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TCR-Induced Activation of LFA-1 Involves Signaling through Tiam1

Mikaela Grönholm,1 Farhana Jahan,1 Silvia Marchesan,1 Ulla Karvonen, Maria Aatonen, Suneeta Narumanchi, and Carl G. Gahmberg

Adhesion is pivotal for most leukocyte functions, and the β2 integrin family of adhesion molecules plays a central role. The integrins need activation to become functional, but the molecular events resulting in adhesion have remained incompletely understood. In human T cells, activation through the TCR results in specific phosphorylation of the T758 on the β2 chain of LFA-1. We now show that this phosphorylation leads to downstream binding of 14-3-3 proteins, followed by engagement of the guanine nucleotide exchange factor protein Tiam1 and Rac1 activation. Downregulation of the signaling molecules inhibits LFA-1 activity. Activation by the chemokine stromal cell-derived factor-1a also results in T758 phosphorylation and integrin activation. Thus, TCR and chemokine activation converges on LFA-1 phosphorylation, followed by similar downstream events affecting adhesion. The Journal of Immunology, 2011, 187: 3613–3619.

Leukocyte adhesion to cells and surrounding molecules is of critical importance for immune functions. Adhesion is required when T cells make contacts with the endothelium as they exit the bloodstream, or with APCs during Ag recognition. Leukocyte adhesion is dependent on the integrin LFA-1 (αLβ2, CD11a/CD18), which binds to ICAMs (1, 2). LFA-1 is a heterodimeric transmembrane protein that mediates bidirectional signaling over the plasma membrane. The integrin is inactive in resting cells and does not bind extracellular ligands. However, when T cells are stimulated, for example, through the TCR or chemokine receptors, intracellular signaling pathways are induced, resulting in LFA-1 activation. This is termed inside-out signaling. After ligand binding, integrins are able to transmit signals into the cell interior to change cell behavior, during so-called outside-in signaling (3, 4). LFA-1 activation is a complex process involving activation by phosphorylations (5), changes in integrin conformation and complex formation (6), as well as changes in clustering and cytoskeletal contacts. The cytoskeleton is intimately involved in the dynamic regulation of the adhesive state of LFA-1, switching from sufficiently strong binding to maintain cell–cell contacts to a state that allows cells to dissociate and move (7–9).

The integrin cytoplasmic domains are short and devoid of catalytic activity. However, integrin function is regulated by protein interactions with the cytoplasmic domains, which in turn are modified by phosphorylation. In particular, T758 on the integrin β2 chain becomes phosphorylated in T cells after TCR stimulation (10), and this leads to the recruitment of 14-3-3 proteins to the β2 polypeptide (5, 11). This association between T758-phosphorylated β2 and 14-3-3 is required for actin cytoskeleton rearrangements, cell spreading, and adhesion to ICAM-1 (5, 12). Phosphorylation of T758 also results in activation of Rac1 and Cdc42 (13), well-known reorganizers of the actin cytoskeleton (14). The 14-3-3 proteins are dimers and are able to interact with two phosphorylated targets at the same time, thus functioning as adapter proteins (15, 16). Among the known binding partners of 14-3-3 are many proteins involved in the regulation of small GTP-binding proteins (17). Thus, it is conceivable that 14-3-3 bound to T758-phosphorylated β2 further interacts with a guanine nucleotide exchange factor (GEF), which activates Rac1 and Cdc42 in T cells, thus affecting adhesion and migration.

Tiam1 (T-lymphoma invasion and metastasis protein) is a Rac-specific GEF (18). It has been shown to interact with 14-3-3 in HeLa cells (19) and transfected COS7 cells, and the interaction is dependent on the N terminus of Tiam1 (20). In T cells, Tiam1 is required for chemokine-induced Rac activation and cell migration (21), but the molecular mechanisms have remained incompletely understood.

In this study, we investigated whether Tiam1 links phosphorylated LFA-1 and 14-3-3 to Rac1 activation. We show that upon TCR-induced activation, a complex is formed among LFA-1, 14-3-3, and Tiam1, which activates Rac1. Down-regulation of Tiam1 inhibits Rac1 activity, cell adhesion, and migration, as well as LFA-1 activation. Furthermore, treatment with the chemokine stromal cell-derived factor-1a, (SDF-1a) also results in T758 phosphorylation and activation of LFA-1, indicating similar signaling.

Materials and Methods

Reagents

Ficoll-Hypaque was purchased from GE Healthcare, Life Bionsciences (Uppsala, Sweden), RPMI 1640, t-glutamine, and penicillin-streptomycin for cell isolation and culture were obtained from Lonza (Basel, Switzerland). FBS was from Thermo Fisher Scientific (Waltham, MA). The peptides (CLFKSATTTVMN and CLFKSApTTTVMN, in which pT is phosphothreonine) were synthesized by TAG Copenhagen (Copenhagen, Denmark). Protein G Sepharose and CNBr-activated Sepharose were from GE Healthcare. The antagonist of 14-3-3 proteins, the R18 peptide PHCVRDLSWLDLEANMCLP, was from TOCRIS Bioscience.

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Abbreviations used in this article: GEF, guanine nucleotide exchange factor; PAK-PBD, p21 activated kinase-p21 binding domain; RIPA, radio immunoprecipitation assay; SDF-1a, stromal cell-derived factor-1a; siRNA, small interfering RNA.

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(Ellisville, MO), and, as a control, the P621 peptide VDVSDGSTDLD-VIGA was used.

The mAbs R2E7B and R7E4 against the human β2 subunit of leukocyte integrin have been described previously (22). IB4, which recognizes only heterodimeric forms of β2 integrins, was a gift of M. Arnaout (Massachusetts General Hospital, Boston, MA). The β2 integrin Abs KIM127 and mAb24 were gifts of M. Robinson (Celttech, Slough, U.K.) and N. Hogg (Imperial Cancer Research Fund, London, U.K.), respectively. The 14-3-3 K-19 and Tiam1 C-16 Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), the Rac Ab from BD Transduction Laboratories (Franklin Lakes, NJ), and the actin Ab A5060 from Sigma-Aldrich (St. Louis, MO). HRP-linked Abs against mouse and rabbit Ig were purchased from GE Healthcare.

Cell cultures

Buffy coats were obtained from the Finnish Red Cross Blood Transfusion Service (Helsinki, Finland) and were used for the isolation of T cells, as described previously (23). Briefly, PBMCs were isolated by Ficoll-Hypaque gradient centrifugation. T cells were further enriched by using nylon-wool columns. Enriched T cells were suspended in RPMI 1640 medium supplemented with 10% FCS. JE6.1 cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 medium.

Immunoprecipitation

Human T cells were cultured with anti-CD3 (10 μg/ml MEM 57; ImmunoTools, Friesoythe, Germany) or left untreated. Human T cells or JE6.1 cells were cultured on ice for 15 min before radioimmunoprecipitation assay buffer (RIPA 2%) (50 mM Tris-HCl [pH 7.8], 150 mM NaCl, 1% Triton X-100, 1% Nonidet P-40, 15 mM MgCl2, and 5 mM EDTA). Cells were lysed at 13,400 rpm for 30 min at 4˚C. The supernatants were mixed with the Tiam1 (C-16), 14-3-3 (K-19), CD11/18 (IB4), or IgG Abs (2 μg) and incubated for 4 h at 4˚C with constant stirring. Prewashed protein G Sepharose beads were added and incubated for another 4 h at 4˚C. Sepharose beads were washed four times with RIPA 1% buffer (50 mM Tris-HCl [pH 7.8], 150 mM NaCl, 0.5% Triton X-100, 0.5% Nonidet P-40, 15 mM MgCl2, and 5 mM EDTA), and bound proteins were eluted with Laemmli sample buffer, run on SDS-PAGE, and analyzed by silver staining (24) for mass spectrometry or immunoblotted for 14-3-3 (K-19), Tiam1 (C-16), and the β2 integrin chain (R2E7B). The depletion immunoprecipitation was performed, as above, and Tiam1 was depleted from the JE6.1 cell lysate in two separate immunoprecipitations, the first for 2 h, the second for 4 h. As a control, the same experiment was done with IgG. The remaining lysates were used for immunoprecipitation with β2 (IB4), 14-3-3 (K-19), or IgG Abs.

Affinity chromatography

The 14-3-3 protein was purified, as described (25), and coupled to Ni-NTA Sepharose. Human T cells were either left untreated or were stimulated with anti-CD3 (OKT3, 10 μg/ml) and PhosSTOP Phosphatase inhibitor mixture (Roche, Basel, Switzerland). T cell lysates were mixed with the affinity matrix for 1 h and washed extensively with 500 mM NaCl. Bound proteins were eluted with SDS and analyzed by Western blotting with Abs against 14-3-3 (K-19), Tiam1 (C-16), and the β2 integrin chain (R2E7B). The depletion immunoprecipitation was performed, as above, and Tiam1 was depleted from the JE6.1 cell lysate in two separate immunoprecipitations, the first for 2 h, the second for 4 h. As a control, the same experiment was done with IgG. The remaining lysates were used for immunoprecipitation with β2 (IB4), 14-3-3 (K-19), or IgG Abs.

Adhesion assay

Soluble rICAM-1 (6 μg/ml; R&D Systems, Minneapolis, MN) was coated on flat-bottom 96-well microtiter plates by overnight incubation at 4˚C. The wells were blocked with 2% dry milk for 80 min at 37˚C. T cells were suspended in RPMI 1640 with 40 mM HEPES, 0.1% BSA, and 2 mM MgCl2 and treated with 100 μM Tiam inhibitor, NSC23766 (TOCRIS Bioscience), for 2 h or left untreated. T cells were activated with OKT3 (10 μg/ml) and allowed to adhere for 30 min at 37˚C, after which unbound cells were removed by gentle washing. The binding was quantified by ELISA.

Migration assay and peptide transfection

For the chemokine-induced T cell migration assay, 3 μm pore-size Transwell membranes were incubated with soluble ICAM-1 or BSA (15 ng/ml) overnight at 4˚C, washed once with PBS, and placed in the wells of a 24-well plate, containing 600 μl buffer (RPMI 1640/10% FBS) with 15 ng/ml chemokine SDF-1α (R&D Systems). Forty thousand JE6.1 or T cells were placed on the membrane and allowed to migrate toward SDF-1α at 37˚C for 2 h. Migrated cells were collected and counted. For transfection experiments, 40,000 T cells in 100 μl buffer (RPMI 1640/5% FBS) were transfected in 48-well plates. For transfection, 2 μg nonphosphorylated or T758-phosphorylated β2 peptide was incubated for 20 min at room temperature with 1 μl enhancer and 1 μl Turbofect transfection reagent (Fermentas; Thermo Fischer Scientific) in 50 μl RPMI 1640 and added to cells. After 5-min incubation, the transfected cells were loaded onto Transwell filters, and migration assays were performed, as above. In some cases, cells were preincubated with the β3 adhesion-blocking R7E4 Ab (25 μg/ml) or the Tiam1 inhibitor (100 μM) for 2 h at 37˚C. To block the interaction between 14-3-3 proteins and their targets, cells were transfected with the R18 peptide or the control peptide P621 (2 μg/transfection) prior to migration.

Immunofluorescence

JE6.1 or T cells were treated with 100 μM Tiam inhibitor for 2 h or left untreated. Cells were activated by anti-CD3 (10 μg/ml MEM-57) for 30 min or 150 ng/ml SDF-1α for 5 min and spun down on cytosine glasses. Cells were fixed in 3.5% paraformaldehyde and stained with Tiam1 (C-16), CD18 (R7E4 mAb), the extended form of LFA-1 (KIM127 mAb), or the extended open form of LFA-1 (mAb24) and Alexa Fluor secondary Abs (Invitrogen, Eugene, OR), and images were acquired by a Zeiss Axiophot microscope equipped with an AxioCam-cooled CCD camera (Carl Zeiss, Esslingen, Germany).

Quantifications

Quantifications were done using Image J (v10.2) software from at least three independent experiments. Student t test was used for calculation of p values. In the figures, the mean ± SD is given.

Results

Tiam1 forms a complex with 14-3-3 and the β2 integrin in T cells

LFA-1 phosphorylated on T758 of the β2 peptide was incubated for 2 h at 37˚C for 4 transfection, 2 μg nonphosphorylated or T758-phosphorylated β2 peptide was incubated for 20 min at room temperature with 1 μl enhancer and 1 μl Turbofect transfection reagent (Fermentas; Thermo Fischer Scientific) in 50 μl RPMI 1640 and added to cells. After 5-min incubation, the transfected cells were loaded onto Transwell filters, and migration assays were performed, as above. In some cases, cells were preincubated with the β3 adhesion-blocking R7E4 Ab (25 μg/ml) or the Tiam1 inhibitor (100 μM) for 2 h at 37˚C. To block the interaction between 14-3-3 proteins and their targets, cells were transfected with the R18 peptide or the control peptide P621 (2 μg/transfection) prior to migration.

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Quantifications

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Results

Tiam1 forms a complex with 14-3-3 and the β2 integrin in T cells

LFA-1 phosphorylated on T758 of the β2 chain binds 14-3-3 proteins (5, 11) and activates Rac1 (13), but the link between the phosphorylated integrin and Rac has remained unknown. To study whether Tiam1, a known Rac GEF, can connect signals from LFA-1 through 14-3-3 to Rac, we did coimmunoprecipitation experiments from JE6.1 Jurkat cells. Immunoprecipitated Tiam1 pulled down 14-3-3, and vice versa (Fig. 1A). To study whether 14-3-3...
can bind Tiam1 while in complex with LFA-1, immunoprecipitations were performed with an Ab recognizing the LFA-1 β2 chain. Immunoprecipitated β2 pulled down both 14-3-3 and Tiam1 (Fig. 1B). Tiam1 was further depleted from JE6.1 cell lysates, and subsequent immunoprecipitations with β2 and 14-3-3 were performed. A complex between β2 and 14-3-3 could be detected in Tiam1-depleted lysates, indicating that they can interact also without the presence of Tiam1 (Fig. 1C). We next performed the coimmunoprecipitations from purified blood T cells, which were unstained or TCR-stimulated by anti-CD3 (Fig. 1D). A complex between Tiam1, 14-3-3, and integrin β2 chain could be seen in both nonstimulated and stimulated cells, with slightly more coprecipitating proteins in stimulated cells. However, we could not detect β2 in the 14-3-3 immunoprecipitate. As an adapter protein, 14-3-3 binds several different proteins in cells; thus, the association with β2 may not be easily detectable.

**FIGURE 1.** Tiam1 forms a complex with 14-3-3 and the β2 integrin in T cells. A, Lysates from JE6.1 cells were immunoprecipitated with Abs toward Tiam1 and 14-3-3 or control IgG. Bound proteins were run on SDS-PAGE and immunoblotted with Tiam1 and 14-3-3 Abs. B, The experiment was performed as in A, but the immunoprecipitations were performed with anti–14-3-3 or the integrin β2 Ab (IB4). Bound proteins were detected with Abs toward 14-3-3, integrin β2 (R2E7B), or Tiam1. C, Tiam1 was depleted from JE6.1 lysates by two immunoprecipitations of which the second is shown (Tiam depl. sol., upper panel) or with IgG as a control (IgG depl. sol., lower panel). These lysates were used for immunoprecipitations, as in B, D, Lysates from unstimulated T cells (left) or stimulated with anti-CD3 (α-CD3, MEM57) were immunoprecipitated, as in A. IP, immunoprecipitation; sol., soluble fraction of lysate.

**FIGURE 2.** The 14-3-3 and Tiam1 binding is enhanced by phosphorylation of integrin β2 and blocked by the 14-3-3-blocking peptide R18. A, Lysates from T cells in the absence or presence of anti-CD3 (α-CD3, OKT3) were run over a column with Sepharose-bound 14-3-3. Bound proteins were run on SDS-PAGE and immunoblotted with Tiam1 and 14-3-3 Abs. B, A peptide of the β2 chain containing nonphosphorylated or phosphorylated T758 was pulled down from total cell lysates with PAK-PBD agarose. The introduction of Tiam1-targeting siRNA reduced Rac1 activity, whereas control siRNA did not affect the activity of Rac1. The levels of total Rac1 were not affected by siRNA transfection (Fig. 3A). Furthermore, inhibition of Tiam1 activity by the Tiam inhibitor caused a reduction in Rac1 activity in JE6.1 and T cells (Fig. 3B). Thus,

**TCR stimulation and β2 T758 phosphorylation enhance Tiam1 binding to 14-3-3**

Stimulation of T cells through the TCR is known to cause phosphorylation of T758 on LFA-1 (10) and 14-3-3 binding (5, 11). We sought to determine whether this chain of events affects Tiam1 association to the integrin and 14-3-3. Lysates from T cells treated with anti-CD3, or not, were run over a Sepharose column with purified 14-3-3-3-3 attached (Fig. 2A). Bound proteins were analyzed by SDS-PAGE and detected by Abs to Tiam1 and 14-3-3. Significantly more full-length Tiam1 binds 14-3-3 from stimulated than from unstimulated T cells. This is also true for a 75-kDa protein band, which was recognized by the C-terminal Tiam1 Ab and which we identified as a Tiam1 fragment by mass spectrometry (data not shown).

We next immobilized nonphosphorylated or T758-phosphorylated β2 chain peptides on Sepharose and added JE6.1 cell lysate. Bound 14-3-3 and Tiam1 were detected with specific Abs. More 14-3-3 and Tiam1 associated with the phosphorylated than the nonphosphorylated β2 peptide, indicating that phosphorylation of the β2 chain enhances 14-3-3, but also Tiam1 binding (Fig. 2B). The 14-3-3 interaction with its cellular ligands can be blocked by the R18 peptide, which binds to the phosphopeptide-binding groove in 14-3-3 (26). To assess whether 14-3-3 is required for the association between LFA-1 and Tiam1, the experiment was done in the presence of R18 or the control peptide P621. Blocking of 14-3-3 reduced not only the amount of bound 14-3-3, but also Tiam1 to the β2 peptides, indicating that there is a requirement of 14-3-3 for the Tiam1/LFA-1 association. The control peptide P621 did not block the interactions (Fig. 2B).

**Tiam1 is involved in the activation of Rac1 in T cells**

We then sought to study whether blocking Tiam1 inhibits Rac activation, a result of LFA-1 T758 phosphorylation (13). JE6.1 cells were transfected with siRNA toward Tiam1 or control siRNA (Fig. 3A). The amount of Tiam1 was reduced by the Tiam1-specific siRNA (average reduction from five experiments was 79%), but not by the control siRNA. Active Rac1 was pulled down from total cell lysates with PAK-PBD agarose. The introduction of Tiam1-targeting siRNA reduced Rac1 activity, whereas control siRNA did not affect the activity of Rac1. The levels of total Rac1 were not affected by siRNA transfection (Fig. 3A). Furthermore, inhibition of Tiam1 activity by the Tiam inhibitor caused a reduction in Rac1 activity in JE6.1 and T cells (Fig. 3B). Thus,
based on our results, the presence of functional Tiam1 is a prerequisite for Rac activation in T cells.

**Inhibition of Tiam1 reduces T cell adhesion to ICAM-1**

We next examined the effects of Tiam1 on T cell adhesion, a Rac-dependent process (27). As transfection of control siRNA alone affected cell adhesion, evidently due to the transfection conditions, we could not use siRNA for adhesion and migration experiments. Instead, JE6.1 cells were treated with the Tiam inhibitor or left untreated, and the TCR was activated with anti-CD3 (α-CD3, OKT3) for 30 min. Cells were allowed to adhere to ICAM-1 (black bars) or BSA (gray bars) for 30 min, and the amount of adhered cells was measured. The experiments were repeated three times in duplicate. Adhesion of nontreated cells on BSA was given the value 100. The mean ± SD is given. Student t test was used for calculation of p values. **p < 0.01.

**Tiam1 is required for phospho-T758 β2 integrin-induced T cell migration on ICAM-1**

In addition to T cell adhesion, Rac activation is crucial for transendothelial migration (28). We therefore studied the effect of Tiam1 inhibition on the migration of JE6.1 cells (Fig. 4A) and T cells (Fig. 4B) in a Transwell migration assay toward the chemokine SDF-1α. We first examined whether migration of T cells in this system is dependent on LFA-1. Cells were incubated with the R7E4 Ab that specifically binds to β2, thereby inhibiting β2 activity (22). A drastic decrease of migration confirmed that β2 function is critical for T cell migration under these experimental conditions. We then studied whether Tiam1 is required for migration. When cells were treated with the Tiam inhibitor, migration was reduced to the same levels as in β2 integrin-blocked cells. Treatment with both the β2-blocking Ab and Tiam inhibitor further reduced migration. These results show that Tiam1 is required for LFA-1–dependent SDF-1–induced migration.

In addition to TCR activation, SDF-1α is also known to affect integrin conformation and activation, which are required for T cell migration (29). We therefore next looked at the phosphorylation state of T758 in T cells activated with SDF-1α in the presence of okadaic acid, an inhibitor of serine/threonine phosphatases. Activation of cells with SDF-1α resulted in a strong increase of T758 phosphorylation (Fig. 4C).

To prove the connection between the T758-phosphorylated LFA-1 and Tiam1 in migration, cells were transfected with a nonphosphorylated or T758-phosphorylated β2 peptide to activate downstream signaling, and a migration assay was performed. Transfection in itself caused a slight increase in the migration of T cells as compared with untransfected cells (Fig. 5A). However, the amount of migrating cells almost doubled when the cells were transfected with the phosphorylated T758 β2 peptide, an effect that was not seen with the nonphosphorylated β2 peptide. To elucidate the role of Tiam1 in this process, T758 β2–peptide-transfected cells were then incubated with the Tiam inhibitor, which inhibited the increase in migration (Fig. 5B). This suggests that Tiam1 activity is critical for SDF-1α–induced T cell migration exerted by T758-phosphorylated LFA-1. Finally, to assess the role of 14-3-3-3 for migration, 14-3-3 interactions were blocked by the transfected R18 peptide. Blocking 14-3-3 protein associations led to reduced migration by 30%, an effect not seen with the control peptide P621. Inhibition was stronger in the presence of the Tiam1 inhibitor (Fig. 5C). These results show that LFA-1, 14-3-3, and Tiam1 regulate LFA-1–dependent T cell migration.

**Inhibition of Tiam1 affects SDF-1α–induced Tiam1 localization and LFA-1 activation**

The subcellular localizations of Tiam1 and LFA-1 were then studied in JE6.1 cells. Colocalization of Tiam1 and LFA-1 was enhanced in both anti-CD3– and SDF-1α–treated cells into caplike structures. In the presence of the Tiam1 inhibitor, most of the cap structures disappeared, and colocalization was present in clusters (Fig. 6A, 6B). The same distribution pattern was seen in T cells (data not shown). To study the effect of Tiam1 inhibition on LFA-1 activation, cells were stained with the KIM127 mAb, which recognizes the extended form of LFA-1, or the mAb24, which reacts with the extended, open form of LFA-1. Interestingly, the amount of KIM127 fluorescence was increased in the presence of the Tiam inhibitor. A much higher increase was seen in anti-CD3– and SDF-1α–treated cells. This increase was blocked by the Tiam inhibitor, indicating that Tiam1 also affects the activation state of LFA-1. Likewise, mAb24 reactivity also increased in anti-CD3–activated cells, but not in SDF-1α–activated cells. In contrast to KIM127 activity, the effect of Tiam1 on mAb24 activity was minor (Fig. 6C, 6D). In both anti-CD3– and SDF-1α–activated cells, Tiam1 colocalized with the extended form of LFA-1 (Fig. 6E).
Discussion

A number of cytoplasmic proteins affect LFA-1–dependent signaling and adhesion, but our current picture of these events is still far from complete. When T cells are stimulated through the TCR, intracellular signaling events are triggered, resulting in LFA-1 activation. Many different signaling pathways and proteins have been implicated in LFA-1 activation, including protein and lipid kinases, calcium-binding proteins, and small GTPases. The cytoplasmic part of the β2 chain associates with several binding partners, including talin, α-actinin, filamin, cytohesin, and kindlin (9). Changes in the phosphorylation pattern of LFA-1 enable it to regulate these interactions, resulting in subsequent downstream effects. Phosphorylation of T758 on the LFA-1 β2 chain is known to favor 14-3-3 binding and filamin release from LFA-1, but it appears to affect neither α-actinin binding (30) nor the ability of the talin head to activate the integrin (11, 25). Furthermore, T758 phosphorylation enhances Rac1 activity and cell adhesion (13, 17). The complete signaling pathway from LFA-1 that leads to cytoskeletal reorganization is, however, still not characterized. In our aim to identify these components, we searched for a candidate Rac GEF, which could activate Rac, downstream of the T758 phosphorylated LFA-1. The previously characterized Rac1 activation and increase of cell adhesion caused by T758 phosphorylation were blocked by Tiam1-specific siRNA or a Tiam inhibitor. This indicates that Tiam1

FIGURE 4. Inhibition of Tiam1 reduces T cell migration on ICAM-1. JE6.1 cells (A) or T cells (B) were pretreated with 100 μM Tiam inhibitor for 2 h or left untreated in the presence or absence of the LFA-1–blocking Ab R7E4 (20 μg/ml). Cells were allowed to migrate over ICAM-1- or BSA-coated Transwell membranes toward SDF-1α for 4 h. Migrated cells were counted. The experiment was performed three times in duplicate. The mean ± SD is given. Student t test was used for calculation of p values. **p < 0.01, ***p < 0.001. C, Lysates from T cells activated with SDF-1α or anti-CD3 (α-CD3, MEM57) in the presence or absence of okadaic acid (OA) were run on SDS-PAGE and immunoblotted with the pT758 β2 Ab, which recognizes the phosphorylated form of β2 (upper panel) or a β2 Ab (R2E7B, lower panel).

FIGURE 5. Phospho-T758-integrin β2 enhances T cell migration on ICAM-1. A. The nonphosphorylated or phosphorylated β2 peptide was transfected into T cells, and cells were allowed to migrate over ICAM-1–coated Transwell membranes toward SDF-1α. B, Untransfected or phospho-β2 peptide-transfected cells were left untreated or treated with Tiam inhibitor and allowed to migrate, as in A. C, T cells were transfected with the control peptide P621 or the 14-3-3–blocking peptide R18 with or without the Tiam inhibitor. Migration was performed, as in A. The average of three experiments is shown. Untransfected cells were given the value 100. The mean ± SD is given. Student t test was used for calculation of p values. *p < 0.05, ***p < 0.001.
regulates these cytoskeletal changes. Another Rac GEF, Vav1, is implicated in LFA-1–mediated Rac1 activation (31), and previous studies identify a role for Tiam1 and the Par polarity complex in T cell polarization and trafficking (21, 32). Tiam1 interactions with different scaffold proteins are essential to couple distinct upstream signals to localized Rac activation and specific downstream pathways (33). Whether there is cross talk between LFA-1/14-3-3/Tiam1/Rac1-signaling, the Vav1/Rac1 interaction, and the Tiam1/Par complex or whether they represent distinct signaling pathways needs to be elucidated.

T cells migrate under the direction of chemotactic stimulants (34, 35). Activation of chemokine receptors leads to the activation of LFA-1, and several signaling events have been correlated with this activation, including the activation of Rac (29, 36–38). We now show that SDF-1α stimulation of T cells results in strong phosphorylation on T758 and subsequently a large increase in migration. This migration is LFA-1 dependent, as migration on ICAM-1, a LFA-1 ligand, is markedly increased compared with migration on membranes coated with BSA. In addition, inactivation of LFA-1 with an Ab that specifically blocks LFA-1 binding to ICAMs reduced migration to control levels. Transfection of the phospho-T758 peptide also increased T cell migration toward SDF-1α. We showed that this LFA-1–dependent migration on ICAM-1 was blocked by the Tiam inhibitor, indicating that Tiam1 functions downstream of LFA-1–induced signaling needed for migration.

Blocking of 14-3-3 binding to the β2 integrin in TCR-stimulated cells by the R18 peptide inhibits cell adhesion and spreading on ICAM-1 (5). Thus, the LFA-1/14-3-3 interaction is needed for the effect of LFA-1 on the cell cytoskeleton. We now show that association between LFA-1 and Tiam1 is also 14-3-3 dependent, because the presence of R18 reduced the association between the phospho-T758 peptide and Tiam1. In addition, R18 reduced T cell migration toward SDF-1α, further verifying the involvement of 14-3-3 in cell migration. Taken together, these data have identified a signaling complex of LFA-1, 14-3-3, and Tiam1 that regulates Rac1 activation and, thus, downstream cytoskeletal reorganization affecting cell adhesion and migration. Our data also show that the presence of the extended form of LFA-1 increased in anti-CD3 and SDF-1α–activated cells as detected by the KIM127 Ab. This increase was partially blocked by the Tiam inhibitor. Interestingly, the Tiam inhibitor alone increased KIM127 reactivity in cells, although at lower levels than in activated cells. The
presence of the extended open LFA-1 (mAb24) increased after anti-CD3, but not SDF-1α activation, and was not sensitive to Tiam1 activity. Elucidation of the complex cross talk between different signaling pathways in the regulation of LFA-1 still needs further work.

Signaling from two receptor complexes, the TCR and the chemokine receptor, leads to LFA-1 activation. We showed that stimulation of T cells with either anti-CD3 or SDF-1α resulted in phosphorylation of T758 on the LFA-1 β2 chain. This, in turn, activated downstream signaling through Tiam1, leading to cytoskeletal rearrangements, and affected cell adhesion and migration.

The role of Tiam1 in the adhesion-complex formation and downstream signaling has previously been identified in several cell types. Tiam1 induces the formation of specific αβ/β-containing adhesive complexes at the cell periphery in neuroblastoma cells (39). Tiam1 is a key molecule in αβ/β-mediated activation of Rac1, which is essential for proper production and secretion of laminin, a requirement for the spreading and migration of keratinocytes (40). Tiam1/Rac signaling also controls the establishment and maintenance of E-cadherin-based cell–cell adhesions (41).

Stam et al. (42) showed that T lymphoma invasion is driven by Tiam1 through Rac activation. The role of Tiam1 is increasingly being identified in human cancers and experimental cancer models. Learning more about Tiam1 regulation and its involvement in different signaling pathways is of critical importance for understanding its role in cellular functions and for the development of targeted drug interventions.

Disclosures

The authors have no financial conflicts of interest.

References