Role of Integrin-linked Kinase in Leukocyte Recruitment*

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Chemokines modulate leukocyte integrin avidity to coordinate adhesion and subsequent transendothelial migration, although the sequential signaling pathways involved remain poorly characterized. Here we show that integrin-linked kinase (ILK), a 59-kDa serine-threonine protein kinase that interacts principally with β1 integrins, is highly expressed in human mononuclear cells and is activated by exposure of leukocytes to the chemokine monocyte chemoattractant protein-1. Biochemical inhibitor studies show that chemokine-triggered activation of ILK is downstream of phosphoinositide 3-kinase. In functional assays under physiologically relevant flow conditions, overexpression of wild-type ILK in human monocytic cells diminishes β1 integrin/vascular cell adhesion molecule-1-dependent firm adhesion to human endothelial cells. These data implicate ILK in the dynamic signaling events involved in the regulation of leukocyte integrin avidity for endothelial substrates.

Current models of leukocyte accumulation from the vasculature suggest that the process occurs through a series of sequential steps (1). The selectin family (E-, P-, and L-selectin) of adhesion molecules predominantly governs the initial rolling interaction between leukocytes and vascular endothelium. Subsequent firm arrest occurs via leukocyte integrin interactions with endothelial immunoglobulin superfamily members, such as vascular cell adhesion molecule-1 (VCAM-1). Chemokines enhance leukocyte accumulation by the rapid conversion of initial leukocyte tethering to firm adhesion. After leukocyte arrest, chemokines then drive transendothelial migration of leukocytes into the surrounding tissues.

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† The abbreviations used are: VCAM-1, vascular cell adhesion molecule-1; s, soluble; ILK, integrin-linked kinase; MCP-1, monocyte chemotactant protein-1; PI3K, phosphoinositide 3-kinase; GSK-3, glycogen synthase kinase-3; HUVEC, human umbilical vein endothelial cell; MOI, multiplicity of infection; WT, wild-type; KD, kinase-deficient; EGFP, enhanced green fluorescent protein.

Specific chemokines including monocyte inflammatory protein α and monocyte chemoattractant protein-1 (MCP-1) induce rapid activation of leukocyte integrins such as α4β1, also known as VLA-4. Subsequently, there is rapid deactivation and release of the same integrin heterodimer from its counterligand, VCAM-1 (2). In contrast to chemokines, nonphysiological leukocyte agonists such as phorbol 12-myristate 13-acetate lock integrins into prolonged high affinity states (2). The signaling pathways via which chemokines dynamically modulate integrin avidity for endothelial and matrix ligands to coordinate leukocyte adhesion and release are poorly understood.

Here we investigated the role of integrin-linked kinase in chemokine-triggered signaling. ILK was originally identified in a search for proteins capable of interacting with β1 integrins using a yeast two-hybrid screen with the β1 integrin cytoplasmic domain as bait (3). ILK expression has been documented in the heart, skeletal muscle, kidney, and pancreas (3). Sequencing of ILK revealed a 59-kDa protein serine-threonine kinase with four ankyrin repeats in its N terminus. The C terminus is the β integrin-interacting domain and also contains the kinase catalytic domain. Interposed between the ankyrin repeats and the kinase domain is a pleckstrin homology domain thought to be important for the binding of lipid second messengers such as those generated by PI3K (4). In vitro, ILK can phosphorylate synthetic peptides corresponding to β1 integrin cytoplasmic domains (3). Other potential substrates include the kinases Akt and glycogen synthase kinase 3 (GSK-3) (5). From a functional perspective, ILK overexpression in epithelial cells disrupts cell-extracellular matrix as well as cell-cell interactions. Recent data from studies in fibroblasts suggest a potential role for ILK in cell motility via its interaction with the focal adhesion protein PINCH (6). However, physiologically relevant cellular ligands that modulate ILK activity remain poorly understood (7, 8). Furthermore, a role for ILK in the dynamic processes involved in leukocyte recruitment has not previously been postulated.

Based on evidence that ILK is associated with β integrins and is activated in a PI3K-dependent manner in other systems, we studied its role in leukocyte adhesion. Here we show that ILK is highly expressed in human mononuclear leukocyte subsets and is activated by exposure of leukocytes to chemokines. Chemokine-triggered activation is sustained for several minutes and is dependent on PI3K. Interestingly, overexpression of ILK in human monocytic cells diminishes β1 integrin/VCAM-1-dependent firm adhesion to human vascular endothelial cells. These data implicate ILK in the dynamic signaling events involved in the regulation of leukocyte integrin avidity for endothelial substrates.
ILK in Leukocyte Recruitment

EXPERIMENTAL PROCEDURES

Materials—RPMI 1640 medium, Dulbecco's modified Eagle's medium, and Dulbecco's phosphate-buffered saline with or without Ca²⁺ and Mg²⁺ were purchased from BioWhittaker, Inc. Fetal bovine serum was obtained from Hyclone Inc. Recombinant MCP-1, interleukin 8, and sVCAM-1 were purchased from R&D Systems. Biochemical inhibitors wortmannin and LY294002 were obtained from Alexis Corp.

Cell Culture—HEK 293 cells were obtained from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Human umbilical vein endothelial cells (HUVECs) were isolated and cultured in M199 with 20% fetal bovine serum, endothelial cell growth factor (25 μg/ml; Biomedical Technologies, Stoughton, MA), porcine intestinal heparin (50 μg/ml; Sigma), and antibiotics. After infection with adenoviral vectors, HUVECs were cultured as described above, but the serum concentra-
tion was reduced to 10%. For experimental use in the flow plate apparatus, HUVECs (passage 1–2) were plated at confluence in 0.5-cm² chambers on fibronectin-coated plastic tissue culture slides. On the day of the flow adhesion assay, a fluorescence immunolocalization was performed on HUVECs infected in parallel to document transgene expression and to rule out nonspecific activation of the endothelial monolayer, as described previously (9). THP-1 cells were obtained from American Type Culture Collection. Cells (1 × 10⁶) were infected at an MOI of 10–50 in 0.5 ml of serum-free RPMI 1640 medium in a 12-well dish for 2 h, and the volume was subsequently increased to 2.0 ml, and serum was added to reach 10%. Experiments were performed, and transgene expression was evaluated 24 h after infection.

Flow Cytometry—Monocytes were washed once with RPMI 1640 medium/5% fetal calf serum, incubated with the indicated fluorescence-tagged (non-fluorescein isothiocyanate for EGFFP-transduced cells) primary antibodies for 30–60 min on ice, washed twice with RPMI 1640 medium/5% fetal calf serum, and fixed with 1% formaldehyde. An isotype-matched, fluorescence-labeled, non-binding antibody was included as a control. Fluorescence was then analyzed using a BD Pharmingen, fluorescein-activated cell sorter set to detect fluorescein, forward scatter, and size.

Leukocyte Isolation—Human leukocyte subsets were purified from healthy human donors by Ficoll-Hypaque density gradient centrifuga-
tion at 15 °C (LSM; Organon Teknika, Durham, NC). Neutrophils were purified from the lower fraction after dextran sedimentation and hypotonic lysis of red cells. Monocytes or lymphocytes were purified from the mononuclear band by magnetic bead purification, using a negative selection strategy (monocyte and lymphocyte isolation kits; Miltenyi Biotech) (11). Leukocyte subset suspensions were consistently >92% pure as determined by light scatter and cell surface antigen analysis.

Adhesion Assays under Flow—We employed a commercially available parallel plate laminar flow chamber (Immunetics, Cambridge, MA) (11, 17). For biochemical inhibitor studies, the monocytes were incubated with the indicated compound or vehicle (Me₂SO) for 30 min at 37 °C and then diluted with perfusion media to 10⁶ cells/ml. Where indicated, chemokines were added to the monocyte reservoir (room temperature). Monocyte firm adhesion (≥3 s) was quantified for each coverslip 1 min before and 1 min after the addition of the chemokine. The cells were perfused at an estimated shear stress of 2.0 dynes/cm² (flow rate, 0.78 ml/min). The entire period of perfusion was recorded on videotape.

ILK Activity—Human monocytic THP-1 cells or freshly isolated primary human leukocyte subsets were left untreated or stimulated with 100 ng human MCP-1 at 37 °C for the indicated time periods. Cells were lysed in an ice-cold buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.6, 1% Triton X-100, 1 mM sodium orthovanadate, and 4 μM microcystin in the presence of protease inhibitors (Roche Molecular Biochemicals). Precleared cell lysates were incubated with a polyclonal anti-ILK antibody (Upstate Biotechnology) and protein A beads overnight at 4 °C. Immune complexes were washed twice with lysis buffer followed by two washes with 1 ml of kinase buffer containing 150 mM NaCl, 25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothre-
itol, 0.1 mM sodium orthovanadate, and 10 mM MgCl₂. Washed pellets were incubated with kinase buffer supplemented with 200 μM ATP and 1 μg of the commercially available GSK-3 substrate peptide (Cell Signaling) for 30 min at 30 °C. The kinase reaction was terminated by the addition of SDS sample buffer, and the supernatants were boiled for 5 min at 100 °C and resolved by SDS-PAGE (10–12% gels). Membranes were probed with phospho-GSK-3 (Ser9) (Cell Signaling) and Akt/phospho-Akt-Ser473 antibodies overnight at 4 °C according to the manufacturer's instructions (Cell Signaling).

Statistical Analysis—Data are expressed as the mean ± S.D. Statistical comparison of means was performed by two-tailed unpaired Student's t test. The null hypothesis was rejected at p < 0.05.

RESULTS

ILK Is Expressed in Human Mononuclear Cells—ILK expression has been documented by mRNA and protein analysis in a variety of cell types and tissues, including heart, skeletal muscle, kidney, and pancreas (3). We first performed Western analysis to examine whether ILK protein is expressed in purified human leukocyte subsets. As seen in Fig. 1, human whole peripheral blood mononuclear cells, specifically the monocyte and T-cell subsets, prominently expressed the 59-kDa ILK protein. In contrast, resting human neutrophils appeared to have no ILK.

Chemokines Activate ILK—In light of the association of ILK and β integrins in other cell types, we investigated the effects of chemokines on ILK activity in human leukocytes. We em-

ployed the monocytic THP-1 cell line that mimics much of the phenotype of human monocytes and is readily manipulated by somatic gene transfer techniques for signaling studies (11). As seen in Fig. 2a, the chemokine MCP-1 activated ILK at physiologically relevant concentrations. 10–100-fold higher doses of interleukin 8 also activated ILK, confirming that other chemokines can also activate ILK. Chemokine activation of ILK con-
sistently peaked at ~3 s, lessened, and then increased and became sustained for ~3 min (Fig. 2b). Importantly, we saw marked chemokine-triggered ILK activation in freshly isolated human monocytes as well (Fig. 2c). Because cell-matrix inter-
actions have been shown to activate ILK in other systems (3), we next assessed ILK activation in THP-1 cells bound to VCAM-1, an important counterligand of β integrins in leuko-
cyte recruitment. VCAM-1 binding transiently activated ILK in our model (Fig. 2d), although to a far lesser degree than che-
monocytes were stimulated with MCP-1 (100 nM, 30s, 37 °C) and interleukin 8 for 30 s (37 °C). After termination of the kinase reaction, samples were resolved by SDS-PAGE, and membranes were probed with an anti-phospho-GSK-3 antibody before the kinase assay had no effect on ILK activity. We next assessed the specificity of the observed ILK activation by chemokines, we lysates immunoprecipitated with the ILK antibody for kinase-deficient ILK, first confirming expression by Western blotting (Fig. 4A). Protein analysis revealed both endogenous 59-kDa ILK and the slightly larger, exogenous, tagged construct. Total ILK activity was measured using the GSK-3 substrate as described above. In the bottom panels, whole THP-1 cell lysates were prepared before immunoprecipitation, and Western blotting was performed with the indicated antibody. Representative data from one of at least three experiments are shown.

**Fig. 2.** MCP-1 activates ILK in human monocytic cells. a, human monocytic THP-1 cells were left untreated or stimulated with the indicated concentration of MCP-1 or interleukin 8 for 30 s (37 °C). Whole cell lysates were immunoprecipitated with anti-ILK antibody, and kinase assays were subsequently performed using a GSK-3 substrate peptide. After termination of the kinase reaction, samples were resolved by SDS-PAGE, and membranes were probed with an anti-phospho-GSK-3(Ser9) antibody. b, THP-1 cells were stimulated with MCP-1 (100 nM, 30 s, 37 °C) for the indicated time period. c, freshly purified human monococytes were stimulated with MCP-1 (100 nM, 30 s, 37 °C). d, THP-1 cells were incubated with recombinant sVCAM-1 (25 μg/ml, 37 °C) for the indicated time period. Where indicated, THP-1 cells were stimulated with MCP-1 (100 nM, 30 s, 37 °C). Representative data from one of at least three experiments are shown.

mokine stimulation. As a readout of ILK activity for these studies, we employed an *in vitro* kinase assay using a GSK-3 fusion protein substrate because GSK-3 has been shown to be a target of ILK activation in other systems (5, 18). To verify the specificity of the observed ILK activation by chemokines, we assessed lysates immunoprecipitated with the ILK antibody for potentially contaminating kinases such as Akt (19). Akt is present in whole cell lysates from THP-1 cells; importantly, however, Western blot analysis of the ILK immunoprecipitates revealed no Akt. In addition, pretreating ILK precipitates with anti-Akt antibody before the kinase assay had no effect on ILK phosphorylation of the GSK-3 fusion protein (data not shown).

Chemokines activate several PI3K isoforms in leukocytes. These include the “classical” PI3K-α (p85/p110, IA) isoform as well as PI3K-γ (p101/p110, IB), which is thought to be activated exclusively by G protein-coupled receptors (11, 20–22). A membrane-targeted, constitutively active PI3K construct stimulates ILK activity in NIH 3T3 cells (5). We next tested whether chemokines such as MCP-1 activate ILK in a PI3K-dependent manner. As shown in Fig. 3, MCP-1 activated ILK, and pretreatment of monocyes with low-dose wortmannin or LY294002 markedly attenuated this activity. Pertussis toxin also abrogated ILK activation, consistent with Gαs-coupled chemokine receptor signaling.

**ILK Activation Inhibits β, Integrin-dependent Adhesion**—Because biochemical characterization suggested ILK activation by chemokines, we used somatic gene transfer to probe potential downstream targets and assess the functional effects on leukocyte recruitment. We manipulated THP-1 cell signaling using adenoviral constructs carrying the cDNAs for wild-type and kinase-deficient ILK, first confirming expression by Western blotting (Fig. 4A). Protein analysis revealed both endogenous 59-kDa ILK and the slightly larger epitope-tagged ILK protein. We next assessed the *in vitro* ILK kinase activity of monocytic THP-1 cells transduced with these constructs. We also assessed phosphorylation of Akt and GSK-3 in whole cell lysates in chemokine-activated and ILK-transduced THP-1 cells. As noted previously, ILK can phosphorylate Akt and GSK-3 in other systems (5, 19). Furthermore, Akt (23) and GSK-3 (24) are downstream substrates of PI3K activation triggered by chemokines. As seen in Fig. 4B, basal activity of uninfected THP-1 cells was low and was unaffected by control adenoviral infection. As compared with AdEGFP- and AdKDILK-transduced THP-1 cells, we saw increased ILK activity in the AdWTILK-transduced THP-1 cells, to levels comparable to that seen with MCP-1 stimulation. In whole THP-1 cell lysates, MCP-1 led to phosphorylation of both Akt and GSK-3, as did AdWTILK transduction.

We performed flow cytometry experiments on transduced monocytic cells in parallel with biochemical and functional
assays. We saw no effect of adenoviral gene transfer of control or ILK constructs on surface expression of $\beta_1$ or $\beta_2$ integrins that are critical for monocyte-endothelial interactions (Fig. 5). Having confirmed that the WT ILK construct conferred increased activity at an MOI of 50 as measured by in vitro kinase assays, we then investigated the functional consequences of this activity in monocytic cells in our vascular flow model. As seen in Fig. 6, uninfected and AdEGFP-transduced THP-1 cells show moderate baseline adhesion to HUVEC monolayers coexpressing E-selectin and VCAM-1. In this model, E-selectin enhances initial leukocyte-endothelial tethering, whereas firm adhesion is entirely blocked by monoclonal antibody blockade of $\alpha_\beta_1$ integrins interacting with their VCAM-1 counterligand. Firm adhesion of both uninfected and AdEGFP-transduced THP-1 cells was similarly enhanced by MCP-1 (100 nM). We saw a significant decrease in VCAM-1-dependent firm adhesion of the WT ILK-transduced THP-1 cells, whereas adhesion of KD ILK-expressing cells was comparable to that of uninfected and control adenovirus-transduced cells. MCP-1 stimulation did not overcome the inhibition conferred by AdWTILK transduction.

**DISCUSSION**

The emigration of circulating blood leukocytes into subendothelial tissues involves multiple steps. Leukocyte firm attachment and transendothelial migration are mediated by integrins, immunoglobulin superfamily members, matrix proteins, and chemokines. The signaling processes by which chemokines dynamically modulate integrins to orchestrate leukocyte adhesion and cell release are poorly understood.

Integrin-linked kinase is a focal adhesion serine-threonine kinase associated principally with $\beta_1$ integrins. ILK is emerging as an important transducer of cell-matrix signaling in a number of physiological contexts. A role for ILK in the dynamic processes involved in leukocyte recruitment, however, has not previously been postulated. Our data show that ILK is highly expressed in human mononuclear subsets. Chemokines markedly activate ILK in both human monocytes and the THP-1 cell line, which faithfully recapitulates monocyte biology. Leukocyte engagement of the endothelial adhesion molecule VCAM-1 also activates ILK. MCP-1-triggered activation of ILK is dependent on PI3K in monocytes. Both MCP-1-activated THP-1 cells and AdILK-transduced THP-1 cells share Akt and GSK-3 as downstream substrates. Finally, a WT ILK construct diminishes baseline VCAM-1-dependent adhesion and strikingly inhibits the MCP-1-triggered augmentation of monocyte arrest.

These data are consistent with a growing body of evidence associating ILK with integrin-triggered signaling (‘outside-in
signaling") as well as intracellular signaling cascades that culminate in integrin activation ("inside-out signaling") (25). In other systems, epithelial cell adhesion to matrix proteins triggers ILK activation (3). Consistent with these prior findings, leukocyte adhesion to VCAM-1 also activated ILK in our studies. Interestingly, however, MCP-1-triggered activation far exceeded that seen upon leukocyte-matrix interactions.

PI3K is a key intermediary in cell motility, specifically in chemokine-triggered leukocyte recruitment (11, 26–28). The obligate pathways downstream of PI3K remain poorly understood (23, 29). Very recent investigation in fibroblasts suggests a role for ILK in shape change and motility (6). However, the relevant cellular ligands remain undefined. The present work therefore extends prior studies because it implicates members of the chemokine superfamily as potent agonists of ILK in a PI3K-dependent cascade modulating leukocyte adhesion.

Our functional data suggest that ILK may play a role in the sequential modulation of integrin affinity and avidity, which has been proposed as a crucial mechanism to facilitate leukocyte adhesion and transendothelial migration (2). CC chemokines such as MCP-1 transiently increase and then subsequently reduce VLA-4-mediated binding of monocytes to VCAM-1 (2), and these data suggest a role for ILK in the latter process. Because ILK negatively modulates β1 integrin-independent adhesion of epithelial cells, it is not entirely surprising that it plays a similar role in leukocyte adhesion in our system. Interaction between ILK and negative modulators of integrin avidity in other systems, such as H-ras (30), is presently unclear. β1 Integrin-associated proteins that subserve similar functions in human neutrophils also merit investigation. An unanswered question of obvious interest is how the functional effects of other chemokines correlate with their effects on ILK stimulation. Finally, future investigations must address the relevant downstream targets of PI3K involved in leukocyte recruitment, particularly in the context of ILK. Whereas our studies suggest that phosphorylation of Akt and GSK-3 is seen by both chemokine-triggering and transduction with ILK, they do not prove the functional relevance of these observations. Precise definition of the contribution of specific pathways will require additional investigation by engineering new dominant negative constructs or perhaps in genetically manipulated murine models.

Rapid pulses of stimuli, such as those seen with chemokine stimulation, may be important for activating the specific signaling pathways that coordinate cell adhesion and migration. A limitation of the present studies in evaluating a physiological role for ILK in leukocyte recruitment is the persistent signal conferred by the adenoviral constructs. We recognize that a number of potentially confounding pathways could be activated by persistent ILK stimulation in our studies. Newer systems that employ small molecule agonists of chimeric proteins turned on in seconds or minutes (31) or transgenic animals alluded to above will be helpful to further dissect a role for ILK in leukocyte recruitment.

Our working model suggests that chemokines such as MCP-1 can convert initial monocyte tethering to firm arrest following a brief, potentially subsecond exposure (17, 32). Once the leukocyte has engaged the endothelium, ILK activation persists over several minutes. Sustained ILK activation, in turn, may represent a counter-regulatory mechanism to decrease adherence via as yet uncharacterized effects on focal adhesions. This prepares the cell’s response to subsequent directional cues. In summary, we conclude that ILK is involved in the dynamic signaling events by which chemokines control leukocyte integrin avidity for endothelial substrates.

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