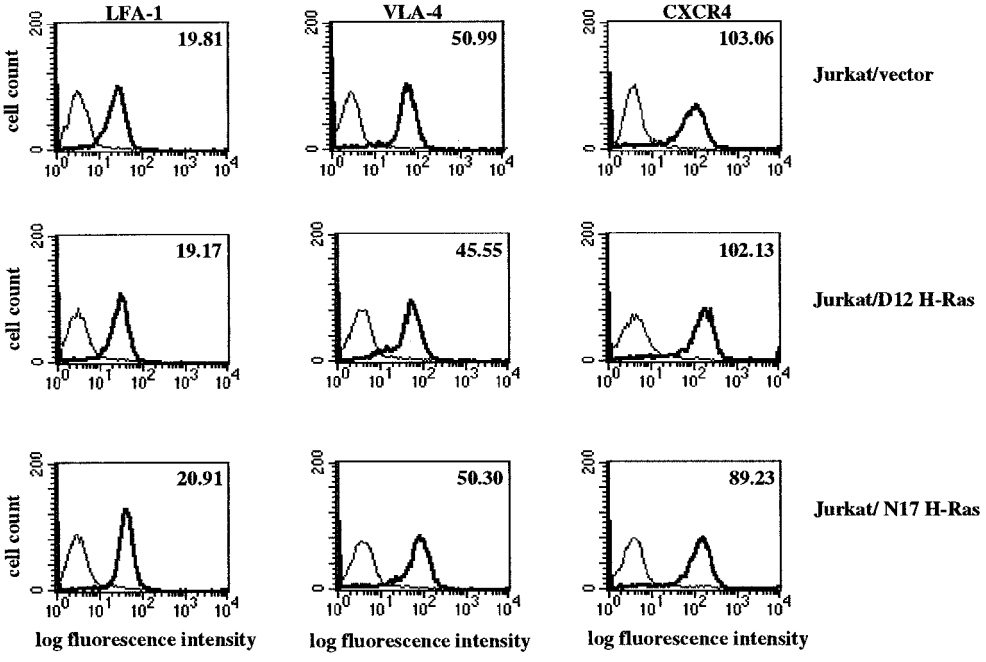


**Figure 3.** Expression of LFA-1, VLA-4, and CXCR4 on Jurkat cell transfectants. Jurkat cells transfected with vector alone, active (D12) or inactive (N17) H-Ras were stained with specific mAbs to LFA-1, VLA-4, and CXCR4 (bold lines) and the corresponding isotype controls (thin lines). Surface expression was measured by FACScan with appropriate gates. Shown are representative histograms with the respective specific mean fluorescence intensities.

### *H-Ras May Act as Negative Regulator of LFA-1 Affinity Induction via Raf-1/ERK Kinase*

The ability of H-Ras to suppress integrin activation has been described in Chinese hamster ovary cell transfectants (Hughes *et al.*, 1997). In leukocytes stimulated by T-cell receptor engagement or IL-3, H-Ras has been found to mediate an induction of integrin avidity via cytoskeletal changes; however, a direct involvement of the Raf-1/ERK kinase has not been demonstrated (O'Rourke *et al.*, 1998; Tanaka *et al.*, 1999). This may be due to a difference in the affinity state of the integrin, depending on cellular environment. Hence, we characterized the mutant cell clone J19, expressing LFA-1 in a constitutively active form. As previously described (Weber *et al.*, 1999a), surface expression of LFA-1, comparison of purified LFA-1 in adhesion assays, and DNA sequencing of $\alpha$L and $\beta$2 cytoplasmic domain cDNA generated by reverse transcription-polymerase chain reaction confirmed that the LFA-1 molecule expressed by the J19 cells was unchanged to that of the wild-type Jurkat clone (Jn.9), suggesting the presence of a signaling defect (our unpublished data). Analysis of the level of phosphorylated ERK in the Jurkat and J19 cells revealed a significantly reduced level of phosphorylated ERK in the J19 cells, suggesting a defect in the H-Ras/Raf-1/ERK pathway (Figure 7A). In contrast, we did not observe a significant difference in the levels of JNK and p38 phosphorylation or in the total expression of the MAP kinases in Jurkat and J19 cells (Figure 7A). To determine whether the levels of phosphorylated ERK could be restored by H-Ras, we overexpressed dominant active D12H-Ras in J19 cells (Figure 7B). Transfection of J19 cells with dominant active D12 H-Ras at least partially restored the levels of phosphorylated ERK without affecting expression of total or phosphorylated JNK and p38 kinase (Figure 7A). Flow cytometric analysis confirmed equivalent levels of LFA-1 on J19 cells and J19/D12 H-Ras transfectants (Figure 8A). In adhesion assays, the binding of unstimulated J19/D12 H-Ras transfectants to immobilized ICAM-1 was significantly lower than that observed with the mutant J19 cells (Figure 8B). To investigate whether the J19 cells express LFA-1 in a high-affinity form, we performed soluble binding assays to ICAM-1.Ig. Consistent with a previous report (Stewart *et al.*, 1996), we found that the binding of soluble ICAM-1 in the presence of $Mg^{2+}$ and $Ca^{2+}$ low on resting Jurkat cells but can be increased by removing $Ca^{2+}$ in am Mg/EGTA buffer (Figure 8C). Specific binding was confirmed by preincubation with a blocking mAb to LFA-1 (Figure 8C). Notably, we observed that J19 cells bound ICAM-1 more efficiently than wild-type cells even in the presence of $Ca^{2+}$, indicating the expression of receptors in a high-affinity form (Figure 8C). However, transfection of J19 cells with D12 H-Ras reduced the binding of soluble ICAM-1.Ig (Figure 8C). In addition, we investigated the effects of the H-Ras mutants on the binding of soluble ICAM-1 to wild-type Jurkat cells induced by SDF-1$\alpha$. In accordance with a previous study (Constantin *et al.*, 2000), we found that wild-type Jurkat cells rapidly bound soluble ICAM-1 in response to SDF-1$\alpha$ (Figure 8D). Moreover, the rapid increase in LFA-1–dependent binding of soluble ICAM-1.Ig stimulated by SDF-1$\alpha$ at 1 min was inhibited by dominant active D12 H-Ras but not by dominant inactive N17 H-Ras (Figure 8D). These experiments infer that active H-Ras may impair the induction of high-affinity receptors by SDF-1$\alpha$. Together, our data indicate that H-Ras can negatively regulate the high-affinity form of LFA-1, thereby reducing cell adhesion to ICAM-1.

### *Activation of LFA-1 by SDF-1$\alpha$ Depends on PI3-K, whereas Down-Regulation Is Mediated by ERK*

Known effectors of H-Ras, e.g., Raf-1/ERK kinase pathway and PI3-K, have been implicated in integrin regulation. Al-

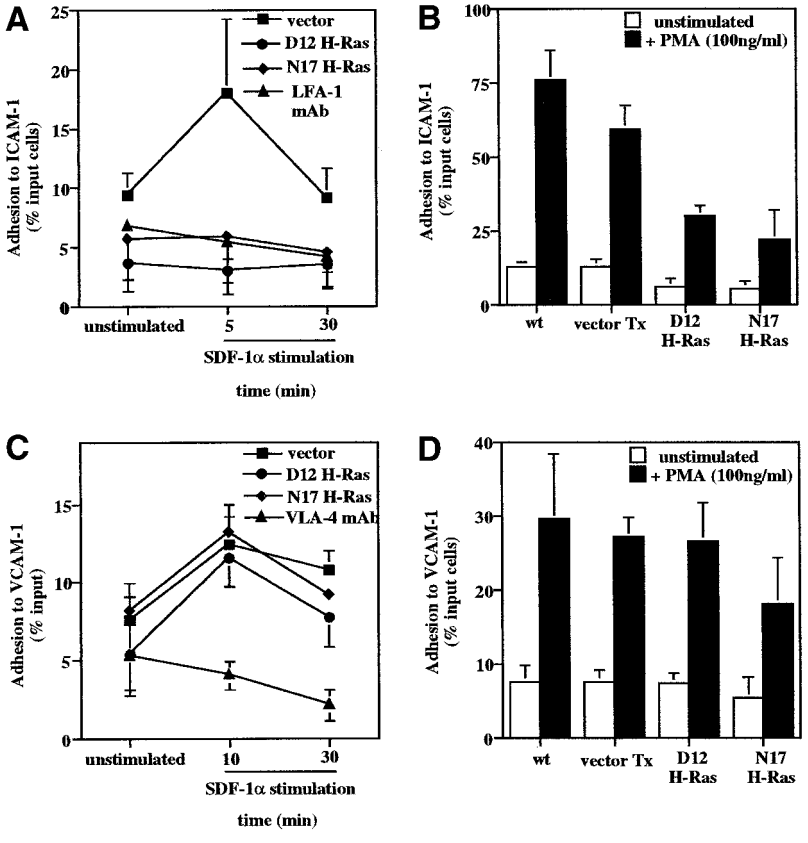


**Figure 4.** Adhesion of Jurkat/D12 and Jurkat/N17 transfectants to ICAM-1 and VCAM-1. BCECF-AM–labeled Jurkat cell transfectants were stimulated with SDF-1$\alpha$ (1 $\mu$g/ml) (A and C) or PMA (100 ng/ml) (B and D) and allowed to adhere to ICAM-1 (A and B) or VCAM-1 (C and D) for indicated times. Nonadherent cells were removed by a flick wash. For mAb inhibition, vector-transfected cells were preincubated with the anti-LFA-1 mAb (TS1/22) or the anti-VLA-4 mAb (HP1/2) or the respective isotype control for 30 min on ice before being added to the assay. Fluorescence of input and adherent cells was measured in a fluorescence plate reader. Data represent the mean ± SD of at least four separate experiments performed in triplicate.

though the Raf-1/ERK kinase pathway may mediate a suppression in integrin affinity, PI3-K has been shown to be involved in the activation of integrins (Hughes *et al.*, 1997; Capodici *et al.*, 1998; Nagel *et al.*, 1998; Constantin *et al.*, 2000). Because the transient regulation of LFA-1 avidity by SDF-1$\alpha$ was impaired by both the active and inactive forms of H-Ras, we investigated whether this pattern of regulation was mediated by sequential signals via PI3-K and the Raf-1/ERK kinase pathway. Adhesion assays with Jurkat cells to immobilized ICAM-1 demonstrated that inhibition of PI3-K with wortmannin resulted in an impairment of the initial increase in LFA-1–mediated adhesion; however, at later time points, the levels of adhesion observed were similar to that of untreated Jurkat cells (Figure 9A). In contrast, inhibition of MEK kinase with PD 98059 did not affect the level of adhesion at early time points; however, the subsequent decrease in adhesion was impaired (Figure 9C). Thus, it appears that SDF-1$\alpha$ activation of LFA-1 involves an early up-regulation of adhesion via PI3-K and a subsequent down-regulation of adhesion was mediated by the Raf-1/ERK kinase pathway. The striking parallel between the effects on LFA-1 avidity regulation exerted by stable expression of dominant inactive and active H-Ras mutants and by treatment with pharmacological inhibitors of H-Ras effectors is further indicative of the notion that the effects observed were unlikely due to alterations in gene regulation or adaptations induced by the transfections. In contrast, adhesion assays on immobilized VCAM-1 revealed that the transient regulation of VLA-4 avidity was not significantly altered by inhibition of either PI3-K or ERK at different substrate densities of VCAM-1 tested (Figure 9, B and D; our unpublished data). It has been reported that PI3-K might activate MEK kinase and subsequently ERK (King *et al.*, 1997). This suggests that the down-regulation of LFA-1 avidity induced by ERK may occur as a result from the early activation of PI3-K. Notably, in combination, inhibition of PI3-K and MEK kinase resulted in a gradual increase in adhesion to ICAM-1 but did not affect the regulation of adhesion to VCAM-1 (Figure 9, E and F). This indicates that SDF-1$\alpha$ induced regulation of LFA-1 avidity requires a sequential involvement of both pathways downstream of H-Ras.

## DISCUSSION

In this report, we demonstrate that the small GTPase H-Ras is involved in the regulation of integrin avidity and leukocyte chemotaxis induced by the CXC chemokine SDF-1$\alpha$. Furthermore, investigation of the downstream effectors of H-Ras revealed that the sequential action of PI3-K and ERK participated in mediating the transient regulation of LFA-1 avidity by chemokines.

It has previously been reported that H-Ras can negatively regulate integrin activation via the Raf-1/MEK/ERK kinase pathway (Hughes *et al.*, 1997). In this study, it was demonstrated that H-Ras impairs expression of the $\alpha$IIb$\beta$3 activation epitope recognized by the reporter mAb PAC1 in Chinese hamster ovary transfectants, suggesting that it may

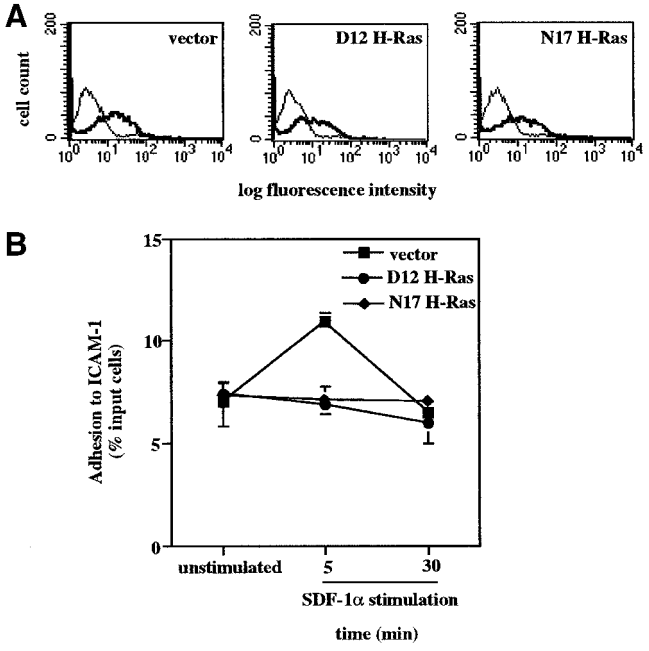


**Figure 5.** Adhesion of Jurkat cells transiently transfected with H-Ras mutants on ICAM-1. (A) Cells were transiently cotransfected with vector, or D12 or N17 H-Ras together with a cDNA-encoding GFP. Expression of GFP was measured by flow cytometry. (B) Adhesion of vector, D12 and N17 H-Ras Jurkat transfectants to ICAM-1. Transfectants labeled with BCECF-AM were stimulated with SDF-1α (1 μg/ml) and allowed to adhere to ICAM-1 for indicated times. Nonadherent cells were removed by a flick wash. Fluorescence of input and adherent cells was measured in a fluorescence plate reader. Data represent the mean ± SD of three experiments.

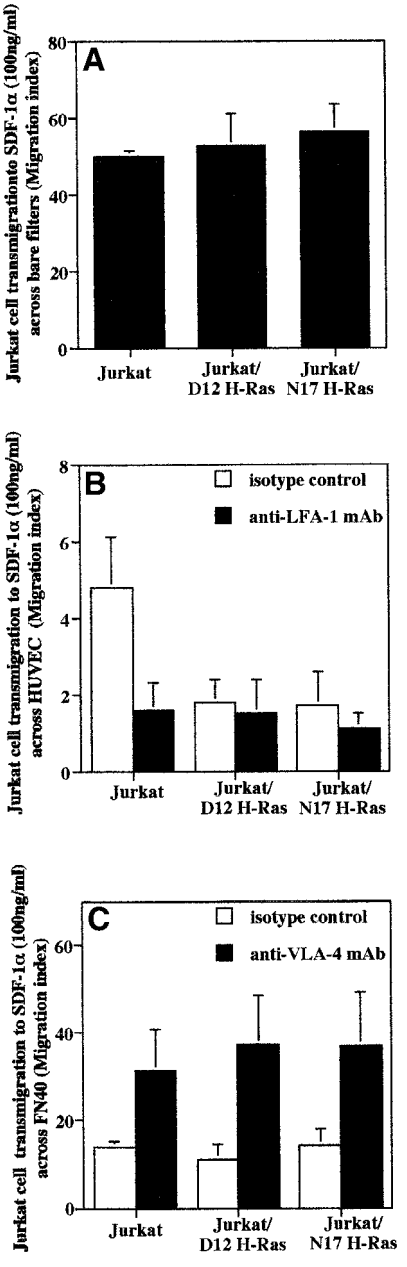


**Figure 6.** Transmigration of Jurkat cell transfectants to SDF-1α. Jurkat cell transfectants were allowed to transmigrate across bare filters (A), filters coated with endothelial cells (B) or the 40-kDa fragment (FN40) of fibronectin (C) to an SDF-1α gradient for 3 h (A and C) or 4 h (B). For mAb inhibition, cells were pretreated with LFA-1 mAb TS1/22, VLA-4 mAb HP1/2, or isotype control (all 10 μg/ml) for 30 min on ice which were kept present during the assays (B). Migrated cells and input were counted by flow cytometry with the use of standard beads. Data are expressed as migration index and shown are the mean ± SD of at least three separate experiments performed in duplicate.

function to suppress the affinity of the integrin (Hughes *et al.*, 1997). Our results now provide three separate lines of evidence to support this concept. First, characterization of the mutant J19 cells, which express LFA-1 in a high-affinity state revealed a reduced amount of phosphorylated ERK, suggestive of a signal transduction defect. Transfection of J19 cells with the active form of H-Ras restored the level of phosphorylated ERK and reduced constitutive LFA-1 affinity, indicating that H-Ras may negatively regulate the activity of LFA-1 in leukocytes when expressed in a high-affinity state. Second, expression of active D12 H-Ras inhibited the rapid increase in LFA-1 binding to soluble ICAM-1 induced by SDF-1α. Third, specific inhibition of MEK kinase with PD 98059 inhibited the down-regulation of LFA-1–mediated adhesion in response to SDF-1α. Together, these findings suggest that the H-Ras/ERK pathway can suppress a high-avidity state of LFA-1 induced by chemokines in leukocytes, and may also imply an active role for H-Ras in the down-regulation of LFA-1 activity by chemokines.

We found that both active and inactive forms of H-Ras impaired the modulation of LFA-1 avidity by SDF-1α. Although the down-regulation of LFA-1 avidity appeared to be mediated by the ERK pathway, activation of PI3-K was responsible for the rapid increase in LFA-1–dependent adhesion. Notably, our results indicate that N17 H-Ras only slightly impaired the increase in ERK phosphorylation but almost completely abrogated Akt phosphorylation in response to SDF-1α. These differences may reflect that SDF-1 is a very potent stimulus for ERK phosphorylation as evident

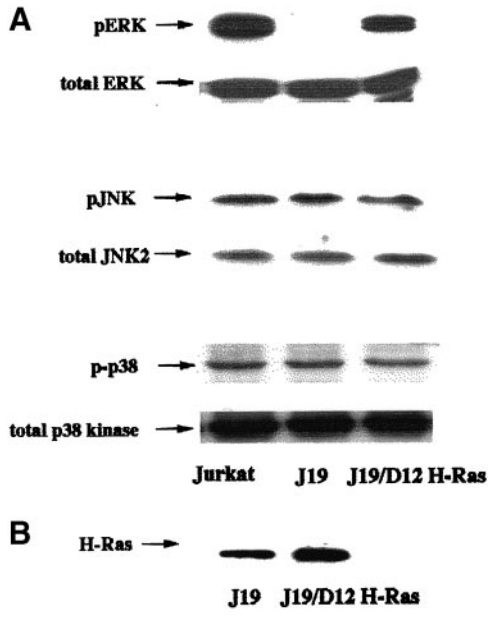


**Figure 7.** Transfection of active H-Ras in the mutant J19 cells restores expression of phosphorylated ERK. J19 cells were transfected with dominant active D12 H-Ras. (A) Cell lysates from wild-type Jurkat, J19/vector, and J19/D12 H-Ras were separated by 10% SDS-PAGE and specific mAbs were used to detect phosphorylated and total ERK, JNK, and p38 kinase (A) and total H-Ras (B). Shown are representative blots from three separate experiments.

in wild-type Jurkat cells, whereas PI3-K activation may be more susceptible to dominant inactive H-Ras. This was consistent with a report (Shibayama *et al.*, 1999) demonstrating that pERK was still up-regulated upon IL-3 stimulation in cells expressing dominant inactive H-Ras. Alternatively, an upstream regulator of ERK may bypass H-Ras. Nevertheless, it should rather be emphasized that the effects of N17 H-Ras are most likely due to the inhibition of PI3-K activation. A recent report has shown that activation of LFA-1 by chemokines involves a rapid increase in both lateral clustering and affinity changes (Constantin *et al.*, 2000). Although PI3-K appeared to be important in mediating lateral mobility, it did not play a direct role in inducing the high-affinity state of LFA-1, although PI3-K–dependent lateral mobility may facilitate the induction of high-affinity receptors. Notably, our findings that dominant inactive N17 H-Ras, which predominantly inhibits PI3-K activation, did not interfere with the induction of soluble ICAM-1 binding by SDF-1$\alpha$ would support that PI3-K is not involved in the up-regulation of LFA-1 affinity.

As a direct effector of H-Ras, PI3-K has been implicated in integrin activation and cell adhesion (Rodriguez-Viciana *et al.*, 1994; Kolanus and Seed, 1997; Jones *et al.*, 1998). PI3-K has been demonstrated to activate LFA-1–mediated adhesion by inducing the membrane recruitment of cytohesin-1, which directly interacts with the $\beta$2 subunit (Nagel *et al.*, 1998). It has also been shown that activation of PI3-K may be important for the activation of the Raf-1/ERK pathway (King *et al.*, 1997; Chaudhary *et al.*, 2000). Because inhibition of PI3-K has been shown to impair ERK phosphorylation by chemokines (Sotsios *et al.*, 1999), this suggests that a sequential involvement of PI3-K and ERK may play a role in the transient regulation of LFA-1 avidity by chemokines. Thus, although inactive H-Ras impairs activation of PI3-K necessary for the early up-regulation in LFA-1 avidity, overexpression of dominant active H-Ras may predominantly induce the Raf-1/ERK kinase pathway impairing LFA-1 activation by chemokines. It has also been reported that PI3-K may be involved in the activation of the $\beta$2 integrin Mac-1 by the chemoattractant formyl-methionyl-leucyl-phenylalanine, independently of ERK (Capodici *et al.*, 1998). In addition, active H-Ras can cause sustained LFA-1–specific adhesion to ICAM-1 or endothelial cells, which was mediated by PI3-K and triggered by immobilized or endogenous MIP-1$\alpha$ (Tanaka *et al.*, 1999). In contrast, we and others have found that soluble SDF-1$\alpha$ induced a transient increase in LFA-1 avidity (Campbell *et al.*, 1998; Weber *et al.*, 1999a; Constantin *et al.*, 2000). These differences may suggest chemokine-specificity in signaling pathways, which may then influence integrin regulation.

In contrast to LFA-1, we did not observe any effect of dominant inactive or active H-Ras mutants on VLA-4 regulation or VLA-4–dependent chemotaxis of Jurkat cells induced by SDF-1$\alpha$. Our findings demonstrate that neither the H-Ras/Raf-1/ERK pathway nor PI3-K was involved in VLA-4 activation by SDF-1$\alpha$ and thus support a recent findings in myeloma cells (Sanz-Rodriguez *et al.*, 2001). Our data thus confirm that the mechanisms involved in chemokine-induced activation of $\beta$1 and $\beta$2 integrins may differ (Weber *et al.*, 1996b). It has been proposed that the transient activation of VLA-4 by chemokines is independent of changes in affinity or conformation. Rather, chemokines have been shown to induce a transient avidity regulation of VLA-4 expressed on monocytes and eosinophils in a manner dependent on actin cytoskeletal rearrangements (Weber *et al.*, 1996a,b). Although the involvement of PI3-K in regulation of LFA-1 avidity appears to depend on the substrate density (Constantin *et al.*, 2000), we did not observe any effects of wortmannin or LY29004 on VLA-4 adhesion to very low concentrations of VCAM-1 (our unpublished data). The fact that VLA-4 regulation did not appear to be affected by the pathways studied may also reflect or be due to a preexisting relatively high-avidity state of VLA-4 expressed on unstimulated Jurkat cells. In extension, this may infer that PI3-K and ERK are only involved in the chemokine regulation of integrins (e.g., LFA-1), which are maintained in a default low-affinity state under resting conditions.

Recently, it has been reported that the Src kinase Lck may be associated with a preexistent high-affinity state of VLA-4 (Feigelson *et al.*, 2001). Lck kinase may up-regulate VLA-4 affinity and may thereby facilitate rapid spontaneous, as well as chemokine-induced adhesion of T cells mediated by VLA-4. However, stimulation of ERK in response to chemokines was independent of the presence of lck kinase and this also supports our findings that the H-Ras/Raf/ERK kinase pathway is not involved in VLA-4 avidity regulation by chemokines. Although our findings did not reveal a role for H-Ras in VLA-4 activation, this does not exclude that VLA-4 plays a crucial role in leukocyte recruitment in vivo and in vitro. In contrast to stimulation with chemokines, H-Ras has been found to signal VLA-4 activation induced by IL-3, which was however not dependent on PI3-K (Shibayama *et*

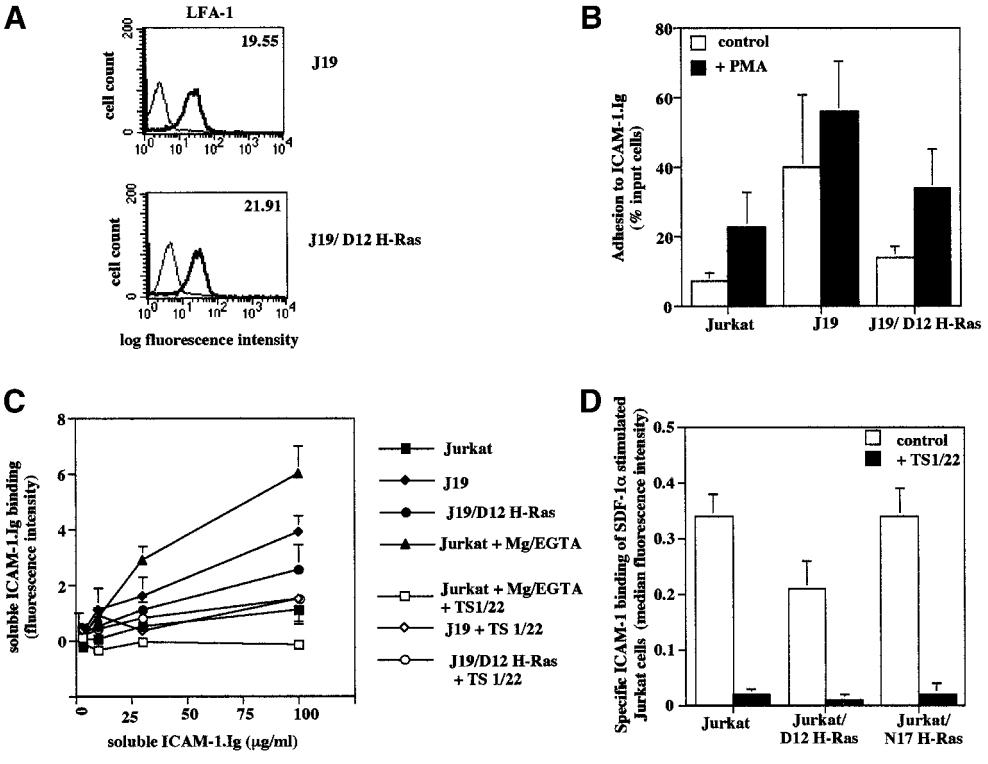


**Figure 8.** Transfection of active H-Ras restores low constitutive affinity of LFA-1 in the mutant J19 cells and impairs ICAM-1 binding upon SDF-1$\alpha$ stimulation. (A) Expression of LFA-1 on J19 cell transfectants. J19 and J19/D12 H-Ras cell transfectants were stained with specific mAb to LFA-1 (bold lines) and the corresponding isotype controls (thin lines). Surface expression was measured by FACScan with appropriate gates. Data shown are representative histograms and specific mean fluorescence intensities. (B) Jurkat, J19/vector and J19/D12 H-Ras cells were stimulated with PMA or left untreated and allowed to adhere to immobilized ICAM-1. Nonadherent cells were removed by a flick wash. Fluorescence of input and adherent cells was measured in a fluorescence plate reader. Data represent the mean ± SD of four separate experiments performed in triplicate. (C) Jurkat, J19/vector, and J19/D12 H-Ras cells were incubated with increasing concentrations of soluble ICAM-1. Ig in TBS/2 mM $Mg^{2+}$/1 mM $Ca^{2+}$/0.5% BSA at 37°C for 1 h, washed, and stained with FITC-conjugated anti-human IgG. Jurkat cells were also incubated in TBS/10 mM $Mg^{2+}$/1 mM EGTA/0.5% BSA to induce the maximal binding to soluble ICAM-1.Ig. Binding was confirmed to be specific for LFA-1 by preincubation with the blocking LFA-1 mAb TS1/22. Shown is the mean fluorescence intensity. Data represent mean ± SD of three separate experiments. (D) Jurkat cells and transfectants were incubated with soluble ICAM-1.Ig (100 $\mu$g/ml) and FITC-conjugated antihuman Ig for 30 min, stimulated with SDF-1$\alpha$ (1 $\mu$g/ml) for 1 min, fixed, and analyzed in a FACScan. Induction of ICAM-1 binding was expressed as specific median fluorescence intensity stimulated by SDF-1$\alpha$. The presence of LFA-1 mAb TS1/22 inhibited induction of ICAM-1 binding, revealing that it was mediated by LFA-1. Data represent mean ± SD of three separate experiments.

*al.*, 1999) and has been implicated in LFA-1 activation after T-cell receptor engagement (O'Rourke *et al.*, 1998). This infers that the involvement of H-Ras may not only be integrin specific but also fundamentally dependent on the type of stimulus.

We observed that expression of either the active or inactive forms of H-Ras impaired transendothelial migration to SDF-1$\alpha$, which was largely dependent on LFA-1, as demonstrated by blocking with LFA-1 mAb (Weber *et al.*, 1997a). Both PI3-K and ERK have been implicated in leukocyte chemotaxis (Turner *et al.*, 1995; Weber *et al.*, 1998a; Sotsios *et al.*, 1999). Given the role of H-Ras in LFA-1 avidity regulation, this confirms the requirement for a dynamic regulation of LFA-1 during chemotaxis across unstimulated endothelium (Weber *et al.*, 1996b, 1997a). Another mechanism implicating H-Ras during cell migration is the effects on actin cytoskeletal remodeling. H-Ras may induce cell spreading and active H-Ras has been shown to increase the chemotactic migration of skeletal myoblasts (Rodriguez-Viciana *et al.*, 1997; Suzuki *et al.*, 2000). However, it has also been reported that active Raf-1 induced rounding of cells of fibronectin and that a reduction in H-Ras activity may be important in the initiation of migration (Lee *et al.*, 1996; Hughes *et al.*, 1997). Our findings suggest that a cyclical activity of H-Ras is necessary for leukocyte chemotaxis, which may reflect the dynamic regulation of LFA-1 but does not exclude effects on the actin cytoskeleton. This extends findings that the regulation of another small GTPase, i.e., cdc42, is critical for chemokine-induced transendothelial migration of leukocytes by mediating actin-based filopodia formation and polarization rather than by affecting integrin avidity (Weber *et al.*, 1998a).

Cell migration requires the coordination of multiple interdependent cellular events involving numerous signaling molecules. During this process, activation and deactivation of integrins are important in the dynamic adhesive steps critical for cell migration. Furthermore, integrins may trigger various downstream cellular signaling pathways that are involved in cell adhesion. It has been proposed that the differential regulation of specific integrins by chemokines contributes to the individual events during leukocyte chemotaxis, i.e., although VLA-4 mediates lateral migration, LFA-1 is critical for transendothelial migration. We demonstrate here that H-Ras may positively or negatively regulate LFA-1 avidity via activation of PI3-K and the Raf/ERK kinase pathway, respectively. Given the importance of LFA-1 during transendothelial migration, these data reiterate the complex processes involved for successful leukocyte trafficking.

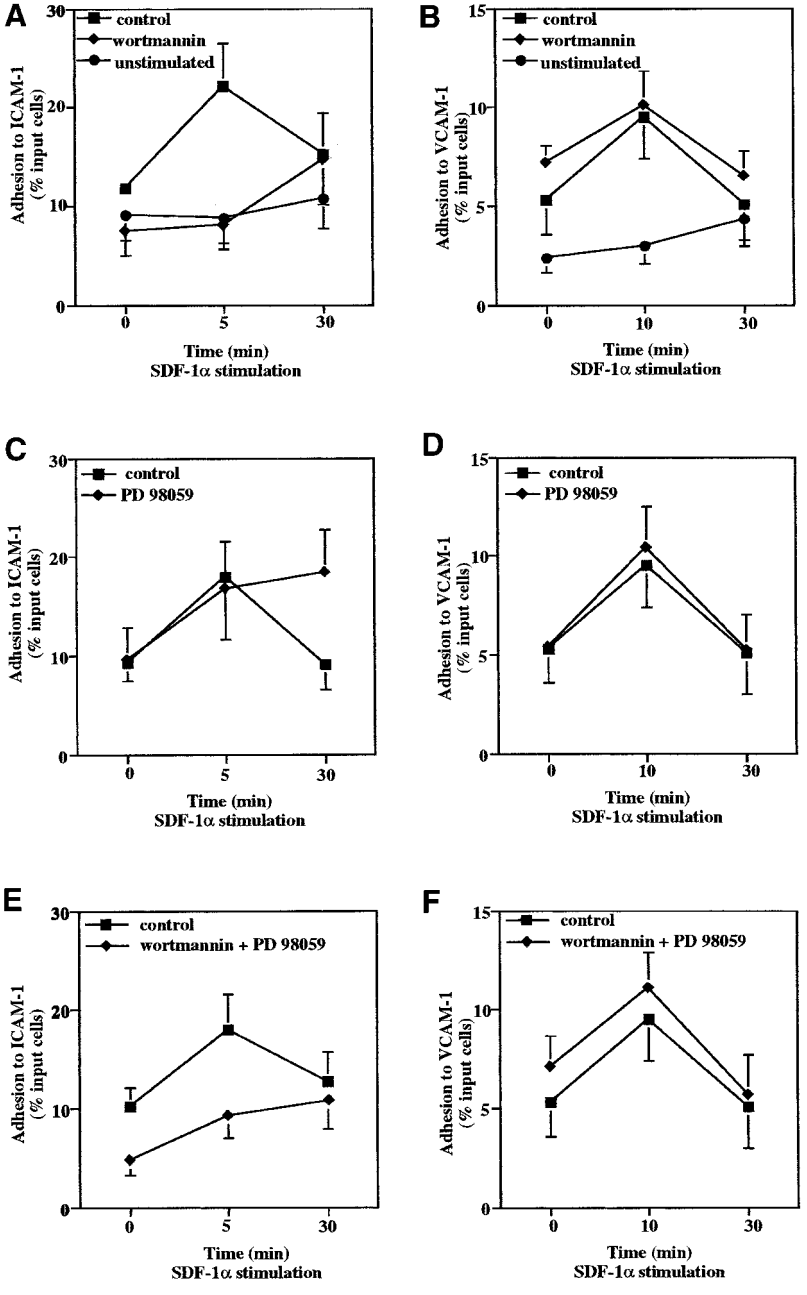


**Figure 9.** Involvement of PI3-K and the Raf-1/ERK kinase pathway in chemokine-induced integrin regulation. Jurkat cells were stimulated with SDF-1α (1 μg/ml) or left unstimulated and allowed to adhere for indicated times to immobilized ICAM-1 (A, C, and E) and VCAM-1 (B, D, and F). For inhibition of PI3-K and MEK kinase, cells were preincubated with wortmannin (A and B) and PD 98059 (C and D), respectively, or both (E and F) for 15 min at 37°C. Nonadherent cells were removed and fluorescence of input and adherent cells was measured in a fluorescence plate reader. Data represent the mean ± SD of at least four separate experiments performed in triplicate.

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## REFERENCES

Baggiolini, M. (1998). Chemokines and leukocyte traffic. Nature *392*, 565–568.

Campbell, J.J., Hedrick, J., Zlotnik, A., Siani, M.A., Thompson, D.A., and Butcher, E.C. (1998). Chemokines and the arrest of lymphocytes rolling under flow conditions. Science *279*, 381–384.

Capodici, C., Hanft, S., Feoktistov, M., and Pillinger, M.H. (1998). Phosphatidylinositol 3-kinase mediates chemoattractant-stimulated, CD11b/CD18-dependent cell-cell adhesion of human neutrophils: evidence for an ERK-independent pathway. J. Immunol. *160*, 1901–1909.

Chaudhary, A., King, W.G., Mattaliano, M.D., Frost, J.A., Diaz, B., Morrison, D.K., Cobb, M.H., Marshall, M.S., and Brugge, J.S. (2000). Phosphatidylinositol 3-kinase regulates Raf1 through Pak phosphorylation of serine 338. Curr. Biol. *10*, 551–554.

Clark, E.A., and Brugge, J.S. (1995). Integrins and signal transduction pathways: the road taken. Science *268*, 233–238.

Constantin, G., Majeed, M., Giagulli, C., Piccio, L., Kim, J.-Y., Butcher, E.C., and Laudanna, C. (2000). Chemokines trigger imme-

diate β2 integrin affinity and mobility changes: differential regulation and roles in lymphocyte arrest under flow. Immunity *13*, 759–769.

Dedhar, S., and Hannigan, G.E. (1996). Integrin cytoplasmic interactions and bidirectional transmembrane signaling. Curr. Opin. Cell. Biol. *8*, 657–669.

Diamond, M.S., and Springer, T.A. (1994). The dynamic regulation of integrin adhesiveness. Curr. Biol. *4*, 506–517.

Feigelson, S.W., Grabovsky, V., Winter, E., Chen, L.L., Pepinsky, R.B., Yednock, T., Yablonski, D., Lobb, R., and Alon, R. (2001). The Src kinase p56Lck upregulates VLA-4 integrin affinity: implications for rapid spontaneous and chemokine-triggered T cell adhesion to VCAM-1 and fibronectin. J. Biol. Chem. *276*, 13891–13901.

Ganju, R.K., Brubaker, S.A., Meyer, J., Dutt, P., Yang, Y., Qin, S., Newman, W., and Groopman, J.E. (1998). The α-chemokine, stromal cell-derived factor-1α, binds to the transmembrane G-protein-coupled CXCR-4 receptor and activates multiple signal transduction pathways. J. Biol. Chem. *273*, 23169–23175.

Geiger, C., Nagel, W., Boehm, T., van Kooyk, Y., Figdor, C.G., Kremmer, E., Hogg, N., Zeitlmann, L., Dierks, H., Weber, K.S., and Kolanus, W. (2000). Cytohesin-1 regulates Symbol" § 122 integrin-mediated adhesion through both ARF-GEF function and interaction with LFA-1. EMBO J. *19*, 2525–2536.

Giancotti, F.G., and Ruoslahti, E. (1999). Integrin signaling. Science *285*, 1028–1032.

Hato, T., Pampori, N., and Shattil, S.J. (1998). Complementary roles for receptor clustering and conformational change in the adhesive and signaling functions of integrin αIIbβ3. J. Cell Biol. *141*, 1685–1695.

Hesselgesser, J., Liang, M., Hoxie, J., Greenberg, M., Brass, L.F., Orsini, M.J., Taub, D., and Horuk, R. (1998). Identification and characterization of the CXCR4 chemokine receptor in human T cell lines: ligand binding, biological activity, and HIV-1 infectivity. J. Immunol. *160*, 877–883.

Humphries, M.J. (1996). Integrin activation: the link between ligand binding and signal transduction. Curr. Opin. Cell. Biol. *8*, 632–640.

Hughes, P.E., Renshaw, M.W., Pfaff, M., Forsyth, J., Keivens, V.M., Schwartz, M.A., and Ginsberg, M.H. (1997). Suppression of integrin activation: a novel function of a Ras/Raf-initiated MAP kinase pathway. Cell. *88*, 521–530.

Hwang, S.T., Singer, M.S., Giblin, P.A., Yednock, T.A., Bacon, K.B., Simon, S.I., and Rosen, S.D. (1996). GlyCAM-1, a physiologic ligand for L-selectin, activates β2 integrins on naive peripheral lymphocytes. J. Exp. Med. *84*, 1343–1348.

Hynes, R.O. (1992). Integrins: versatility, modulation and signaling in cell adhesion. Cell. *69*, 11–25.

Jones, S.L., Knaus, U.G., Bokoch, G.M., and Brown, E.J. (1998). Two signaling mechanisms for activation of αMβ2 avidity in polymorphonuclear neutrophils. J. Biol. Chem. *273*, 19556–19666.

King, W.G., Mattaliano, M.D., Chan, T.O., Tsichlis, P.N., and Brugge, J.S. (1997). Phosphatidylinositol 3-kinase is required for integrin-stimulated AKT and Raf-1/mitaogen-activated protein kinase pathway activation. Mol. Cell. Biol. *17*, 4406–4418.

Kolanus, W., and Seed, B. (1997). Integrins and inside-out signal transduction: converging signals from PKC and PIP3. Curr. Opin. Cell. Biol. *9*, 725–731.

Lee, T., Feig, L., and Montell, D.J. (1996). Two distinct roles for Ras in a developmentally regulated cell migration. Development *122*, 409–418.

Lub, M., van Kooyk, Y., van Vliet, S.J., and Figdor, C.G. (1997). Dual role of the actin cytoskeleton in regulating cell adhesion mediated by the integrin lymphocyte function-associated molecule-1. Mol. Biol. Cell *8*, 341–351.

Nagel, W., Zeitlmann, L., Schilcher, P., Geiger, C., Kolanus, J., and Kolanus, W. (1998). Phosphoinositide 3-OH kinase activates the β2 integrin adhesion pathway and induces membrane recruitment of cytohesin-1. J. Biol. Chem. *273*, 14853–14861.

Nelson, P.J., and Krensky, A.M. (1998). Chemokines, lymphocytes and viruses: what goes around, comes around. Curr. Opin. Immunol. *10*, 265–270.

O'Rourke, A.M., Shao, H., and Kaye, J. (1998). A role for p21ras/MAP kinase in TCR-mediated activation of LFA-1. J. Immunol. *161*, 5800–5803.

Peled, A., Grabovsky, V., Habler, L., Sandbank, J., Arenzana-Seisdedos, F., Petit, I., Ben-Hur, H., Lapidot, T., and Alon, R. (1999). The chemokine SDF-1 stimulates integrin-mediated arrest of CD34(+) cells on vascular endothelium under shear flow. J. Clin. Invest. *104*, 1199–1211.

Rodriguez-Viciana; P., Warne, P.H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M.J., Waterfield, M.D., and Downward, J. (1994). Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature *370*, 527–532.

Rodriguez-Viciana; P., Warne, P.H., Khwaja, A., Marte, B.M., Pappinm D., Das, P., Waterfield, M.D., Ridley, A., and Downward, J. (1997). Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. Cell. *98*, 457–467.

Sadhu, C., Masinowsky, B., and Staunton, D.E. (1998). Differential regulation of chemoattractant-stimulated β2, β3 and β7 integrin activity. J. Immunol. *160*, 5622–5628.

Sanchez-Madrid, F., Krensky, A.M., Ware, C.F., Robbins, E., Strominger, J.L., Burakoff, S.J., and Springer, T.A. (1982). Three distinct antigens associated with human T lymphocyte-mediated cytolysis: LFA-1, LFA-2 and LFA-3. Proc. Natl. Acad. Sci. USA *79*, 7489–7493.

Sanz-Rodriguez, F., Hidalgo, A., and Teixido, J. (2001). Chemokine stromal cell-derived factor-1α modulates VLA-4 integrin-mediated multiple myeloma cell adhesion to CS-1/fibronectin and VCAM-1. Blood. *97*, 346–351.

Shibayama, H., Anzai, N., Braun, S.E., Fukuda, A., Mantel, C., and Broxmeyer, H.E. (1999). H-Ras is involved in the inside-out signaling pathway of interleukin-3-induced integrin activation. Blood *5*, 1540–1548.

Simon, S.I., Burns, A.R., Taylor, A.D., Gopalan, P.K., Lynam, E.B., Sklar, L.A., and Smith, C.W. (1995). L-selectin (CD62L) cross-linking signals neutrophil adhesive functions via the Mac-1 (CD11b/CD18) β2-integrin. J. Immunol. *155*, 1502–1514.

Simon, S.I., Hu, Y., Vestweber, D., and Smith, C.W. (2000). Neutrophil tethering on E-selectin activates β2 integrin binding to ICAM-1 through a mitogen-activated protein kinase signal transduction pathway. J. Immunol. *164*, 4348–4358.

Smith, C.W., Marlin, S.D., Rothlein, R., Toman, C., and Anderson, D.C. (1989). Co-operative interactions of LFA-1 and Mac-1 with intercellular adhesion molecule-1 in facilitating adherence and transendothelial migration of human neutrophils in vitro. J. Clin. Invest. *83*, 2008–2017.

Sotsios, Y., Whittaker, G.C., Westwick, J., and Ward, S.G. (1999). The CXC chemokine stromal cell-derived factor activates a Gi-coupled phosphoinositide 3-kinase in T lymphocytes. J. Immunol. *163*, 5954–5963.

Springer, T.A. (1990). Adhesion receptors of the immune system. Nature *346*, 425–434.

Springer, T.A. (1994). Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell *76*, 301–314.

Stewart, M.P., Cabanas, C., and Hogg, N. (1996). T cell adhesion to intercellular adhesion molecule-1 (ICAM-1) is controlled by cell spreading and the activation of integrin LFA-1. J. Immunol. *156*, 1810–1817.

Suzuki, J., Yamazaki, Y., Guang, L., Kaziro, Y., and Koide, H. (2000). Involvement of Ras and Ral in chemotactic migration of skeletal myoblasts. Mol. Cell. Biol. *20*, 4658–4665.

Tanaka, Y., Minami, Y., Mine, S., Hirano, H., Hu, C., Fujimoto, H., Fujii, K., Saito, K., Tsukuda, J., van Kooyk, Y., Figdor, C.G., Kataoka, T., and Eto, S. (1999). H-Ras signals to cytoskeletal machinery in induction of integrin-mediated adhesion of T cells. J. Immunol. *163*, 6209–6216.

Tilton, B., Ho, L., Oberlin, E., Loetscher, P., Baleux, F. Clark-Lewis, I., and Thelen, M. (2000). Signal transduction by CXC chemokine receptor 4: stromal cell-derived factor 1 stimulates prolonged protein kinase B and extracellular signal-regulated kinase 2 activation in T lymphocytes. J. Exp. Med. *192*, 313–324.

Turner, L., Ward, S.G., and Westwick, J. (1995). RANTES-activated human T lymphocytes. J. Immunol. *155*, 2437–2444.

van Kooyk, Y., van Vliet, S.J., and Figdor, C.G. (1999). The actin cytoskeleton regulates LFA-1 ligand binding through avidity rather than affinity changes. J. Biol. Chem. *274*, 26869–26877.

van Kooyk, Y., and Figdor, C.G. (2000). Avidity regulation of integrins: the driving force in leukocyte adhesion. Curr. Opin. Cell. Biol. *12*, 542–547.

Vicente-Manzanares, M., Rey, M., Jones, D.R., Sancho, D., Mellado, M., Rodriguez-Frade, J.M., del Pozo, M.A., Yanez-Mo, M., de Ana, A.M., Martinez-A, C., Merida, I., and Sanchez-Madrid, F. (1999). Involvement of phosphatidylinositol 3-kinase in stromal cell-derived factor-1$\alpha$–induced lymphocyte polarization and chemotaxis. J. Immunol. *163*, 4001–4012.

Weber, C., Kitayama, J., and Springer, T.A. (1996a). Differential regulation of $\beta$1 and $\beta$2 integrin avidity by chemoattractants in eosinophils. Proc. Natl. Acad. Sci. USA *93*, 10939–10944.

Weber, C., Alon, R., and Springer, T.A. (1996b). Sequential regulation of $\alpha$4$\beta$1 and $\alpha$5$\beta$1 integrin avidity by CC chemokines in monocytes: implications for transendothelial chemotaxis. J. Cell Biol. *134*, 1063–1073.

Weber, K.S., Klickstein, L.B., and Weber, C. (1999). Specific activation of leukocyte $\beta$2 integrins lymphocyte function-associated antigen-1 and Mac-1 by mediated by distinct pathways via the $\alpha$ subunit cytoplasmic domains. Mol. Biol. Cell *10*, 861–873.

Weber, K.S.C., Klickstein, L.B., Weber, P.C., and Weber, C. (1998a). Chemokine-induced monocyte transmigration requires cdc42-mediated cytoskeletal changes. Eur. J. Immunol. *28*, 2245–2251.

Weber, C., Lu, C.-F., Casasnovas, J., and Springer, T.A. (1997a). Role of the $\alpha$L$\beta$2 integrin avidity in transendothelial chemotaxis of mononuclear cells. J. Immunol. *159*, 3968–3975.

Weber, C., and Springer, T.A. (1998b). VLA-4 avidity regulation by chemokines facilitates lateral migration of monocytes. J. Immunol. *161*, 6825–6834.

Weber, K.S.C., York, M.R., Springer, T.A., and Klickstein, L.B. (1997b). Characterization of lymphocyte function-associated antigen 1 (LFA-1)-deficient T cell lines: the $\alpha$L and $\beta$2 subunits are interdependent for cell surface expression. J. Immunol. *158*, 273–279.

Yauch, R.L., Felsenfeld, D.P., Kraeft, S.K., Chen, L.B., Sheetz, M.P., and Hemler, M.E. (1997). Mutational evidence for control of cell adhesion through integrin diffusion/clustering, independent of ligand binding. J. Exp. Med. *186*, 1347–1355.

Zeitlmann, L., Knorr, T., Knoll, M., Romeo, C., Sirim, P., and Kolanus, W. (1998). T cell activation induced by novel gain-of-function mutants of Syk and ZAP-70. J. Biol. Chem. *273*, 15445–15452.

Zhang, Z., Vuori, K., Wang, H., Reed, J.C., and Ruoslahti, E. (1996). Integrin activation by R-ras. Cell *85*, 61–69.