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Signal-Transducing Adaptor Protein-2 Regulates Stromal Cell-Derived Factor-1α-Induced Chemotaxis in T Cells

Yuichi Sekine,* Osamu Ikeda,* Satoshi Tsuji,* Chikako Yamamoto,* Ryuta Muromoto,* Asuka Nanbo,* Kenji Oritani,† Akihiko Yoshimura,‡ and Tadashi Matsuda*‡

Signal-transducing adaptor protein-2 (STAP-2) is a recently identified adaptor protein that contains pleckstrin and Src homology 2-like domains, as well as a YXXQ motif in its C-terminal region. Our previous studies revealed that STAP-2 regulates integrin-mediated T cell adhesion. In the present study, we find that STAP-2 expression affects Jurkat T cell migration after stromal cell-derived factor-1α (SDF-1α)-treatment. Furthermore, STAP-2-deficient T cells exhibit reduced cell migration after SDF-1α-treatment. Importantly, overexpression of STAP-2 in Jurkat T cells induces activation of small guanine triphosphatases, such as Rac1 and Cdc42. Regarding the mechanism for this effect, we found that STAP-2 associates with Vav1, the guanine-nucleotide exchanging factor for Rac1, and enhances downstream Vav1/Rac1 signaling. These results reveal a novel STAP-2-mediated mechanism for the regulation of SDF-1α-induced chemotaxis of T cells via activation of Vav1/Rac1 signaling. *The Journal of Immunology, 2009, 183: 7966–7974.

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C hemokines, a group of chemotactically active cytokines, are involved in chemotaxis and transendothelial migration of leukocytes during immune and inflammatory reactions (1–4). Several chemokine receptors are transiently expressed on lymphocytes during distinct stages of development. In general, naïve lymphocytes use the CCR7, CXCR4, and CXCR5 receptors to recirculate into the secondary lymphoid tissues. Effector and memory lymphocytes express a variety of patterns of adhesion molecules and chemokine receptors that allow them to function in active sites of infection or inflammation. Thus, directed leukocyte migrations are key events to organize functional immune systems, and chemokines are prominent in this homing pathway.

Stromal cell-derived factor-1α (SDF-1α; CXCL12)‡ is a member of the CXC subfamily of chemokines and interacts with a seven-transmembrane G protein–coupled receptor, CXCR4 (1, 2, 5, 6). Targeted disruption of the SDF-1α gene or the CXCR4 gene in mice has revealed that both have critical roles in the fetal development of the hematopoietic, cardiovascular, and cerebellar systems (7, 8). Binding of SDF-1α to CXCR4 has been reported to activate a number of intracellular signal transduction pathways and effector molecules, including the Rho family proteins, RhoA, Rac1, and Cdc42 (9, 10). Furthermore, CXCR4 is also known as a coreceptor for T cell-tropic HIV strains (11).

We have recently cloned a novel adaptor molecule, signal-transducing adaptor protein-2 (STAP-2), whose human homolog is identical to a recently cloned adaptor molecule, BKS, a substrate of Brk (breast tumor kinase) (12, 13). STAP-2 contains an N-terminal pleckstrin homology (PH) region, a YXXQ motif in its C-terminal region and a region distantly related to the SH2 domain and the proline-rich, tyrosine phosphorylation motifs. STAP-2 is expressed in a variety of tissues and cells, such as lymphocytes, macrophages, and hepatocytes, and its abundant expression pattern has suggested a wide range of functions in vivo. Indeed, we have reported that STAP-2 can modulate STAT3 and STAT5 transcriptional activity, as well as FceRI- and TLR-mediated signals (12, 14–16). It is noteworthy that thymocytes and peripheral T cells from STAP-2-deficient mice show enhanced IL-2– or TCR-dependent cell growth and enhanced integrin-mediated adherence to fibronectin (14, 17). These observations indicate that STAP-2 might be important in the regulation of T cell function. In the present study, we have found that STAP-2-deficient T cells show reduced chemotaxis toward SDF-1α. As a mechanism of this response, we propose a novel function of STAP-2, involving direct interaction with and activation of Vav1/Rac1 signaling.

Materials and Methods

Reagents, Abs, and mice

Recombinant human SDF-1α was purchased from PeproTech. Recombinant murine CCL19 was purchased from R&D Systems. Expression vectors for Vav1 and Vav1 deletion mutants were provided by Dr. X. R. Bustelo (Consejo Superior de Investigaciones Científicas, University of Salamanca, Salamanca, Spain) (18). HA-tagged Rac1 N17 and Rac1 V12 were provided by Dr. K. Kaibuchi (Nagoya University). GST-Rac-binding domain (GST-RBD) construct was provided by Dr. K. Mizuno (Tohoku University). Epitope-tagged STAP-2, GST fusion STAP-2 mutants (GST-STAP-2 PH (1–147 aa), GST-STAP-2 SH2 (148–243 aa), GST-STAP-2 C (692–1052 aa)) constructs were described previously (14, 19). Anti-Myc, anti-IA, anti-GST, anti-Rac1, anti-Cdc42, and anti-Vav1 Abs were obtained from Santa Cruz Biotechnology. Anti-actin mAb was purchased from Chemicon International. Anti-phosphotyrosine (PY) mAb (PY20) was purchased from BD Biosciences. Anti-phospho-ERK (pERK) and anti-ERK Ab were purchased from Cell signaling Technology. Anti-STAP-2 Ab was purchased from Everest Biotech. Anti-pVav1 (phosphor-Vav1

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Results

STAP-2 enhances chemotactic activity of T cells to SDF-1α

Our previous studies have indicated that STAP-2 regulates IL-2- or TCR-dependent cell growth and integrin-mediated adherence to fibronectin in T cells (14, 17). These findings led us to investigate the effects of STAP-2 on chemokine-mediated chemotaxis of T cells, an important aspect of an immune response. To verify the roles of STAP-2 in SDF-1α-induced T cell chemotaxis, we used a Jurkat T cell line, which strongly expresses the SDF-1α receptor CXCR4. We first established Jurkat cells overexpressing STAP-2 (Jurkat/STAP-2 cells). As shown in Fig. 1A, Jurkat/STAP-2 cells showed higher chemotactic activity toward SDF-1α compared with control Jurkat/pCDNA3 cells. FACS analysis did not reveal differences in levels of CXCR4 expression between Jurkat/pCDNA3 and Jurkat/STAP-2 cells (data not shown). We also tested whether similar results are obtained with normal T cells. To this end, we employed PHA-P/IL-2-activated PBL, which respond strongly to SDF-1α. PHA-P/IL-2-activated PBL were transfected by Amaxa Nucleofection with pCDNA3 or Myc-tagged STAP-2 expression vector and analyzed chemotactic activity toward SDF-1α. As shown in Fig. 1B, expression of STAP-2 enhanced SDF-1α-induced T cell chemotaxis in normal human T cells. We next used siRNA to reduce the endogenous expression of STAP-2 to examine a role of endogenous STAP-2 on SDF-1α-induced T cell chemotaxis. Covariance analysis of the STAP-2 expression level with migration rate revealed that knockdown of STAP-2 expression was highly correlated with enhanced chemotaxis of T cells. These findings suggest that STAP-2 plays a negative role in SDF-1α-induced chemotaxis of T cells. This could be relevant to human and mouse hematopoietic organs, the populations of T cells (CD3, CD4, and CD8), B cells

Immunoprecipitation, immunoblotting, and pull-down assay using GST fusion protein

The immunoprecipitation and Western blotting assays were performed as described previously (20). Briefly, cells were harvested and lysed in a lysis buffer (50 mM Tris-HCl (pH 7.4), 0.15 M NaCl, containing 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM PMSF). The immunoprecipitates from cell lysates were resolved on SDS-PAGE and transferred to polyvinylidene difluoride transfer membrane (PerkinElmer). The filters were then immunoblotted with each Ab. Immunoreactive proteins were visualized using an ECL detection system (Millipore). Pull-down assay using GST fusion proteins was performed as previously described (17).

Affinity precipitation assays using GST-RBD

Active GTP-bound forms of Rho family GTPases were detected by affinity precipitation assays, as reported previously (19). GST fusion proteins of GST-RBD were bacterially expressed and purified on glutathione-Sepharose. Jurkat cells were stimulated with SDF-1α (10 ng/ml) for the indicated periods. Cells were lysed with the above lysis buffer and incubated with 10 μg of purified GST-RBD and glutathione-Sepharose beads. The beads were washed three times with lysis buffer. The precipitates were analyzed by immunoblotting with anti-Rac1 or anti-Cdc42 Abs.

Indirect immunofluorescence microscopy and F-actin localization

To analyze the subcellular localization of STAP-2 and Rac1 proteins, we used a stable Jurkat transformant, expressing Myc-STAP-2, that was stimulated with SDF-1α (10 ng/ml) for the indicated periods and spun down onto glass slides and fixed. Immunofluorescence stainings were performed as described (25, 26). The following primary Abs were used: mouse anti-Myc and rabbit anti-Rac1 Abs. Two secondary Abs were used: FITC-conjugated anti-mouse IgG or rhodamine-conjugated anti-rabbit IgG (Chemicon International). For visualization of F-actin localization, Jurkat transfectants were stimulated with SDF-1α (10 ng/ml) for the indicated periods and fixed with 4% parafomaldehyde and treated with 0.2% Triton X-100 in PBS. Cells were then soaked in PBS containing 3% BSA and stained with rhodamine-conjugated phalloidin (Invitrogen) for 1 h. The observation was performed under a Biozero BX-9000 fluorescent microscope (Keyence) equipped with Plan APO VC ×60/1.40 objective lens (Nikon). Image processing was performed using the BZ-Analyzer 3.5 (Keyence).

Statistical analyses

The significance of difference between group means was determined by Student’s t test.

Tyk2 Ab was purchased from Signalway Antibody. The generation of STAP-2-deficient mice was described previously (12). Six- to 8-wk-old male STAP-2-deficient mice (C57BL/6 background) were used for these studies. STAP-2-deficient mice were housed and bred in the Pharmaceutical Sciences Animal Center of Hokkaido University. All animals were maintained under pathogen-free conditions and in compliance with national and institutional guidelines. All protocols were approved by the Hokkaido University animal ethics committee.

Cell culture, cell purification, and treatment

The human T cell leukemia cell line Jurkat was maintained in RPMI 1640 medium supplemented with 10% FCS. Stable Jurkat transfectants expressing pcDNA3 (Jurkat/pCDNA3) or pcDNA3-STAP-2 (Jurkat/STAP-2) were established as described previously (17) and maintained in the above medium in the presence of G418 (0.5 mg/ml). Human T cell lymphoma HUT78 was maintained in RPMI 1640 medium supplemented with 10% FCS. Human embryonic kidney carcinoma cell line 293T was maintained in DMEM containing 10% FCS, and the indicated plasmids were transfected with the standard calcium precipitation protocol (20). For isolation of splenic T cells, single-cell suspensions of splenocytes from 6- to 8-wk-old mice were prepared as described previously (17). Splenic T cells were purified (>95% CD3+ cells) by negative selection by EasySep mouse T cell enrichment kit (StemCell Technologies). Human PBL were obtained from healthy human donors. PBL were isolated by using Lymphoprep (AxisShield) and allowed to attach to a plastic dish to deplete monocytes. After 2 h of incubation at 37°C, nonadherent cells were collected and used as PBL. PBL were grown in RPMI 1640 medium containing 10% FCS in the presence of PHA (PHA-P) (10 μg/ml) (Wako Pure Chemical) and recombinant human IL-2 (20 U/ml; Sigma-Aldrich) for 5 days. PBL (5 × 10⁷) were nucleofected with pcDNA3 empty vector (3 μg) or Myc-tagged STAP-2 (3 μg) using a human T cell Nucleofector kit (Nucleofector program T-23; Amaxa Biosystems) (21).

Small interfering RNA (siRNA), RT-PCR, and quantitative real-time PCR

Silencing of STAP-2 or Vav1 expression in Jurkat/pCDNA3 or Jurkat/STAP-2 cells was achieved by their specific siRNAs. Jurkat/pCDNA3 or Jurkat/STAP-2 cells (4 × 10⁴) were nucleofected with control (nonsilencing; Qiagen, catalog no. 1022076), STAP-2 (22), or Vav1 siRNA (Santa Cruz; SC-29517) using a cell line Nucleofector kit V (Amaxa Biosystems). At 24 h after transfection, cells were treated or untreated with SDF-1α (10 ng/ml) for the indicated periods and lysed. To confirm a reduction of endogenous STAP-2, total RNAs from the transfected cells were prepared by using Iso-Gen (Nippon Gene) and used in RT-PCR. RT-PCR was performed using RT-PCR high-Plus-kit (Toyobo). Quantitative real-time PCR analyses of STAP-2, as well as the control G3PDH mRNA transcripts, were conducted using an assay-on-demand gene-specific fluorescently labeled TaqMan MGB probe in an ABI Prism 7000 sequence detection system (Applied Biosystems).

Chemotaxis assays

In vitro T cell migration assay was conducted as previously described (23, 24). Briefly, the transwell filters (5 μm pore filter; BD Biosciences) were placed in the lower chamber containing 500 μl of complete medium with or without SDF-1α. Murine splenic T cells or Jurkat T cells were resuspended in 100 μl of RPMI 1640 medium and allowed to migrate toward the underside of the top chamber. After 1–3 h of incubation at 37°C in 5% CO₂, the numbers of migrated cells of the lower chamber were counted with a phase contrast microscope. The results are expressed as the number of cells migrated to the bottom chamber. Each experiment was performed more than three times in triplicate. Chemotaxis of T cells to SDF-1α was blocked by placing SDF-1α in the upper chamber or in both the upper and the lower chambers, demonstrating that the effects seen (migration to the lower chamber) were caused by chemotaxis and not by chemokinesis.

Immunoprecipitation, immunoblotting, and pull-down assay using GST fusion protein

The immunoprecipitation and Western blotting assays were performed as described previously (20). Briefly, cells were harvested and lysed in a lysis buffer (50 mM Tris-HCl (pH 7.4), 0.15 M NaCl, containing 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM PMSF). The immunoprecipitates from cell lysates were resolved on SDS-PAGE and transferred to polyvinylidene difluoride transfer membrane (PerKinElmer). The filters were then immunoblotted with each Ab. Immunoreactive proteins were visualized using an ECL detection system (Millipore). Pull-down assay using GST fusion proteins was performed as previously described (17).
FIGURE 1. STAP-2 regulates SDF-1α-induced T cell chemotaxis.

A. The migration of Jurkat/pcDNA3 or Jurkat/STAP-2 T cells (2 × 10⁶) in response to SDF-1α (1, 10 ng/ml) was studied in a Transwell assay. Data are mean values of independent experiment with SDs (n = 6). *, p < 0.0001, **, p < 0.0001. B. PBL (5 × 10⁶) were nucleofected with pcDNA3 or Myc-tagged STAP-2 by nucleofection with a human T cell Nucleofector kit according to the manufacturer’s optimized protocol. The cells were harvested after 48 h, and total cell lysate (TCL) was blotted with anti-Myc or anti-actin Ab. The migration of vector or STAP-2-transfected PBL (2 × 10⁶) in response to SDF-1α (50 ng/ml) was studied in a Transwell assay. Data are mean values of independent experiment with SDs (n = 4). *, p < 0.05. C. Jurkat T cells (4 × 10⁶) were nucleofected with control or STAP-2 siRNA by nucleofection with a cell line Nucleofector kit V according to the manufacturer’s optimized protocol. The cells were harvested after 24 h, and total RNA samples isolated from these cells were subjected to RT-PCR analysis using STAP-2 primers. STAP-2 expression levels were also quantified by quantitative real-time PCR (QT-PCR). Data represent the levels of STAP-2 mRNA normalized to that of a G3PDH internal control and are expressed relative to the value of control. Data represent the mean of duplicate PCR determinations, which in general varied by 10%. Shown is a representative experiment, which was repeated at least three times with similar results. The migration of control or STAP-2 siRNA-treated Jurkat T cells (2 × 10⁶) in response to SDF-1α (1, 10 ng/ml) was studied in a Transwell assay. Data are mean values of independent experiments with SDs (n = 7). *, p < 0.05, **, p < 0.005. D. The migration of splenic T cells (1 × 10⁶) from wild-type or STAP-2-deficient mice in response to SDF-1α (1, 10, or 50 ng/ml) was studied in a Transwell assay. Data are mean values of independent experiments with SDs (n = 5). *, p < 0.01, **, p < 0.05. E. The migration of splenic T cells (1 × 10⁶) from wild-type or STAP-2-deficient mice in response to CCL19 (100 or 500 ng/ml) was studied in a Transwell assay. Data are mean values of independent experiment with SDs (n = 4). *, p < 0.005.
(B220, CD43), and myeloid cells (Mac1) in STAP2-deficient mice were identical to those in wild-type mice. FACS analysis also revealed that T cell development in thymus and the percentage of CD3-positive T cells in spleen were normal in those mice (data not shown). Importantly, T cells from spleens of STAP-2-deficient mice were reduced in their chemotactic response to SDF-1/H9251 (Fig. 1D). FACS analysis demonstrated no alteration in the levels of CXCR4 expression between STAP-2-deficient and wild-type T cells (data not shown). Therefore, STAP-2 has an ability to up-regulate the chemotactic activity of T cells toward SDF-1/H9251.

We also tested whether STAP-2 influences T cell chemotaxis induced by another chemokine, CCL19, which is also known to induce murine T cell migration (27). As shown in Fig. 1E, a significant decrease in the CCL19-induced T cell chemotaxis was observed in STAP-2-deficient T cells compared with those from wild-type mice, although no significant differences in the CCR7, a CCL19 receptor mRNA, expression levels were observed among the them (data not shown). Therefore, endogenous STAP-2 is likely to regulate chemokine-induced T cell migration.

**STAP-2 enhances the activation of small GTPases**

To delineate the molecular mechanisms of STAP-2-mediated up-regulation of SDF-1α-induced T cell chemotaxis, we investigated the effects of STAP-2 on the activation of ERK after SDF-1α stimulation. As shown in Fig. 2A, using an anti-phospho-ERK Ab to assay activation status, similar levels of SDF-1α-induced ERK activation in both Jurkat/pcDNA3 and Jurkat/STAP-2 cells were observed.

We then examined the activation of Rho GTPases, such as RhoA, Rac1 and Cdc42, because overexpression of mutants of all three Rho GTPases was shown to inhibit SDF-1α-induced T cell chemotaxis (10). When we employed a pull-down assay using GST-RBD to measure the amount of the active form of Rac1 and Cdc42, we observed that SDF-1α-induced activation of Rac1 and Cdc42 occurred within 5 min in Jurkat/pcDNA3 cells (Fig. 2B, upper panel). In Jurkat/STAP-2 cells, Rac1 and Cdc42 were in the activated form, independently of SDF-1α, and much larger amounts of Rac1 and Cdc42 were activated after SDF-1α stimulation compared with that in Jurkat/pcDNA3 cells. Of importance, STAP-2 was coprecipitated with GST-RBD in parallel with Rac1 activation (Fig. 2B, lower panels). Therefore, STAP-2 can modify the activation of Rac1 and Cdc42.

**Colocalization of STAP-2 with Rac1 after SDF-1α stimulation**

We then tested whether STAP-2 interacts with Rac1 in vivo. Expression vectors for HA-tagged Rac1 N17 (dominant negative form) or Rac1 V12 (dominant active form) together with GST-tagged STAP-2 were transfected into 293T cells and then the transfected cells were lysed, pulled down with glutathione-Sepharose, and immunoblotted with anti-HA (upper panel) or anti-GST Ab (middle panel). TCL was blotted with anti-HA Ab (lower panel). Densitometric quantification of the above results was also shown. Relative intensity of STAP-2 bound Rac1 was normalized to total Rac1 of the same sample.

![FIGURE 2. STAP-2 enhances the activation of small GTPases in Jurkat T cells. A, Jurkat/pcDNA3 or Jurkat/STAP-2 cells (1 x 10⁶) were stimulated without or with SDF-1α (10 ng/ml) for the indicated periods. The cells were then lysed, and total cell lysate (TCL) was blotted with anti-pERK, anti-ERK, or anti-Myc Ab. B, Jurkat/pcDNA3 or Jurkat/STAP-2 cells (1 x 10⁶) were stimulated without or with SDF-1α (10 ng/ml) for the indicated periods. Rac1-GTP was pulled down using GST-RBD and blotted with anti-Rac1, anti-Cdc42, or anti-Myc Ab. TCL was also blotted with anti-Rac1, anti-Cdc42, anti-Myc, or anti-GST Ab. Densitometric quantification of the above results was also shown. Relative intensities of Rac1-GTP or Cdc42-GTP were normalized to total Rac1 or Cdc42 of the same sample. C, 293T cells (1 x 10⁶) were transfected with GST-fused STAP-2 (10 μg) together with or without HA-Rac1 N17 or HA-Rac1 V12 (10 μg). At 48 h after transfection, the cells were lysed, pulled down with GST-Sepharose, and blotted with anti-HA (upper panel) or anti-GST Ab (middle panel). TCL was blotted with anti-HA Ab (lower panel). Densitometric quantification of the above results was also shown. Relative intensity of STAP-2 bound Rac1 was normalized to total Rac1 of the same sample.](http://www.jimmunol.org/)

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immunocytochemistry. As we found with precipitation analysis, STAP-2 was colocalized with Rap1 in the uropod of Jurkat/STAP-2 cells; cells displayed a slightly polarized morphology after SDF-1α stimulation (Fig. 3A). Furthermore, we observed a substantial accumulation of F-actin in the cortical ring of SDF-1α-stimulated Jurkat/STAP-2 cells that was much more prominent than that in SDF-1α-stimulated Jurkat/pcDNA3 cells (Fig. 3B). Therefore, STAP-2 is likely to strongly interact with activated Rac1, followed by F-actin assembly.

**STAP-2 constitutively associates with Vav1 in T cells**

Among the representative GDP/GTP exchange factors for Rac1, the expression of Vav1 is observed exclusively in hematopoietic cells (28–30). We therefore examined whether Vav1 was involved in STAP-2-dependent activation of Rac1. We first tested interactions between Vav1 and STAP-2. As shown in Fig. 4A, in Jurkat/STAP-2 cells, Vav1 was associated with STAP-2 in the absence of SDF-1α stimulation, indicating that STAP-2 constitutively interacts with a GDP/GTP exchange factor, Vav1, in T cells. To exclude the possibility that the interaction between STAP-2 and Vav1 was due to overexpression, we used coimmunoprecipitation to examine direct binding between endogenous STAP-2 and Vav1 in human T cell lymphoma HUT78 cells, which express both proteins at high levels. An anti-STAP-2 Ab coimmunoprecipitated Vav1 independently of SDF-1α stimulation, indicating that the binding of Vav1 to STAP-2 occurs at physiological expression levels (Fig. 4B).

To determine the domains of STAP-2 involved in its association with Vav1, a series of STAP-2 deletion mutants, fused with GST (GST-STAP-2 PH, GST-STAP-2 SH2, and GST-STAP-2 C), were employed (Fig. 4C). The respective mutants, together with Vav1, were transiently expressed in 293T cells. The binding potentials of these proteins with GST-STAP-2 were examined by pull-down assays with glutathione-Sepharose, followed by Western blot analysis with anti-Vav1 or anti-GST Ab. The precipitates for the GST-STAP-2 protein failed to interact with the C-terminal deletion of Vav1 protein (ΔN–ΔC), indicating that STAP-2 associates with the C-terminal SH3-SH2-SH3 domains of Vav1 (Fig. 4F). Similarly, we further tested which region in the C-terminal SH3-SH2-SH3 domains of Vav1 is involved in its interaction with STAP-2. As shown in Fig. 4G, the ΔN–ΔC–ΔSH3N mutant showed a strong binding potential with STAP-2; however, both of the ΔN–ΔC–ΔSH2 and ΔN–ΔC–ΔSH3C mutants showed a marked reduction of binding potential with STAP-2. Therefore, the C-terminal SH2-SH3 domains play an important role in interactions with STAP-2.

Reduction of endogenous Vav1 abrogates STAP-2/Rac1 complex formation, resulting in a reduction of chemotactic activity in Jurkat/STAP-2 cells

To assess the functional relevance between Vav1 and STAP-2 in SDF-1α-mediated Rac1 activation, we used siRNA to reduce the endogenous expression of Vav1 in Jurkat/STAP-2 cells. A reduction of ~50% of the Vav1 protein was observed following Vav1 siRNA transfection (data not shown). Activated Rac1 was markedly decreased by reduction of endogenous Vav1 (Fig. 5A). Importantly, SDF-1α-induced binding of STAP-2 with Rac1 was also decreased by a reduction of endogenous Vav1 (Fig. 5B). Furthermore, Jurkat/STAP-2 cells expressing the Vav1 siRNA showed significantly lower chemotaxis than did those expressing a control siRNA (Fig. 5C). Therefore, Vav1 is involved in the interactions between STAP-2 and Rac1, which precede the enhanced chemotaxis in Jurkat/STAP-2 cells.

Reduction of endogenous STAP-2 abrogates Rac1 activation and tyrosine phosphorylation of Vav1

We next examined the effect of STAP-2 on tyrosine phosphorylation status of Vav1. As shown in Fig. 6A, tyrosine phosphorylation of Vav1 was reduced in Jurkat/pcDNA3 cells after SDF-1α stimulation. However, Vav1 was tyrosine phosphorylated in Jurkat/STAP-2 cells without SDF-1α stimulation. These results were supported by the fact that enhanced activation of small GTPases was observed in Jurkat/STAP-2 cells. To further characterize the molecular mechanisms underlying the interactions among Vav1, STAP-2, and Rac1 in SDF-1α-mediated signaling, we knocked down STAP-2 expression in Jurkat cells, as described above.

**FIGURE 3.** Colocalization of STAP-2 with Rac1 after SDF-1α stimulation. A, Jurkat/STAP-2 T cells (6 × 10^6^) were placed in suspension for the indicated periods with SDF-1α (10 ng/ml) and respread onto coverslips. The cells were fixed and reacted with anti-Myc and anti-Rac1 Abs, and visualized with FITC- or rhodamine-conjugated secondary Ab. B, Jurkat/pcDNA3 and Jurkat/STAP-2 T cells (6 × 10^6^) were placed in suspension for the indicated periods with SDF-1α (10 ng/ml) and respread onto coverslips. The cells were fixed and reacted with rhodamine-conjugated palludin.
FIGURE 4. STAP-2 associates with Vav1. A, Jurkat/pcDNA3 or Jurkat/STAP-2 cells (2 × 10^6) were stimulated without or with SDF-1α (10 ng/ml) for the indicated periods. The cells were lysed, immunoprecipitated with anti-Vav1 Ab, and immunoblotted with anti-Myc or anti-Vav1 Ab (upper panels). Total cell lysate (TCL) was blotted with anti-Myc or anti-Vav1 Ab (lower panels). B, Human T cell lymphoma, HUT78 cells (2 × 10^6), were lysed, immunoprecipitated with control IgG or anti-Vav1 Ab, and immunoblotted with anti-STAP-2, anti-Vav1, or anti-PY Ab (upper panels). TCL was also blotted with anti-STAP-2 Ab (bottom panel). C, Domain structures of STAP-2 and GST-fused mutant fragments are schematically shown. D, 293T cells (1 × 10^6) were transfected with Vav1 (10 μg) together without or with GST or GST-fused STAP-2 deletion mutants (10 μg). At 48 h after transfection, the cells were lysed, pulled down with glutathione (GSH)-Sepharose, and blotted with anti-Vav1 (upper panel) or anti-GST Ab (middle panel). TCL was also blotted with anti-Vav1 Ab (lower panel). E, Domain structure of Vav1 and its mutant fragments are schematically shown. CH indicates calponin homology domain; Ac, acidic domain; DH, Dbl homology domain. The ΔN mutant has a deletion of the N-terminal 1–186 aa. F, 293T cells (1 × 10^6 cells/well) were transfected with Vav1 wild-type or its deletion mutants (10 μg) with or without GST-fused STAP-2 (10 μg). At 36 h after transfection, the cells were lysed, pulled down with GSH-Sepharose, and immunoblotted with anti-Vav1 (upper panel) or anti-GST Ab (middle panel). TCL was also blotted with anti-Vav1 Ab (lower panel). G, 293T cells (1 × 10^6 cells/well) were transfected with a series of Vav1 C-terminal deletion mutants (20 μg) with GST-fused STAP-2 (10 μg). At 36 h after transfection, the cells were lysed, pulled down with GSH-Sepharose, and immunoblotted with anti-Vav1 (upper panel) or anti-GST Ab (middle panel). TCL was also blotted with anti-Vav1 Ab (lower panel).
the body. Systems, but also the localization of immune cells throughout the body.CXCR4 signaling is an important new observation and this function...suppress the release of NO and the expression of iNOS...2-deficient mice showed a marked reduction of SDF-1 in vivo...of cytokines and the promotion of cell survival. In the present study, we have found that an adaptor molecule, STAP-2, is a novel regulator of SDF-1...of hematopoietic compartments, such as bone marrow, both SDF-1 and CXCR4-deficient mice have severely reduced numbers of lymphocytes and myeloid progenitors. SDF-1/CXCR4 is critical for the homing of hematopoietic stem cells into bone marrow and for their retention within an appropriate, supportive niche. Additionally, interaction between SDF-1 and CXCR4 is an essential cell signaling system for immune cell trafficking and surveillance. SDF-1 can guide the movement of immune cells in vivo, and it can...bind to both Vav1 and Rac1. Most importantly, down-regulation of endogenous Vav1 protein reduced the binding between STAP-2 and Rac1, resulting in decreased Rac1 activation. Recently, it has been shown that STAP-2 constitutes a complex formation between STAP-2 and Rac1, resulting in decreased Rac1 activation. Interaction between STAP-2 and Rac1 is required for the regulation of the tyrosine phosphorylation status of Vav1 and for further activation of Rac1 in SDF-1α-stimulated Jurkat/STAP-2 cells. Small GTP-binding proteins of the Rho family, including Rho, Rac, and Cdc42, play a central role in the dynamic organization of the actin-based cytoskeleton. Rho regulates the formation of contractile actin-myosin filaments, which form stress fibers and maintain focal adhesions at the trailing edge of migrating cells. Rac is involved in forming actin-rich membrane ruffles and lamellipodia at the leading edge of migrating cells. Cdc42 is critical in cell polarity and filopodia formation, which control the direction of cell movement. Thus, the coordinated activation of Rho members represents a key regulatory event for the migration of cells along a chemotactant gradient. Additionally, the PI3K pathway and the MAPK cascade are also important signals for cell migration. Our manipulation of SDF-1/CXCR4 expression in Jurkat cells clearly indicated the involvement of SDF-1/CXCR4 in the regulation of Rho members. That is, overexpression of SDF-1/CXCR4 enhanced, and knockdown of SDF-1/CXCR4 suppressed, the activation of Rac1 and Cdc42 after SDF-1α stimulation. Rho GTPases are activated by the recruitment of tyrosine-phosphorylated GDP/GTP exchange factors to the membrane, allowing their conversion from the inactive, GDP-bound state to the active, GTP-bound state. Among GDP/GTP exchange factors, the hematopoietic cell-specific protein Vav1 has been shown to catalyze guanine nucleotide exchange of the Rho family proteins. A glycerophosphoinositol, GroPIns2P, can activate Vav1 through an Lck-dependent pathway. Wiskott-Aldrich syndrome protein and its partner, Wiskott-Aldrich syndrome protein-interacting protein, play important roles in actin reorganization by influencing signaling downstream of Rho GTPases. Here, we showed that SDF-1α constitutively interacted with Vav1 and enhanced tyrosine phosphorylation of Vav1 and that SDF-1α could bind to both Vav1 and Rac1. Most importantly, down-regulation of endogenous Vav1 protein reduced the binding between SDF-1α and Rac1, resulting in decreased Rac1 activation. Recently, it has been shown that SDF-1α induces tyrosine phosphorylation of Pyk2, as well as Pyk2 association with ZAP-70 and Vav in Jurkat cells. (Fig. 1B). As shown in Fig. 6B, a reduction of endogenous STAP-2 resulted in significant decrease of the active form of Rac1 before and after SDF-1α-stimulation. Additionally, T cells from STAP-2-deficient mice showed a marked reduction of SDF-1α-induced tyrosine phosphorylation of Vav1 (Fig. 6C). Therefore, interaction between STAP-2 and Vav1 is required for the regulation of the tyrosine phosphorylation status of Vav1 and for further activation of Rac1 in SDF-1α-stimulated Jurkat/STAP-2 cells.
and inflammation. A candidate for therapeutic drug development to modulate infection hypothesis. Therefore, our data suggest that STAP-2 is a novel enhancer of SDF-1 infection under control in vivo, and the present finding that STAP-2 might function to strengthen host defense, as well as to bring in- ducers (12). All previously obtained results indicated that STAP-2 acute phase gene expression was augmented by STAP-2 in hepa- rons, which is associated with fibronectin after PMA treatment in T cells (17) and after TLR4- mune and inflammatory responses. STAP-2 enhances cell adhesion is the possibility that Pyk2 is involved in enhanced tyrosine phos- phorylation by STAP-2. Further detailed examination will be re- quired for this issue. We have reported that STAP-2 has a possibility to regulate im- mune and inflammatory responses. STAP-2 enhances cell adhesion to fibronectin after PMA treatment in T cells (17) and after TLR4- mediated NF-κB activation in macrophages (16), STAP-2 also inhibits EBV LMP1-mediated NF-κB signaling (22), which is related to the growth of infected cells. Furthermore, IL-6-induced acute phase gene expression was augmented by STAP-2 in hepa- tocytes (12). All previously obtained results indicated that STAP-2 might function to strengthen host defense, as well as to bring in- fecture under control in vivo, and the present finding that STAP-2 enhances SDF-1α-induced chemotaxis in T cells is in line with this hypothesis. Therefore, our data suggest that STAP-2 is a novel candidate for therapeutic drug development to modulate infection and inflammation.

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Disclosures
The authors have no financial conflicts of interest.

References

FIGURE 6. Reduction of endogenous STAP-2 abrogates Rac1 activation and tyrosine phosphorylation of Vav1. A. Jurkat/pcDNA3 or Jurkat/ STAP-2 cells (1 × 10⁵) were stimulated without or with SDF-1α (10 ng/ml) for the indicated periods. The cells were lysed, immunoprecipitated with anti-Vav1 Ab, and immunoblotted with anti-PY or anti-Vav1 Ab (upper panels). Total cell lysate (TCL) was also blotted with anti-Myc or anti-Vav1 Ab (lower panels). B. Control or STAP-2 siRNA-treated Jurkat T cells (2 × 10⁵) were stimulated without or with SDF-1α (10 ng/ml) for 15 min. The cells were lysed, and Rac1-GTP was pulled down using GST-RBD and blotted with anti-Rac1 Ab (upper panel). TCL was also blotted with anti-phosphoVav1 or anti-Vav1 Ab. Similar results were obtained in three independent experiments. C. Isolated T cells (6 × 10⁶) from wild- type or STAP-2-deficient mice were stimulated with or without SDF-1α (10 ng/ml) for indicated time. Then, the cells were lysed and immunoblot- ted with anti-phosphoVav1 or anti-Vav1 Ab. Similar results were obtained in three independent experiments.


