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Src Homology 2-Domain Containing Leukocyte-Specific Phosphoprotein of 76 kDa Is Mandatory for TCR-Mediated Inside-Out Signaling, but Dispensable for CXCR4-Mediated LFA-1 Activation, Adhesion, and Migration of T Cells

Jessica Horn,* Xiaojian Wang,** Peter Reichardt,† Theresa E. Stradal,‡ Nicole Warnecke,* Luca Simeoni,* Matthias Gunzer,* Deborah Yablonski,‡ Burkhart Schraven,* and Stefanie Kliche3

Engagement of the TCR or of chemokine receptors such as CXCR4 induces adhesion and migration of T cells via so-called inside-out signaling pathways. The molecular processes underlying inside-out signaling events are as yet not completely understood. In this study, we show that TCR- and CXCR4-mediated activation of integrins critically depends on the membrane recruitment of the adhesion- and degranulation-promoting adapter protein (ADAP)/Src kinase-associated phosphoprotein of 55 kDa (SKAP55)/Rap1-interacting adapter protein (RIAM)/Ral module. We further demonstrate that the Src homology 2-domain containing leukocyte-specific phosphoprotein of 76 kDa (SLP76) is crucial for TCR-mediated inside-out signaling and T cell/APC interaction. Besides facilitating membrane recruitment of ADAP, SKAP55, and RIAM, SLP76 regulates TCR-mediated inside-out signaling by controlling the activation of Rap1 as well as Rac-mediated actin polymerization. Surprisingly, however, SLP76 is not mandatory for CXCR4-mediated inside-out signaling. Indeed, both CXCR4-induced T cell adhesion and migration are not affected by loss of SLP76. Moreover, after CXCR4 stimulation, the ADAP/SKAP55/RIAM/Rap1 module is recruited to the plasma membrane independently of SLP76. Collectively, our data indicate a differential requirement for SLP76 in TCR- vs CXCR4-mediated inside-out signaling pathways regulating T cell adhesion and migration. The Journal of Immunology, 2009, 183: 5756–5767.

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Resting T cells are not adhesive because LFA-1 is presented in a closed, inactive conformation. Upon triggering of the TCR or of chemokine receptors (see below), a conformational change is induced within LFA-1 that augments its affinity for ICAM-1. In addition, clustering of LFA-1 molecules on the surface of T cells enhances avidity for ICAM-1 binding. The molecular events leading to integrin activation have collectively been termed inside-out signaling (6).

Gain-of-function and loss-of-function studies have demonstrated that additional signaling proteins are involved in TCR-mediated integrin activation. These include talin (6, 7), the Wiskott-Aldrich syndrome-Verprolin-homologous protein WAVE2 (8, 9), Rap1 (6, 10, 11), and its downstream targets regulator of adhesion and cell polarization enriched in lymphoid tissues or Rap1-interacting adapter protein (RIAM) (12–14), adhesion- and degranulation-promoting adapter protein (ADAP) (15, 16), and Src kinase-associated phosphoprotein of 55 kDa (SKAP55) (17, 18). Moreover, components of the LAT/Gads/SLP76 signaling platform are also critically involved in TCR-mediated activation of LFA-1. These include PLCγ1, Vav1, Itk, and ADAP (13, 19, 20). How exactly these molecules orchestrate TCR-mediated activation of integrins is not completely understood. However, we have recently shown that the formation of a signaling module consisting of ADAP/SKAP55 complex together with RIAM is required for plasma membrane targeting of Rap1 in response to TCR stimulation, and therefore for TCR-mediated activation of integrins (17, 21). In line with these data is the observation that ADAP- and SKAP55-deficient mouse T cells show a severe defect in integrin activation in response to TCR-mediated stimuli (15, 16, 18).

Currently, it is proposed that following T cell activation, tyrosine-phosphorylated ADAP binds to the Src homology 2 domain of SLP76, thereby leading to membrane recruitment of the ADAP/SKAP55/RIAM module and integrin activation. In line with this idea is the observation that disruption of the interaction between SLP76 and Gads blocks TCR-mediated adhesion to ICAM-1 (22). Furthermore, it was shown that mutation of those tyrosine residues within ADAP, which are believed to mediate the interaction between ADAP and SLP76, blocks TCR-mediated activation of LFA-1 (23). Thus, it appears as if SLP76 would also play a major role during TCR-mediated activation of integrins by facilitation membrane targeting of the ADAP/SKAP55/RIAM module. However, to date, an inducible interaction between endogenously expressed ADAP/SKAP55/RIAM module and SLP76 in response to TCR engagement has not been demonstrated biochemically. Hence, the molecular signaling events underlying the function of SLP76 for TCR-mediated integrin activation are not completely elucidated.

Importantly, integrins not only become activated after stimulation of the TCR, but also after triggering of chemokine receptors, such as CXCR4. Signaling via CXCR4 is induced by stromal cell-derived factor-1α (or CXCL12) and induces affinity and avidity regulation of LFA-1 (6). This process is important for firm T cell adhesion, T cell polarization, chemokinesis, and chemotaxis (24). Molecules involved in CXCR4-mediated integrin activation and/or chemotaxis are talin (25), Rap1 (6, 26, 27), and its downstream targets regulator of adhesion and cell polarization enriched in lymphoid tissues and Mst1 (28–30), Itk (20), Vav1 (20, 31, 32), Rac (31), and members of the Wiskott-Aldrich syndrome protein family (33, 34). Moreover, we and others have shown that overexpression of ADAP enhances chemotaxis of T cells in response to CXCL12 (35, 36). These findings suggest an important role of ADAP for CXCR4-induced migration of T cells. However, the molecular basis of how ADAP is integrated into CXCR4 signaling is to date unclear. Based on its central role in TCR-mediated signaling processes, SLP76 would be an attractive candidate that could facilitate membrane targeting of ADAP after CXCR4 stimulation. Indeed, it was shown recently that SLP76 regulates CXCR4-induced Ca2+ flux and ERK1/2 phosphorylation (31). Conversely, we have demonstrated that disruption of the SLP76/Gads association, albeit impairing TCR-mediated signaling processes, does not affect CXCR4-induced Ca2+ flux and chemotaxis (22). Thus, it is unclear whether SLP76 is also important for CXCR4-mediated activation of integrins and chemotaxis.

In this study, we have addressed critical questions regarding the function of SLP76 during TCR vs CXCR4-mediated signaling. We show that SLP76 is indeed a critical regulator of TCR-mediated inside-out signaling events in T cells, and we demonstrate that SLP76 is mandatory for induction of TCR-mediated adhesion, affinity/avidity regulation of LFA-1, and the interaction between T cells and B cells. Furthermore, we show that SLP76 is required for TCR-induced Rap1 activation, Rac-mediated actin dynamics, and recruitment of both talin and the ADAP/SKAP55/RIAM module to the plasma membrane and to the IS. Surprisingly, however, SLP76 is not mandatory for CXCR4-mediated activation of LFA-1. In addition, SLP76 is dispensable for adhesion and migration of T cells in response to CXCL12. Finally, we demonstrate that the ADAP/SKAP55/RIAM/Rap1 module is crucial for T cell adhesion and migration in response to CXCR4 triggering, but is recruited to the plasma membrane independently of SLP76. Our findings show that SLP76 acts as a key player during TCR-mediated inside-out signaling, whereas the adapter protein appears to be dispensable for chemokine-dependent processes that regulate adhesion and migration of T cells.

Materials and Methods
Reagents and Abs
All tissue culture reagents were from Biochrom, and all chemicals were from Roth, unless mentioned otherwise. Staphylococcal enterotoxin B, D, and E were purchased from Toxin Technology, respectively. Indo-1 AM, Blue-7-amino-4-chloromethylcoumarin, DDAO-SE, and Alexa Fluor 633-phalloidin were bought from Molecular Probes. FITC or tetramethylrhodamine isothiocyanate (TRITC) phalloidin were bought from Sigma-Aldrich. Phalloidin and anti-phospho-ZAP70 (pY319) rabbit serum (both from Cell Signaling Technologies, Danvers, MA), anti-SKAP55 rat antibody, anti-ADAP sheep serum (41), anti-phospho-SLP76 rabbit serum, anti-SLPI6 mAb (Santa Cruz Biotechnology), anti-LAT mAb (SK13B6) (17), anti-GST rat mAb (provided by C. Erk, Helmholz-Zentrum für Infektionsforschung, Braunschweig, Germany), anti-β-actin mAb (Sigma-Aldrich), anti-phospho-ERK1/2 (T202, Y204) rabbit serum and anti-Vav rabbit serum (both from Cell Signaling Technology), anti-SLP76 mAb (Santa Cruz Biotechnology), anti-LAT mAb (BD Biosciences), anti-ADAP sheep serum (41), anti-phospho-SLP76 mAb (pY145; BD Biosciences), anti-phospho-LAT (pY171) rabbit serum and anti-phospho-ZAP70 (pY319) rabbit serum (both from Cell
RIAM, or Vav1 was evaluated either by Western blotting or on a single-cell flow cytometer and CellQuestPro software (BD Biosciences). Soluble Fc-ICAM-1 binding of T cells after various stimuli was assessed, as previously described (43). Briefly, T cells suspended in binding buffer (HBBS containing 2% FBS) were either left untreated or stimulated with anti-CD3 mAb, CXCL12, or Mg2+/EDTA for 5 min in the presence of 20 μg/ml human rFc-ICAM-1, and bound Fc-ICAM-1 was detected by flow cytometry. Intracellular staining by flow cytometry for SLP76, ADAP, SKAP55, or RIAM was performed, as previously described (44). The specificity of the staining for each serum or mAb was assessed using SLP76-deficient/reconstituted Jurkat T cells or after loss of ADAP, SKAP55, or RIAM expression by vector-based shRNA in Jurkat T cells (please see supplemental Fig. 1). To assess the F-actin content, T cells (1 × 10⁶) were left untreated or stimulated with anti-CD3 mAbs or CXCL12, and reactions were stopped by adding PBS containing 4% paraformaldehyde (PFA), 2 μM calcium/CaCl₂ or Alexa Fluor 488-labeled Tropolonin, and Cy3-conjugated secondary Abs. After 15 min, cells were washed with 1% PFA in PBS and analyzed by flow cytometry. Measurement of TCR-induced calcium release and CD69 up-regulation have been previously described (17). Analysis of protein phosphorylation with phospho-specific Abs by flow cytometry was performed, as described (45).

Conjugate formation, adhesion, migration, and motility assays

Conjugate assays were performed, as described (21). Briefly, superantigen (SA) mixture of staphylococcal enterotoxin B-, D-, and E-pulsed and DDA-pulsed Raji B cells were incubated with an equal number of Jurkat T cells or CFSE-loaded human T cells for 30 min at 37°C. Non-specific aggregates were disrupted; cells were fixed with 1% PFA, and then analyzed by flow cytometry. The percentage of conjugates was defined as the number of double-positive events in the upper right quadrant. Adhesion assays were performed, as previously described (17). Briefly, Jurkat T cells or peripheral human T cells were stimulated with OKT3, PMA, or M1ClI, for 30 min at 37°C before adhesion on Fc-ICAM-1-coated dishes. The bound total or GFP-expressing cell fraction was determined by counting four independent fields by microscopy using an ocular counting reticle. To assess CXCR4-mediated adhesion, peripheral human T cells were incubated for 10 min at 37°C on Fc-ICAM-1-coated dishes immobilized with or without CXCL12; subsequently, nonbound cells were removed by washing with HBBS and bound cells were counted, as described above. Chemotaxis assays were performed, as previously described, using Transwells (Costar) coated with fibronectin (35). After 2 h, the number of migrated cells into the lower chamber was counted and the percentage of GFP-expressing Jurkat T cells was determined by flow cytometry. For live cell imaging of either random or CXCL12-induced motility of T cells on Fc-ICAM-1, self-constructed imaging chambers (46) were coated with Fc-ICAM-1 in PBS at 4°C overnight. Immediately before imaging, cells were left untreated or stimulated with CXCL12 (100 ng/ml), and imaging was performed on a CellIR imaging workstation (Olympus) using an upright microscope stage (BX61) with a ×20 lens. Using an automated X-Y-Z stage, at least two optical fields were chosen for each culture condition. Images were taken every 15–60 s for 2 h. At least 30 cells per observation field were analyzed. Tracking analysis of migrating cells to determine the velocity was done by computer-assisted cell tracking using the Software CellTracker (46).

Immuno-fluorescence microscopy

For B cell/T cell conjugates, SA- and Blue-7-aminoo-4-chloromethylcoumarin-loaded Raji B cells were incubated for 30 min at 37°C with human T cells on poly-(l-lysine)-coated coverslips and fixed with 3.5% PFA in PBS for 10 min. Cells were permeabilized with 0.1% Triton X-100 in PBS, and blocked with 5% horse serum in PBS. The cells were stained with the indicated serum or mAb was assessed using SLP76-deficient/reconstituted Jurkat T cells or after loss of ADAP, SKAP55, or RIAM expression by vector-based shRNA in Jurkat T cells (please see supplemental Fig. 1). To assess the F-actin content, T cells (1 × 10⁶) were left untreated or stimulated with anti-CD3 mAbs or CXCL12, and reactions were stopped by adding PBS containing 4% paraformaldehyde (PFA), 2 μM calcium/CaCl₂ or Alexa Fluor 488-labeled Tropolonin, and Cy3-conjugated secondary Abs. After 15 min, cells were washed with 1% PFA in PBS and analyzed by flow cytometry. Measurement of TCR-induced calcium release and CD69 up-regulation have been previously described (17). Analysis of protein phosphorylation with phospho-specific Abs by flow cytometry was performed, as described (45).

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The online version of this article contains supplemental material.
with FITC-conjugated anti-MEM48 and imaged, as described above. For each experiment, a minimum of at least 40 cells with LFA-1 polarized to one side of the cell was regarded as polarized, whereas those cells showing equal distribution of LFA-1 were considered not to be polarized. The percentage of polarized cells in each field was determined.

Results

Loss of SLP-76 impairs TCR-mediated activation of LFA-1 and T cell-APC conjugation

By using a small peptide that disrupts the constitutive interaction between SLP76 and Gads, we recently provided evidence that formation of the LAT/Gads/SLP76 signaling platform at the plasma membrane is required for TCR-mediated integrin activation (22). However, we formally could not exclude the possibility that the functional effects exerted by the Gads-binding fragment were not due to targeting the SLP76/Gads complex, but rather to disruption of a distinct signaling pathway that regulates integrin activation in response to TCR stimulation. To assess this point more specifically, we reduced the expression of SLP76 in primary human T cells by RNAi. As shown in Fig. 1, A and B, SLP76 siRNA treatment lowered the expression levels of endogenous SLP76 up to 80%, whereas the expression levels of ADAP, SKAP55, and RIAM remained unaffected.
Previously, it had been reported that SLP76 is required for phosphorylation of PLCγ1, activation of ERK1/2, as well as for expression of CD69 upon TCR stimulation. To first address whether loss of SLP76 interferes with these SLP76-mediated functions also in primary human T cells, we analyzed the ability of SLP76-deficient human T cells to activate PLCγ1 and ERK1/2 in response to TCR stimulation by Western blotting and to up-regulate CD69 expression by flow cytometry. As shown in Fig. 1C, loss of SLP76 strongly attenuated TCR-mediated phosphorylation of PLCγ1 at Y783, activation of ERK1/2, and up-regulation of CD69. As expected, more proximal signaling events such as the phosphorylation of ZAP70 at Y119 or tyrosine phosphorylation of LAT (either global or at Y173) were not affected in the absence of SLP76 (Fig. 1C). Note that similar data were obtained, when we suppressed the expression of SLP76 by shRNA in Jurkat T cells (supplemental Fig. 2). Thus, with regard to well-established SLP76-regulated signaling processes, SLP76-deficient primary human T cells behave like their corresponding Jurkat T cell counterparts (3).

Using the siRNA approach, we next analyzed the capability of T cells to adhere to ICAM-1-coated dishes in response to various stimuli. As shown in Fig. 1D and supplemental Fig. 3A, control transfected human T cells and Jurkat T cells readily adhered to ICAM-1 upon TCR or PMA treatment. In contrast, loss of SLP76 substantially attenuated both PMA- and TCR-induced adhesion to ICAM-1 (left panel), whereas the cells showed no defect in their adhesiveness in response to Mn²⁺ (middle panel). The defect in TCR- or PMA-induced adhesion to ICAM-1 was not due to an altered expression of the β₂ integrin (or the TCR) as determined by flow cytometry (Fig. 1E and supplemental Fig. 3B).

Because the interaction of LFA-1 with ICAM-1 is important for the establishment and maintenance of T cell/APC interactions (5), we next analyzed whether SLP76 is required for conjugate formation between human T cells and SA-loaded B cells. Fig. 1F shows that in contrast to control transfected T cells, loss of SLP76 substantially blunted conjugate formation. Similarly, SLP76low Jurkat T cells failed to interact with SA-loaded B cells (supplemental Fig. 3C). Collectively, the experiments shown in Fig. 1 indicate that SLP76 is mandatory for both TCR-mediated adhesion to ICAM-1 and conjugate formation.

**Loss of SLP76 attenuates TCR-mediated affinity/avidity regulation of LFA-1**

TCR-mediated inside-out signaling alters both LFA-1 affinity (conformation) and avidity (clustering) (6). To determine the role of SLP76 for LFA-1 affinity modulation, we assessed the ability of soluble Fc-ICAM-1 to bind to SLP76-proficient and SLP76-deficient T cells after TCR stimulation by means of flow cytometry. Fig. 2A shows that knockdown of SLP76 in human T cells abrogates binding of soluble Fc-ICAM-1 after TCR stimulation (for Jurkat T cells, please see supplemental Fig. 3D). In contrast, both transfectedant cells were allowed to form conjugates with SA-pulsed B cells for 30 min. Fixed cells were stained for the β2 subunit of LFA-1 (green) to determine IS localization of LFA-1. Fluorescence intensity of LFA-1 at the contact zone or the uropod was quantified and calculated as ratio of intensity at the IS vs the uropod. Knockdown efficiency of SLP76 expression was assessed by flow cytometry. Data present the average of three independently performed experiments.

**TCR-mediated activation of Rac and actin dynamics require SLP76**

Reorganization of the actin cytoskeleton is required for TCR-mediated integrin activation and conjugate formation (33, 34), and the small GTPase Rac is a major regulator regulating this process. In T cells, activation of Rac is mediated via the nucleotide exchange factor Vav1, which is recruited to and activated by SLP76 upon T cell activation (32). Therefore, it seemed likely that loss of SLP76 also leads to alterations in actin dynamics upon TCR stimulation. As depicted in Fig. 3, this is indeed the case. TCR-mediated activation of Rac (Fig. 3A), formation of F-actin (Fig. 3B), as well as accumulation of F-actin at the IS (Fig. 3C, upper right panel) are severely impaired in SLP76-deficient T cells. Note that similar data were obtained when the F-actin content after TCR triggering was assessed in SLP76low Jurkat T cells (data not shown). Hence, loss of SLP76 induces a failure to activate Rac and a defect in F-actin remodeling upon TCR stimulation.

Because the scaffolding protein talin links newly synthesized F-actin branches to LFA-1 and, hence, stabilizes the position of LFA-1 in the IS (7, 47), we also investigated whether SLP76 is required for targeting of talin to the IS after TCR stimulation.
Both the activation and the transport of Rap1 to the plasma membrane are known to regulate LFA-1 activation (6, 17, 48). Therefore, we next investigated whether loss of SLP76 affects TCR-mediated activation of Rap1. Fig. 4A demonstrates that control transfected cells exhibited strong activation of Rap1 within 2–10 min after TCR stimulation, whereas in SLP76low Jurkat T cells, TCR-induced Rap1 was strongly suppressed. These results demonstrate that SLP76 is critically involved in Rap1 activation in response to TCR triggering.

We have recently shown that formation and membrane recruitment of a signaling module consisting of the cytosolic adapter proteins ADAP and SKAP55 and the Rap1 effector molecule RIAM (ADAP/SKAP55/RIAM module) regulate TCR-mediated LFA-1 activation through recruitment of Rap1 to the plasma membrane (17, 21). Moreover, an indiscernible association between SLP76 and ADAP following TCR stimulation had been suggested in several previous studies, but the question of whether SLP76 links the TCR via the ADAP/SKAP55/RIAM module to integrin activation has to date not been addressed directly. To clarify this point, we immunoprecipitated SLP76 from resting or TCR-stimulated T cells and subsequently analyzed the precipitates for coprecipitation of ADAP, SKAP55, RIAM, and Rap1 by means of Western blotting. Fig. 4B depicts that SLP76 inductively associates with both the ADAP/SKAP55/RIAM complex and Rap1 upon TCR stimulation of Jurkat T cells (Fig. 4B, left panel) as well as of primary T cells (Fig. 4B, right panel). Analysis of SKAP55 immunoprecipitates that were prepared from either SLP76-proficient or SLP76-deficient T cells further revealed that Rap1 only associates with the ADAP/SKAP55/RIAM module if SLP76 is present (Fig. 4C). Because only active Rap1 can interact with RIAM (12), this finding is most likely due to the attenuated activation of Rap1 in the absence of SLP76 (see Fig. 4A).

The immunoprecipitation data shown in Fig. 4, B, and C, were further substantiated by cellular subtraction experiments that showed that TCR-mediated plasma membrane recruitment of ADAP, SKAP55, RIAM, and Rap1 (Fig. 4D) and, consequently, targeting of SKAP55, RIAM, and Rap1 to the IS (Fig. 4E) were strongly impaired in SLP76low T cells. In summary, the data shown in Fig. 4 indicate that SLP76 is required for both activation and plasma membrane/IS targeting through the ADAP/SKAP55/RIAM/Rap1 module of Rap1 after TCR triggering.

**SLP76 is dispensable for CXCR4-mediated adhesion to ICAM-1 and affinity regulation of LFA-1**

Similar to the TCR, triggering of the chemokine receptor CXCR4 leads to activation of LFA-1 and T cell adhesion to ICAM-1 (6). Above we have shown that SLP76 is mandatory for TCR-mediated integrin activation (Figs. 1 and 2). Therefore, we were interested to investigate whether SLP76 is also required for CXCR4-mediated adhesion. To assess this point, we down-regulated SLP76 expression by siRNA and subsequently analyzed the capability of SLP76low T cells to adhere to ICAM-1 in response to CXCL12 in an adhesion assay. Surprisingly, these experiments revealed that SLP76low T cells were as capable to adhere to Fc-ICAM-1 in response to CXCL12 as their SLP76high counterparts (Fig. 5A). Similarly, neither the binding of soluble Fc-ICAM-1 (Fig. 5B) nor of the conformation-sensitive anti-LFA-1 mAb KIM127 (data not shown) was altered in SLP76low T cells. Hence, we conclude that, in contrast to TCR stimulation, the presence of SLP76 is dispensable for T cell adhesion and affinity regulation in response to CXCL12 stimulation.
SLP76 is dispensable for CXCR4-mediated Rac activation, actin dynamics, and migration

Similar to TCR stimulation, remodeling of the actin cytoskeleton through Rac is crucial for inducing T cell adhesion, polarization, and chemotaxis following CXCR4 stimulation (33). Our above findings led us to ask whether the presence of SLP76 might be dispensable for Rac activation in response to CXCL12 triggering. As shown in Fig. 6A, SLP76low Jurkat T cells indeed display no obvious defect in CXCL12-mediated activation of Rac. Moreover, both SLP76high and SLP76low T cells revealed comparable levels of F-actin formation in response to CXCL12 (Fig. 6B). These data strongly suggest that SLP76 is indeed dispensable for signaling events that are critically involved in CXCR4-mediated activation of Rac and actin polymerization.

To prove whether expression of SLP76 is required for CXCR4-induced T cell chemokinesis, we determined the lateral locomotion of T cells by live cell imaging on Fc-ICAM-1-coated coverslips. As shown in Fig. 6C, both SLP76high and SLP76low T cells showed comparable basal and CXCL12-induced velocities. In contrast, and in line with previously published data (31), Vav1-deficient T cells displayed...
a strongly reduced motility upon CXCR4 triggering (Fig. 6C). It is also important to note that nearly all SPL76low T cells displayed a polarized phenotype upon chemokine stimulation, whereas the majority of Vav1-depleted T cells remained a round, nonpolarized shape (Fig. 6C). Fig. 6D further demonstrates that the absence of SPL76 does not alter CXCR4-induced chemotaxis of human primary T cells in response to a CXCL12 gradient in vitro. In summary, it appears as if SPL76 is not mandatory for migratory steps triggered by CXCL12.

The ADAP/SKAP55/RIAM module is recruited to the plasma membrane independently of SPL76

Upon T cell activation, the Gads/SLP76 complex is recruited to phosphorylated LAT, thereby coupling the TCR to the intracellular signaling machinery (2, 49). Given the above data, we next investigated whether the LAT/Gads/SLP76 signaling platform would be assembled after CXCR4 stimulation. To test this, we stimulated human T cells for various periods of time with CXCL12 and subsequently analyzed global tyrosine phosphorylation of LAT by Western blotting. In contrast to TCR stimulation, phosphorylation of LAT was only very weakly induced upon CXCR4 triggering (Fig. 7A). Similar results were obtained when the phosphorylation status of LAT at Y171 (which is important for Gads binding) was assessed (Fig. 7A). These data suggested that the Gads/SLP76 complex might not associate with LAT following CXCR4 triggering. To investigate this point in more detail, we immunoprecipitated the Gads/SLP76 complex from untreated, TCR-stimulated, or CXCR4-triggered T cells. Fig. 7B shows that an inducible interaction among Gads/SLP76, LAT, and Vav1 was readily detectable after TCR stimulation. In contrast, neither LAT nor Vav1 was found to be associated with the Gads/SLP76 complex in

FIGURE 5. SLP76 is dispensable for CXCL12-mediated adhesion to ICAM-1, affinity maturation of LFA-1. A, Human T cells were transfected with control siRNA (siC) or siRNAs against SLP76 (siSLP76). Cells were left untreated or stimulated with immobilized CXCL12 (10 min) or anti-CD3 mAbs (TCR) for 30 min. Subsequently, cells were analyzed for their ability to adhere to Fc-ICAM-1. Suppression of SLP76 expression was assessed by flow cytometry, and the percentage of SLP76-expressing cells was calculated. Data represent the mean and SE of three independently performed experiments. B, Human T cells transfected as described in A were analyzed for their ability to bind soluble Fc-ICAM-1 upon anti-CD3 (TCR), CXCL12, or Mg²⁺/EGTA (positive control) treatment for 5 min. Suppression of SLP76 expression was evaluated by flow cytometry. One individual experiment of three is shown.

FIGURE 6. The presence of SLP76 is not required for Rac activation, actin polymerization, motility, and chemotaxis in response to CXCL12. A, Jurkat T cells were transfected with either control vector (shC) or SLP76-suppression vector (shSLP76). After 48 h, cells were either left untreated, or stimulated with CXCL12 or anti-CD3 mAbs (TCR) for the indicated time points. Activated Rac was precipitated using the GST-PBD fusion protein. Precipitates and aliquots of whole-cell extracts were analyzed by Western blotting using the indicated Abs. Data are representative of three independent experiments. B, Human T cells were transfected with control siRNA (siC) or siRNA against SLP76 (siSLP76). After 72 h, cells were left untreated, stimulated with anti-CD3 mAbs (TCR, for 5 min) or CXCL12 for 1 min, and stained with FITC-coupled phalloidin. Reduction of SLP76 expression in human T cells was assessed by flow cytometry. Data are representative of three independent experiments. C, Human T cells were transfected with control siRNA (siC), siRNA against SLP76 (siSLP76), or siRNA against Vav1 (siVav1). After 72 h, motility of T cells in the absence or presence of CXCL12 was determined on Fc-ICAM-1-coated coverslips, as described in Materials and Methods. In parallel, transfectants were stimulated on Fc-ICAM-1-coated coverslips with CXCL12 for 30 min. Cells were fixed, permeabilized, and stained with TRITC-phalloidin. Whole-cell extracts were analyzed by Western blotting for the expression of SLP76, Vav1, and β-actin. One individual experiment of three is shown. D, Chemotaxis of human T cells transfected as described in A was addressed using a Transwell assay, as described in Materials and Methods. After 2 h, the number of migrated cells into the lower chamber was counted. Suppression of SLP76 expression was assessed by flow cytometry, and the percentage of SLP76-expressing cells was calculated. Data represent the mean and SE of three independently performed experiments.
response to CXCL12 stimulation (Fig. 7B). These experiments indicate that the LAT/Gads/SLP76 signaling platform is not assembled in response to CXCL12 in T cells.

Above we have shown that the ADAP/SKAP55/RIAM module inducibly interacts with SLP76 upon TCR-mediated T cell activation, and that SLP76 is mandatory for membrane targeting of the ADAP/SKAP55/RIAM module and for activation of Rac or Rap1 (Fig. 4). In marked contrast, CXCL12 stimulation of T cells did not lead to an inducible interaction between the LAT/Gads/SLP76 complex and the ADAP/SKAP55/RIAM module (Fig. 7B). However, the ADAP/SKAP55/RIAM module (and also Rap1) was properly recruited to the plasma membrane in response to CXCR4 stimulation in both SLP76-proficient and -deficient T cells (Fig. 7, C and D). Moreover, SLP76low Jurkat T cells displayed no obvious defect in CXCL12-mediated activation of Rap1 (Fig. 7E). These data suggest that SLP76 is not required for CXCR4-mediated Rap1 activation and plasma membrane targeting of ADAP, SKAP55, RIAM, and Rap1.

To exclude the possibility that ADAP and SKAP55 themselves are dispensable for CXCR4-regulated adhesion and migration, we down-regulated ADAP expression in human T cells by siRNA and subsequently assessed both the adhesiveness and migratory capacity of these cells in response to CXCL12. Fig. 7F shows that loss of ADAP strongly attenuated CXCR4-induced adhesion and chemotaxis in human T cells. Similar data were obtained when we assessed CXCR4-induced migration of ADAPlow Jurkat T cells or of T cells obtained from ADAP-deficient mice (data not shown). In summary, our data show that in contrast to TCR stimulation, SLP76 is not involved in membrane recruitment of the ADAP/SKAP55/RIAM module, the activation of Rac or Rap1, and the induction of adhesion or migration in response to CXCR4 stimulation.

Discussion

Together with the transmembrane adapter protein LAT, the cytosolic adapter protein SLP76 facilitates the formation of a signaling platform (LAT/Gads/SLP76) following TCR stimulation that leads to the activation of multiple intracellular signaling pathways (49). Our previous data suggested that the signaling pathways controlled by the Gads/SLP76 complex also include those regulating adhesion processes (22). In this study, we confirm this hypothesis by showing that suppression of SLP76 expression by RNAi in human T cells or the

FIGURE 7. The SLP76/Gads complex is not associated with LAT or transported to the plasma membrane upon CXCL12 stimulation. A, Human T cells were either left untreated, or stimulated with anti-CD3 mAbs (TCR) or CXCL12 for the indicated periods of time. Lysates were analyzed by Western blotting using the indicated Abs. B, The cell extracts as described in A were used for immunoprecipitation using anti-Gads Ab. Precipitates were analyzed by Western blotting using the indicated Abs. C, Plasma membrane or cytosolic fractions were prepared from untreated (−) or CXCR4-stimulated human T cells (+). The individual fractions were analyzed by Western blotting using the indicated Abs. Fractionation efficiency was assessed by Western blotting using anti-LAT (plasma membrane) or anti-ERK1/2 (cytosol) Abs, respectively. One representative experiment of two is shown. D, Jurkat T cells were transfected with either control vector (shC) or SLP76-suppression vector (shSLP76). After 48 h, plasma membrane or cytosolic fractions were prepared from untreated (−) or CXCR4-stimulated cells (+). The individual fractions were analyzed by Western blotting using the indicated Abs. Fractionation efficiency was assessed by Western blotting using anti-LAT (plasma membrane) or anti-ERK1/2 (cytosol) Abs, respectively. One representative experiment of two is shown. E, Jurkat T cells were transfected with either control vector (shC) or SLP76-suppression vector (shSLP76). After 48 h, cells were either left untreated, or stimulated with CXCL12 or anti-CD3 mAbs (TCR) for the indicated time points. GTP-loaded Rap1 was precipitated using the GST-RalGDS-RBD fusion protein. Precipitates and aliquots of whole-cell extracts were analyzed by Western blotting using the indicated Abs. One representative experiment of three is shown. F, Human T cells were transfected with control siRNA (siC) or siRNA against ADAP (siADAP). After 72 h, transfectants were left untreated or stimulated with CXCL12 (for 10 min) or MnCl2 (for 30 min) and subsequently analyzed for their ability to adhere to Fc-ICAM-1. Chemotaxis of the same cells was analyzed using a Transwell assay, as described in Materials and Methods. After 2 h, the number of migrated cells into the lower chamber was determined. Suppression of ADAP and SKAP55 expression was determined by flow cytometry, and the percentage of ADAP/SKAP55-expressing cells was calculated. Note that loss of ADAP abrogates SKAP55 expression (17). Data represent the mean and SE of three independently performed experiments.
Jurkat T cell line abrogates TCR-mediated conjugate formation, adhesion to ICAM-1, and affinity/avidity maturation of LFA-1. Consistent with previously published data (50, 51), we also found that loss of SLP76 attenuates adhesion to fibronectin (the ligand of β1 integrins; VLA-4) in response to TCR stimulation (J. Horn and S. Kliche, unpublished data). Thus, SLP76 regulates TCR-induced inside-out signaling, leading to the activation of β1 and β2 integrins.

Reorganization of the actin cytoskeleton is critically involved in TCR-mediated integrin activation (33, 34). Moreover, it has previously been demonstrated that overexpression of SLP76 enhances F-actin formation in response to TCR stimulation (52). In addition to these overexpression data, we show in this study that loss of SLP76 attenuates TCR-mediated polymerization of F-actin. Together these findings suggest that SLP76 is one of the (if not the) central molecule that links the TCR to remodeling of the actin cytoskeleton.

The defect in actin dynamics most likely results from an abrogated activation of the small GTPase Rac. Activation of Rac depends on membrane recruitment and activation of the GEF Vav1 and the Tec kinase Itk (20, 32). Both Vav1 and Itk associate with SLP76 upon TCR stimulation, and both molecules are required for F-actin formation at the IS, activation of Rac, clustering of LFA-1, adhesion to ICAM-1, and conjugate formation (20, 32). In this scenario, the role of Vav1 is to generate sufficient pools of activated Rac to promote F-actin polymerization through activation of proteins of the Wiskott-Aldrich syndrome protein/Wiskott-Aldrich syndrome-Verprolin-homologues protein family (33, 34). Our data showing attenuated actin reorganization in SLP76low cells are in line with a model in which loss of SLP76 blocks activation of Rac and consequently signaling events downstream of Rac by impairing the functions of Vav1 and Itk.

We are, however, aware that another group recently reported that TCR-mediated actin remodeling is not attenuated in the SLP76-deficient Jurkat T cell line J14 (53). In addition, no defect in Rac activation upon TCR stimulation was observed in this Jurkat variant (54). The reasons for these discrepant findings in SLP76-deficient Jurkat T cells vs SLP76low human T cells are unclear at present. A possible explanation may come from compensatory mechanisms that developed in line J14 to escape the selection pressure that cannot develop in short-term manipulated T cells.

Besides the reorganization of the actin cytoskeleton, one critical regulator of TCR-mediated conjugate formation and inside-out signaling is the small GTPase Rap1 (6). Several studies have reported that Rap1 activation critically depends on the expression of PLCγ1 (13, 26, 55). PLCγ1 signaling to Rap1 occurs via the nucleotide exchange factor calcium and diacylglycerol-regulated guanine nucleotide exchange factor I that is activated by the second messengers calcium and diacylglycerol (which both are generated following lipid exchange factor PLCγ1). Thus, a defect in PLCγ1 activation in SLP76-suppressed T cells (see Fig. 1C and supplemental Fig. 2, C and D) and consequently a failure to activate CalDAG-GEFI might be largely responsible for the attenuated activation of Rap1 after TCR triggering in the absence of SLP76. However, additional mechanisms controlled by SLP76 may also contribute to the block in Rap1 activation. For example, SLP76 is mandatory for activation and membrane targeting of protein kinase Cθ, which has recently been identified to control TCR-induced Rap1 activity by phosphorylation of Rap1-GEF2 (56, 57). Hence, loss of SLP76 might affect several signaling pathways that are critical for TCR-induced activation of Rap1.

We had previously shown that the ADAP/SKAP55/RIAM complex is required for plasma membrane targeting of Rap1. Moreover, we had demonstrated that this event is crucial for inside-out signaling and for the activation of integrins in response to TCR stimulation. However, loss of ADAP and/or SKAP55 selectively abrogated plasma membrane recruitment of Rap1, although TCR-mediated activation of Rap1 was not affected (17, 21). In contrast, the data presented in this study show that SLP76 is mandatory for both membrane targeting and activation of Rap1 after TCR triggering. The former event most likely involves the ADAP/SKAP55/RIAM module, whereas the activation of Rap1 might be regulated through the PLCγ1- or the protein kinase Cδ-signaling pathways that were discussed above. Hence, our data support a model in which plasma membrane targeting and activation of Rap1 are two interrelated, but distinctly controlled signaling events that are organized at the level of SLP76.

The activation of Rap1 and Rac as well as changes in F-actin dynamics are also mandatory for affinity modulation of LFA-1, T cell adhesion, and migration in response to CXCR4 stimulation (6, 58). Given the central role of SLP76 in TCR-mediated signaling, it was totally unexpected for us to find that loss of SLP76 did not affect these events after CXCR4 stimulation and that the SLP76/Gads complex was not recruited to LAT in response to CXCR4 stimulation. The latter observation is probably due to the fact that the tyrosine residue within LAT that is responsible for recruiting the Gads/SLP76 complex (Y171) is not phosphorylated in response to CXCL12 triggering.

Our data also showed that SLP76 is not involved in the recruitment (and consequently the activation) of downstream effector molecules (e.g., Vav1) that are important for mediating adhesion and migration processes of T cells in response to CXCL12 triggering (58). Indeed, whereas Vav1-deficient T cells failed to polarize in response to CXCL12 stimulation, SLP76low T cells showed no defect in this process. Moreover, CXCL12-induced lateral migration velocity and chemotaxis were unaffected in SLP76low T cells. Hence, it appears as if SLP76 is dispensable for both adhesive and migratory processes in response to CXCL12 stimulation.

In line with the functional data, we found that membrane recruitment of the ADAP/SKAP55/RIAM module that is critical for CXCR4-mediated adhesion and migration of T cells was not affected by loss of SLP76. Although this finding strongly supports our above discussed hypothesis that membrane targeting of Rap1 and activation of this GTPase are distinct processes that are individually regulated by different signaling receptors, it opens the question as to which signaling molecule provides the molecular link between CXCR4 and the ADAP/SKAP55/RIAM module.

In this regard, it has recently been shown that the cytosolic adapter protein Shc is phosphorylated upon CXCR4 stimulation and assembles into a complex that includes Lck, ZAP70, Vav1, and LAT (59). Moreover, Shc-deficient Jurkat T cells show an attenuated migratory capacity and impaired F-actin dynamics upon CXCR4 stimulation, and mutation of critical tyrosine residues of Shc (Y238/240 or Y217) results in defective phosphorylation of Vav1 and Itk (59). Thus, Shc might be an attractive candidate to connect CXCR4 with the Vav1-Itk-dependent pathway of F-actin polymerization and T cell migration (59). It will be important to assess the role of Shc in CXCR4-induced inside-out signaling, adhesion, and Rap1 activation in future studies. Similarly, it needs to be analyzed whether, and if so how, LAT is involved in this process. Experiments are currently set up in our laboratory to assess these points. Nevertheless, our data collectively and surprisingly indicate that, in contrast to TCR signaling, SLP76 is not a key component connecting the chemokine receptor CXCR4 with the activation of integrins and the induction of T cell adhesion and migration.


Supplemental Figure 1. Intracellular staining of SLP76, ADAP, SKAP55, or RIAM for flow cytometry. (A) Jurkat or SLP76-deficient/reconstituted J14 were used for intracellular staining of SLP76 by flow cytometry. Cells were stained with the pre-immun (dashed line) or the anti-SLP76 sheep serum (black line). In addition, lysates of these cell lines were analyzed for the expression of SLP76 by Western blotting using the anti-SLP76 sheep serum. The detection of tubulin was used as loading control (B) Jurkat T-cells were electroporated with the control vector (shC) or the SKAP55 suppression vector (shSKAP55). After 48h, Jurkat T-cells or transfectants were stained with anti-GST (dashed line) or anti-SKAP55 rat mAbs (black line) and analyzed by intracellular flow cytometry of non-gated cells or within the GFP gate. In parallel, cell extracts were analyzed by Western blotting with the indicated antibodies (anti-SKAP55 and β-actin mAbs). (C) Jurkat T-cells were electroporated with the control vector (shC) or the ADAP suppression vector (shADAP). After 48h, Jurkat T-cells and transfectants were stained with the pre-immun (dashed line) or the anti-ADAP sheep serum (black line) and analyzed by intracellular flow cytometry of non-gated or GFP-positive gated cells. Since the presence of ADAP is required for stable expression of SKAP55, transfectants were stained in addition with anti-GST (dashed line) or anti-SKAP55 rat mAbs (black line) and analyzed by intracellular flow cytometry within the GFP gate. In parallel, cell extracts were analyzed by means of Western blotting using the following antibodies anti-ADAP sheep serum, anti-SKAP55 rat mAb, and β-actin mAb. (D) Jurkat T-cells were electroporated with the control vector (shC) or the RIAM suppression vector (shRIAM). After 48h, Jurkat T-cells or transfectants were stained with anti-GST (dashed line) or anti-RIAM rat mAbs (black line) and analyzed by intracellular flow cytometry non-gated cells or within the GFP gate. In parallel, cell extracts were analyzed by Western blotting with the indicated antibodies (anti-RIAM rat and β-actin mAbs).
Supplemental Figure 2. Silencing of SLP76 by shRNA in Jurkat T-cells abrogates TCR-induced PLCγ1 phosphorylation, calcium release, ERK1/2 activation and CD69 upregulation. (A) Jurkat T-cells were electroporated with the indicated amounts of control vector (shC) or SLP76 suppression vector (shSLP76). After 48h, cell extracts were analyzed by means of Western blotting using the indicated antibodies. (B) In parallel, cells transfected with 40 μg of shC or shSLP76 vector were stained for the endogenous expression of SLP76, ADAP, SKAP55, or RIAM analyzed by flow cytometry within the GFP gate. The pre-immune sheep serum was used as isotype control (dashed line). (C) Jurkat T-cells were transfected with either control vector (shC) or SLP76-suppression vector (shSLP76). After 48 h, cells were left either untreated or stimulated with the anti-CD3 mAbs (TCR) for 5 min. Cells were fixed, permeabilized and stained with either Alexa647-conjugated pPLCγ1 Y783 or pZAP-70 Y319 antibodies and analyzed by flow cytometry within the GFP gate. Note, that the total expression of PLCγ1 or ZAP-70 was not affected (data not shown). One representative experiment out of three is shown. (D) The above described transfectants were loaded with Indo-1 and stimulated with anti-CD3 mAbs (TCR) followed by ionomycin (iono). Increases in intracellular calcium were detected by ratiometric ultraviolet laser flow cytometry of cells within the GFP gate. Data are representative of three individual experiments. (E) Jurkat T-cells transfected as described in (C) were stimulated with CD3 mAbs (TCR) for 5 min, fixed, permeabilized, stained with anti-pERK1/2 (T202, Y204) mAbs and analyzed by flow cytometry within the GFP gate. Note, that the total expression of ERK1/2 was not affected (data not shown). One representative experiment out of three is shown. (F) Above described transfectants were cultured on plate-bound anti-CD3 mAbs (TCR) or in the presence of PMA for 18 h, stained with anti-CD69 mAbs and analyzed by flow cytometry. Data are representative of three individual experiments.
Supplemental Figure 3. The presence of SLP76 is critical for adhesion to ICAM-1, conjugate formation and LFA-1 affinity/avidity regulation in Jurkat T-cells. (A) Jurkat T-cells were transfected with either control vector (shC) or SLP76-suppression vector (shSLP76). After 48 h, cells were left unstimulated (non) or stimulated with anti-CD3 mAbs (TCR), PMA, or MnCl₂ for 30 min and subsequently analyzed for adhesion to ICAM-1. Adhesion data represent the mean and S.E. of at least three independently performed experiments. (B) The same transfectants were used to analyze the surface expression of CD18 and the TCR (black line). The anti-GST rat mAb was used as isotype control (dashed line). One representative experiment out of three is shown. (C) Jurkat T-cells transfected as described in (A) were incubated with SEE-pulsed DDAO-SE-labeled Raji B-cells for 30 min. The percentage of conjugate formation between Jurkat T-cell and Raji B-cells was determined by flow cytometry. Data represent the mean and S.E. of three independently performed experiments. Representative histograms of SA-pulsed DDAO-SE B-cells conjugated with GFP-expressing Jurkat T-cells in the presence or absence of SLP76 are shown. (D) Jurkat T-cells were transfected as in (A) and analyzed for their ability to bind soluble Fc-ICAM-1 in response to anti-CD3 mAbs (TCR) and Mg²⁺/EDTA stimulation for 5 min by flow cytometry. One representative experiment out of three is shown. (E) Jurkat T-cells were transfected with pS-C (shC) or pS-SLP76 (shSLP76) in combination with pmCherryC1. After 48 h, cells were stimulated with biotinylated anti-CD3 mAbs and crosslinked with strepavidin for 30 min. Cells were fixed and stained with FITC-conjugated CD18. The percentage of polarized cells was scored in three independent experiments.