Interaction of SAP-1, a Transmembrane-type Protein-tyrosine Phosphatase, with the Tyrosine Kinase Lck

ROLES IN REGULATION OF T CELL FUNCTION*

SAP-1 is a transmembrane-type protein-tyrosine phosphatase that is expressed in most tissues but whose physiological functions remain unknown. The cytoplasmic region of SAP-1 has now been shown to bind directly the tyrosine kinase Lck. Overexpression of wild-type SAP-1, but not that of a catalytically inactive mutant of SAP-1, inhibited both the basal and the T cell antigen receptor (TCR)-stimulated activity of Lck in human Jurkat T cell lines. Lck served as a direct substrate for dephosphorylation by SAP-1 in vitro. Overexpression of wild-type SAP-1 in Jurkat cells also: (i) inhibited both the activation of mitogen-activated protein kinase and the increase in cell surface expression of CD69 induced by TCR stimulation; (ii) reduced the extent of the TCR-induced increase in the tyrosine phosphorylation of ZAP-70 or that of LAT; (iii) reduced both the basal level of tyrosine phosphorylation of p62
tyrosine, as well as the increase in the phosphorylation of this protein induced by CD2 stimulation; and (iv) inhibited cell migration. These results thus suggest that the direct interaction of SAP-1 with Lck results in inhibition of the kinase activity of the latter and a consequent negative regulation of T cell function.

Regulation of protein-tyrosine phosphorylation contributes to many important physiological processes including cell growth, differentiation, and migration as well as glucose metabolism, synaptic transmission, and the immune response (1, 2). The balance between protein-tyrosine phosphorylation and dephosphorylation is precisely determined by the action of protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs) (3–6), although the molecular mechanisms by which these enzymes are coordinately regulated remain largely unknown.

Protein-tyrosine phosphorylation plays central roles in signal transduction by the T cell antigen receptor (TCR) (7, 8). The earliest biochemical events known to be elicited by engagement of the TCR are the activation of the Src family PTKs Lck and Fyn (9, 10). In human T cells, the TCR-mediated activation of Lck results from autophosphorylation of this enzyme on Tyr505 (20). In contrast, phosphorylation of Tyr505 near the COOH terminus of Lck by the Src family kinase Csk negatively regulates Lck activity (11). Activated Lck or Fyn mediates the phosphorylation of CD3 and the ζ chain, which are the signal-transducing subunits of the TCR (7, 8). These modifications occur within immunoreceptor tyrosine-based activation motifs and direct the recruitment of ZAP-70, a Syk family PTK, to the TCR through interaction with its tandem Src homology 2 (SH2) domains (12). The consequent activation of ZAP-70 results in the phosphorylation of various adapter proteins including LAT (for linker of activated T cells), SLP-76, Vav, and phospholipase C-γ (7, 8, 13).

In contrast to protein-tyrosine phosphorylation, the role of protein-tyrosine dephosphorylation in TCR-mediated signal transduction has been only partially resolved (14). SHP-1, a cytoplasmic PTP that contains two SH2 domains, negatively regulates the TCR-mediated signaling pathway (15, 16), and its localization in membrane rafts is required for such regulation (17). In addition, PEP and PTP-PEST, which are related cytoplasmic PTPs, also negatively regulate TCR-mediated signaling (18, 19). Whereas PEP appears to cooperate with Csk to inhibit TCR signaling, PTP-PEST dephosphorylates Shc, p130
Csk, Pyk2, and focal adhesion kinase (7, 8, 14). The receptor-like PTP CD148, also known as DEP-1 (20, 21), negatively regulates the TCR-mediated signaling pathway by catalyzing the dephosphorylation of LAT (22–24). In contrast, CD45, another receptor-like PTP, dephosphorylates Tyr505 of Lck and thereby increases its activity and promotes TCR signaling (25). The molecular mechanism by which the activity of Lck is downregulated through protein-tyrosine dephosphorylation in vivo and the identity of the PTPs that mediate such regulation remain unknown, however.
SAP-1 (for stomach cancer-associated protein-tyrosine phosphatase-1) was originally identified as a PTP expressed in a stomach cancer cell line (26). It is a transmembrane-type PTP with a single catalytic domain in its cytoplasmic region and eight fibronectin type III-like domains in its extracellular region (26). A "substrate-trapping" approach identified p130 

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- use of this enzyme functions as a suppressor of cell growth.

SAP-1 mRNA has been detected in most tissues examined but is especially abundant in the spleen. To explore the biological role of SAP-1 in the immune system, we have attempted to identify molecules that interact with the cytoplasmic region of this protein. We now show that this region of SAP-1 binds directly to Lck. Furthermore, overexpression of wild-type SAP-1 resulted in down-regulation of the kinase activity of Lck and thereby negatively regulated TCR-mediated T cell functions.

EXPERIMENTAL PROCEDURES

**Antibodies**—Rabbit polyclonal antibodies (pAbs) to SAP-1 (26), a mouse monoclonal antibody (mAb) (3G5) to SAP-1 (27), and rabbit pAbs to p62 (30) were generated as described previously. The mouse mAb (154A7) to SAP-1 was also generated by using a recombinant immuno- globulin-Fc fusion protein, which contains three fibronectin type III-like domains of SAP-1 in its extracellular region (amino acids 1–250), as an antigen. The detail for the preparation of this recombinant protein will be described elsewhere. The mAb was purified from culture supernatants of the hybridoma by column chromatography on protein A-Sepharose 4FF (Amersham Biosciences). Two mouse mAbs (anti-T112 and anti-T113) to CD2 (31) were kindly provided by E. L. Reinherz (Dana-Faber Cancer Institute, Boston, MA). Mouse mAbs to CD3 (OKT3), or to the Myc epitope tag (9E10) were purified from the culture supernatants of hybridoma cells. A mouse mAb to Lck, rabbit pAbs to LAT, and a mouse mAb (4G10) to phosphotyrosine were obtained from Upstate Biotechnology. Rabbit pAbs to human Src autophosphorylated on Tyr118 de also recognize the autophosphorylation site of other Src family PTKs, including Lck, were from Cell Signaling Technology. Rabbit pAbs to MAP kinase and to active MAP kinase (pTeEpY) were from Promega; rabbit pAbs to SAP-70 and a horseradish peroxidase-conjugated mouse mAb (PY20) to phosphotyrosine were from Santa Cruz Biotechnology; and a mouse mAb to CD247 (TCR β chain) was from COSMO BIO. A fluorescein isothiocyanate (FITC)-conjugated mouse mAb to CD89 for flow cytometry was obtained from BD Pharmingen. Goat antibodies to mouse immunoglobulins were obtained from Southern Biotechnology Associates, Inc.

**Plasmids for Yeast Two-hybrid Screening and Transient Transfection**—The expression vectors pBTH116HA and pCIneo-myc were kindly provided by Y. Takai (Osaka University, Osaka, Japan). To obtain the expression vectors pBTH116HA and pCIneo-myc, two mouse mAbs (anti-T112 and anti-T113) to CD2 (31) were kindly provided by E. L. Reinherz (Dana-Faber Cancer Institute, Boston, MA). Mouse mAbs to CD3 (OKT3), or to the Myc epitope tag (9E10) were purified from the culture supernatants of hybridoma cells. A mouse mAb to Lck, rabbit pAbs to LAT, and a mouse mAb (4G10) to phosphotyrosine were obtained from Upstate Biotechnology. Rabbit pAbs to human Src autophosphorylated on Tyr118 also recognize the autophosphorylation site of other Src family PTKs, including Lck, were from Cell Signaling Technology. Rabbit pAbs to MAP kinase and to active MAP kinase (pTeEpY) were from Promega; rabbit pAbs to SAP-70 and a horseradish peroxidase-conjugated mouse mAb (PY20) to phosphotyrosine were from Santa Cruz Biotechnology; and a mouse mAb to CD247 (TCR β chain) was from COSMO BIO. A fluorescein isothiocyanate (FITC)-conjugated mouse mAb to CD89 for flow cytometry was obtained from BD Pharmingen. Goat antibodies to mouse immunoglobulins were obtained from Southern Biotechnology Associates, Inc.

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immmobilized on glutathione-Sepharose beads (10 μg of protein/15 μl of packed beads; Amersham Biosciences). The beads were then washed three times with 1 ml of ice-cold lysis buffer, suspended in SDS sample buffer, and subjected to immunoblot analysis with pAbs to Lck.

**Retrovirus Production and Infection—**Full-length cDNAs for wild-type SAP-1 or a catalytically inactive SAP-1 mutant (SAP-1-C/S), in which Cys352 was replaced by serine (27), were inserted into the EcoRI site of the pMX-puro vector (kindly provided by T. Kitamura, University of Tokyo, Tokyo, Japan). The production of retroviruses encoding the SAP-1 proteins and infection of cells with these viruses were performed as described (34). Plat-E packaging cells (35) (kindly provided by T. Kitamura) were maintained under a humidified atmosphere of 5% CO2 and 95% air at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells (2 × 10^6) were transiently transfected with 3 μg of pMX-puro vectors with the use of FuGENE 6. Fresh medium (Dulbecco’s modified Eagle’s medium supplemented with 10% FBS) was added to the cells 16 h after transfection, and supernatants (6 ml) were harvested after incubation for an additional 24 h. Jurkat cells expressing the ecotropic receptor (J.EcoR cells) were kindly supplied by T. Saito (Chiba University, Chiba, Japan) (36). Expression of EcoR in human cells confers susceptibility to infection by the pMX-puro-derived retroviruses, which normally infect only rodent cells (36). Parental J.EcoR cells were infected with each retrovirus-containing culture supernatant supplemented with Polybrene (10 μg/ml) (Sigma), blasticidin (10 μg/ml) (Invitrogen), penicillin (100 units/ml), and streptomycin (100 μg/ml). Colonies were then isolated after 14 days. Several cell lines expressing SAP-1-WT or SAP-1-C/S were assayed for PTP activity with pAbs to SAP-1.

**Assay of PTP Activity—**The PTP activity of SAP-1 immunoprecipitated from Jurkat cells with the mAb 3G5 was assayed with p-nitrophenyl phosphate (pNPP) as a substrate as described previously (26). In brief, J.EcoR cells (1 × 10^6) stably expressing SAP-1-WT or SAP-1-C/S were lysed on ice in 500 μl of ice-cold lysis buffer containing 1 mM phenylmethylsulfonyl fluoride and aprotinin (10 μg/ml). Postnuclear cell lysates were subjected to immunoprecipitation with 2 μg of mAb 3G5 prebound to protein G-Sepharose beads, after which the beads were washed twice with 1 ml of WG buffer and twice with 1 ml of PTP assay buffer (40 mM Mes-NaOH (pH 5.0), 1.6 mM dithiothreitol) before incubation for 30 min at 30 °C with 200 μl of PTP assay buffer containing 25 mM pNPP. The reaction was terminated by addition of 200 μl of 1 N NaOH, and absorbance at 410 nm was measured. Duplicate samples were subjected to immunoblot analysis with pAbs to SAP-1 to determine the amount of SAP-1 protein in the immunoprecipitates.

**In Vitro Dephosphorylation Assay—**Jurkat cells were stimulated with pervanadate (100 μM Na3VO4, 10 μM H2O2 in phosphate-buffered saline (PBS); 37 °C, after which postnuclear cell lysates were prepared and subjected to immunoprecipitation with pAbs to Lck as described above. The resulting precipitates were washed three times with ice-cold dephosphorylation buffer (100 mM Hepes NaOH (pH 7.6), 150 mM NaCl, 2 mM dithiothreitol, 2 mM EDTA). A GST fusion protein of the catalytically inactive mutant SAP-1-C/S was generated and purified as previously described (27). Immunoprecipitates were incubated with 20 μg of GST-SAP-1-WT, GST-SAP-1-C/S, or GST in 400 μl of dephosphorylation buffer first for 1 h at 4 °C and then for 30 min at 30 °C. Reaction mixtures were then subjected to immunoblot analysis with pAbs to phosphorylated Tyr^394 of Src.

**CD69 Expression—**For the assay of CD69 expression, Jurkat cells (1 × 10^6) in 24-well plates were stimulated for 16 h either with immobilized OKT3 or with the combination of 1 μM phorbol 12-myristate 13-acetate (PMA) and 0.5 μM ionomycin. Cells were then stained with an FITC-conjugated mAb to CD69 and analyzed by flow cytometry with an Epics XL instrument (Beckman Coulter).

**Cell Migration Assay—**Cell migration was assayed with a Transwell apparatus as described previously (37). In brief, the cell suspension (2 × 10^5 cells in 100 μl) was transferred to a polycarbonate filter (pore size, 8 μm; Corning) in the upper compartment of the apparatus, and 500 μl of culture medium were placed in the lower compartment. The apparatus was then placed for 3 h at 37 °C in a humidified incubator containing 5% CO2. The number of cells that had migrated through the filter was counted in triplicate with a hemocytometer. Each experiment was performed in triplicate wells.

**Quantitative Image Analysis—**Intensity of an immunoblot band was determined by densitometric analysis that was performed using NIH Image version 1.62.

**RESULTS**

**Interaction of the Cytoplasmic Region of SAP-1 with Lck—**To identify proteins that interact with the cytoplasmic region of SAP-1, we screened a human spleen cDNA library by the yeast two-hybrid method with this region of SAP-1 (residues 778–1117) as the bait (Fig. 1A). Nine positive clones were obtained, among which three contained Lck cDNA and one contained a cDNA for CrkII, an adapter protein that is tyrosine-phosphorylated by Src family PTKs (38). Among the Lck clones, one (clone 9) contained a full-length cDNA whereas the other two (one of which is clone 15) encoded a COOH-terminal region of Lck (amino acids 154–509) containing a portion of the SH2 domain and the kinase domain (Fig. 1B). We further analyzed the precise region of Lck that was responsible for binding to the cytoplasmic region of SAP-1 by yeast two-hybrid analysis. We found that the region of Lck comprising residues 154–242 bound to the cytoplasmic region of SAP-1, whereas the region comprising amino acids 249–509 did not, suggesting that the middle portion of Lck (residues 154–242) is responsible, at least in part, for binding to SAP-1 (Fig. 1B).

We next examined whether the cytoplasmic region of SAP-1 interacts with Lck directly in vitro. Lysates of human Jurkat T cells, which express endogenous Lck, were incubated with an immobilized GST fusion protein containing the cytoplasmic region of SAP-1 (GST-SAP-1-WT) or with GST alone. Lck specifically bound to GST-SAP-1-WT but not to GST (Fig. 2A). We then examined the binding of Lck to SAP-1 in COS-7 cells transfected with expression vectors for full-length Lck and the Myc epitope-tagged cytoplasmic region of SAP-1. Immunoblot analysis revealed that immunoprecipitates prepared from the transfected cells with a mAb to Lck contained the Myc epitope-tagged cytoplasmic region of SAP-1 (Fig. 2B). We next determined whether SAP-1 binds Lck in Jurkat T cells. Reverse transcription-PCR analysis revealed the presence of SAP-1 mRNA in Jurkat cells (data not shown). SAP-1 was immunoprecipitated from Jurkat cell lysates with the mAb 3G5 to SAP-1, and the resulting precipitates were subjected to immunoblot with the mAb 154A7 to SAP-1 or pAbs to Lck. Immunoblot analysis with the mAb 154A7 revealed the expression of SAP-1 protein in the lysates of Jurkat cells (Fig. 2C, upper panel). Moreover, immunoblot of immunoprecipitates from Jurkat cells with the mAb 3G5 to SAP-1 revealed the interaction of SAP-1 with Lck (Fig. 2C, lower panel). These results thus suggest that the cytoplasmic region of SAP-1 binds Lck directly both in vitro and in vivo.

**Generation of Jurkat Cell Lines Overexpressing SAP-1 and Effects of SAP-1 Overexpression on the TCR-mediated Activation of Lck—**Stimulation of the TCR induces the activation of Lck by autophosphorylation on Tyr^394 (39). Given that our data showed that Lck binds to the cytoplasmic region of SAP-1, we next determined whether SAP-1 regulates the TCR-mediated activation of Lck using Jurkat T cell line. To generate Jurkat cell lines that overexpress either wild-type SAP-1 (SAP-1-WT) or the catalytically inactive mutant SAP-1-C/S (27), we subjected cells expressing EcoR (J.EcoR cells) to retrovirus-mediated transfection (36). We obtained several independent cell lines that expressed either SAP-1-WT (J-SAP-1-WT cells) or SAP-1-C/S (J-SAP-1-C/S cells). A J-SAP-1-WT cell line (clone A4) and a J-SAP-1-C/S cell line (clone D2) were chosen for further characterization because they expressed the recombinant proteins at a high level (Fig. 3A). The level of endogenous Lck was not changed by the expression of SAP-1-WT or that of SAP-1-C/S (Fig. 3A). These cell lines were subjected to immunoprecipitation with the mAb 3G5 to SAP-1, and the resulting precipitates were assayed for PTP activity. The PTP activity...
FIG. 1. Yeast two-hybrid screening for molecules that interact with the cytoplasmic region of SAP-1. A, the domain structure of SAP-1 and the region of SAP-1 used as a bait. Numbers indicate amino acid residues. FNs, fibronectin type III-like domains; TM, transmembrane domain. B, domain structure of human Lck indicating portions of the protein encoded either by clones obtained by yeast two-hybrid screening (clones 9 and 15) or by deletion mutants (Lck-cat, clone 15cat) used for determination of the region of Lck responsible for the interaction with SAP-1 by two-hybrid analysis. Numbers indicate NH₂- and COOH-terminal residue positions of each domain of Lck. L40 yeast cells containing HIS3 and LacZ reporter genes were cotransformed with plasmids encoding constructs consisting of the DNA binding domain of LexA fused to the cytoplasmic region of SAP-1 and of the trans-activation domain of GAL4 fused to either full-length Lck or indicated Lck mutants. Transformed cells that grew on histidine-deficient medium (−His) within 3 days were scored as positive. The β-galactosidase (β-gal) activity of each clone was tested by filter assay and scored as positive (blue) or negative (white) after incubation for 2 h. Relative levels of growth on histidine-deficient medium and of β-galactosidase activity are indicated as +, ++, +++, or +++.

We determined the activation state of Lck by immunoblot analysis with pAbs specific for autophosphorylated tyrosine residues of Src family PTKs including Lck; autophosphorylation of these residues results in the activation of these PTKs (40). Lck was thus immunoprecipitated from Jurkat cell lysates, and the resulting precipitates were subjected to immunoblot analysis. The autophosphorylation of Lck was observed even in unstimulated Jurkat cells, and a small increase of this parameter was observed in TCR-stimulated mock-transfected Jurkat cells (Fig. 4A). This result was consistent with the previous observation (24). In contrast, the autophosphorylation of Lck was markedly reduced in both unstimulated and TCR-stimulated J-SAP-1-WT cells (Fig. 4A). Overexpression of SAP-1-C/S did not change both basal and TCR-stimulated Lck activities. The PTP activity of SAP-1 thus appeared to be required for the inhibition of TCR-mediated Lck activation by this protein.

To examine whether SAP-1 directly dephosphorylates Lck in vitro, we immunoprecipitated Lck from lysates of pervanadate-stimulated Jurkat cells. The resulting precipitates were then incubated either with GST alone or with GST fusion proteins of wild-type SAP-1 or SAP-1-C/S, after which the reaction mixtures were subjected to immunoblot analysis with pAbs to the autophosphorylated tyrosine residues of Src family PTKs. Incubation with GST-SAP-1-WT, but not with either GST or GST-SAP-1-C/S, completely abolished the autophosphorylation of Lck (Fig. 4B), suggesting that autophosphorylated Lck is a direct substrate of SAP-1.

Effects of SAP-1 Overexpression on MAP Kinase Activation and CD69 Expression Induced by TCR Stimulation—Given that expression of SAP-1-WT markedly inhibited TCR-induced Lck activation and that activated Lck mediates signaling that leads sequentially to the activation of MAP kinase and up-regulation of cell surface expression of CD69 (7, 41), we next determined effects of overexpression of SAP-1 on these latter two manifestations of TCR stimulation. Immunoblot analysis with pAbs specific for activated MAP kinase revealed that TCR stimulation induced the activation of MAP kinase in mock-transfected Jurkat cells and that this response was markedly inhibited in J-SAP-1-WT cells but not in J-SAP-1-C/S cells (Fig. 5A). Similarly, flow cytometry with a mAb to CD69 revealed that expression of SAP-1-WT, but not that of SAP-1-C/S, greatly inhibited the increase in surface expression of CD69 induced by TCR stimulation (Fig. 5B). The combination of activation of protein kinase C by PMA and Ca²⁺ mobilization by ionomycin also up-regulates the surface expression of CD69 in a manner independent of TCR-mediated early tyrosine phosphorylation events (41). The increase in the surface expression of CD69 induced by the combination of PMA and ionomycin in J-SAP-1-WT cells was similar to that apparent in mock-transfected Jurkat cells or in J-SAP-1-C/S cells (Fig. 5B), suggesting that overexpression of SAP-1 affects upstream events in the
signaling pathway responsible for TCR-induced activation of MAP kinase.

**Effects of SAP-1 Overexpression on Tyrosine Phosphorylation of TCR/H9256, ZAP-70, and LAT Induced by TCR Stimulation**—Activation of either Lck or Fyn results in the tyrosine phosphorylation of immunoreceptor tyrosine-based activation motifs within the TCR \(\zeta\) chain (7–10). ZAP-70 then binds to tyrosine-phosphorylated TCR\(\zeta\) through its SH2 domains (12), thereby tyrosine-phosphorylated by Lck, and activated to catalyze the tyrosine phosphorylation of LAT (13). We therefore examined whether overexpression of SAP-1 affected the tyrosine phosphorylation of TCR\(\zeta\). TCR stimulation resulted in the tyrosine phosphorylation of TCR\(\zeta\) in mock-transfected Jurkat cells (Fig. 6A). The TCR-stimulated tyrosine phosphorylation of this protein was not substantially affected by expression of SAP-1-WT or SAP-1-C/S (Fig. 6A). In contrast, the extent of tyrosine phosphorylation of ZAP-70 induced by TCR stimulation was reduced in J-SAP-1-WT cells, compared with that apparent in mock-transfected cells and J-SAP-1-C/S cells (Fig. 6B). Furthermore, the extent of tyrosine phosphorylation of LAT induced by TCR stimulation was markedly reduced in J-SAP-1-WT cells, compared with that apparent in mock-transfected cells and J-SAP-1-C/S cells (Fig. 6C).

**Effects of SAP-1 Overexpression on Basal and CD2-induced Tyrosine Phosphorylation of p62dok**—Given that overexpression of SAP-1 reduced the autophosphorylation of Lck and the tyrosine phosphorylation of LAT induced by TCR stimulation, we next examined the effect of SAP-1 on overall tyrosine phos-
phorylation in lysates prepared from unstimulated or TCR-stimulated Jurkat cells. Several tyrosine-phosphorylated proteins were detected even in unstimulated mock-transfected Jurkat cells, whereas the tyrosine phosphorylation of various proteins, including 150-, 120-, and 40-kDa molecules, was increased in response to TCR stimulation in these cells (data not shown). Among these tyrosine-phosphorylated proteins, the phosphorylation of a 60-kDa protein was markedly reduced in J-SAP-1-WT cells, in the absence or presence of TCR stimulation, compared with that apparent in mock-transfected cells or J-SAP-1-C/S cells (data not shown). Given that p62\textsuperscript{ dok} (42, 43) is a putative substrate of SAP-1 (27), we examined whether the 60-kDa tyrosine-phosphorylated protein might be p62\textsuperscript{ dok}. Immunoprecipitation of p62\textsuperscript{ dok} with specific antibodies revealed that the extent of its tyrosine phosphorylation, in the absence and presence of TCR stimulation, was markedly reduced in J-SAP-1-WT cells compared with that apparent in mock-transfected cells (Fig. 7A).
increased by CD2 stimulation, and Lck has been implicated in this phosphorylation event (44, 45). We therefore examined the effect of SAP-1 overexpression on the tyrosine phosphorylation of p62 dok induced by CD2 stimulation. Stimulation of CD2 resulted in a small increase in the extent of tyrosine phosphorylation of p62 dok in mock-transfected Jurkat cells. Both the basal and CD2-stimulated tyrosine phosphorylation of p62 dok were markedly reduced in J-SAP-1-WT cells (Fig. 7B). The tyrosine phosphorylation of p62 dok was slightly but consistently inhibited in unstimulated or TCR-stimulated J-SAP-1-C/S cells compared with that apparent in mock-transfected cells (Fig. 7A). Such inhibition was also observed in CD2-stimulated J-SAP-1-C/S cells compared with that apparent in mock-transfected cells (Fig. 7B).

Effect of Overexpression of SAP-1 on Jurkat Cell Migration—Tyrosine phosphorylation of p62 dok is thought to contribute to the positive regulation of cell migration in fibroblasts, melanoma cells, and leukemia cells, and this regulation also appears to require either the Ras GTPase-activating protein or the adapter protein Nck (30, 46, 47). Previous studies also indicate that Lck and the activation of MAP kinase are important for the positive regulation of T cell migration (37, 48, 49). We therefore finally examined the effect of overexpression of SAP-1 on Jurkat cell migration with the use of a Transwell apparatus. The migratory activity of J-SAP-1-WT cells was markedly reduced compared with that of mock-transfected cells, whereas that of J-SAP-1-C/S cells appeared slightly reduced but this effect was not significant (Fig. 8).

DISCUSSION

We have demonstrated that the cytoplasmic region of SAP-1, a transmembrane-type PTP, directly binds Lck, a PTK that has previously been shown to interact with the cytoplasmic tails of CD4 and CD8 (50, 51). Whereas the NH2-terminal region of Lck (amino acids 1–67) mediates its binding to CD4 or CD8, we have now shown that a middle portion of this protein (amino acids 68–140) mediates the interaction with SAP-1.
acids 154–242, corresponding to part of the SH2 domain and the region between the SH2 and catalytic domains) is responsible, at least in part, for its association with SAP-1. Lck thus appears to associate with multiple signaling molecules via distinct molecular regions. The cytoplasmic regions of several transmembrane-type PTPs function as molecular scaffolds. For example, the Drosophila transmembrane-type PTP Dlar binds the cytoplasmic PTK Abl as well as the tyrosine-phosphorylated protein Ena, and the formation of this complex plays an important role in axonal guidance (52). RPTPα/H9262, another transmembrane-type PTP, forms a complex with p120ctn (53), whereas RPTPβ/H9252 or -or/H9253-catenin (54). In addition, the transmembrane-type PTP CD148 (DEP-1) interacts with p120ctn (55). These transmembrane-type PTPs are thus implicated in the regulation of cadherin-mediated cell adhesion (52–55). In addition to Lck, our yeast two-hybrid screening analysis revealed that SAP-1 also binds CrkII (38), an adapter protein that is tyrosine-phosphorylated by Src family PTKs; further analysis is required to determine whether this interaction is physiologically relevant. Thus, like other transmembrane-type

Fig. 6. Effects of overexpression of SAP-1 on the tyrosine phosphorylation of TCRζ, ZAP-70, and LAT induced by TCR stimulation. Mock-transfected or SAP-1-overexpressing Jurkat (J-SAP-1-WT or J-SAP-1-C/S) cells were left untreated or stimulated with OKT3 and cross-linking antibodies, after which cell lysates were subjected to immunoprecipitation with mAbs to TCRζ (aTCRζ) (A), pAbs to ZAP-70 (aZAP-70) (B), or pAbs to LAT (aLAT) (C). Resulting precipitates were then subjected to immunoblot analysis either with horseradish peroxidase-conjugated mAb PY20 to phosphotyrosine (aPY) (A and C, upper panels) or with mAb 4G10 to phosphotyrosine (aPY) (B, upper panel). Duplicate samples were subjected to immunoblot analysis with pAbs to TCRζ (A, lower panel), pAbs to ZAP-70 (B, lower panel), or pAbs to LAT (C, lower panel). Densitometric analysis was performed, ratio of the band intensity of PY to that of TCRζ/ZAP-70/LAT for each lane was calculated, and results were expressed as a percentage of the value with OKT3-stimulated mock-transfected cells. All results are representative of three independent experiments.

Fig. 7. Effects of overexpression of SAP-1 on the tyrosine phosphorylation of p62dok. Mock-transfected or SAP-1-overexpressing Jurkat (J-SAP-1-WT or J-SAP-1-C/S) cells were left untreated or stimulated either with OKT3 and cross-linking antibodies for 5 min (A) or with mAbs (anti-T112 and anti-T113) to CD2 (1:100 dilution) for 10 min (B). Cell lysates were then prepared and subjected to immunoprecipitation with pAbs to p62dok(dok), and the resulting precipitates were subjected to immunoblot analysis with horseradish peroxidase-conjugated mAb PY20 to phosphotyrosine. Duplicate samples were subjected to immunoblot analysis with pAbs to p62dok. Densitometric analysis was performed, ratio of the band intensity of PY to that of p62dok for each lane was calculated, and results were expressed as a percentage of the value with unstimulated mock-transfected cells. All results are representative of three independent experiments.

Fig. 8. Effect of overexpression of SAP-1 on Jurkat cell migration. Mock-transfected or SAP-1-overexpressing Jurkat (J-SAP-1-WT or J-SAP-1-C/S) cells (2 × 10⁶) were applied to polycarbonate filters in upper compartments of a Transwell apparatus. After incubation for 3 h at 37 °C, the number of cells that had migrated into lower compartments was determined. Data are means ± S.E. of triplicates from an experiment that was performed three times with similar results. *, p < 0.05 for the indicated comparison; NS, not significant (p > 0.05) (analysis of variance and Fisher’s PLSD (protected least significance difference) test).
PTPs, SAP-1 may function not only as a PTP but also as a scaffolding protein. We have also demonstrated that overexpression of SAP-1 inhibits both the basal activity of Lck and the activation of this PTK in response to TCR stimulation. Given that SAP-1 mediates the dephosphorylation of autophosphorylated Lck in vitro, it might negatively regulate the kinase activity of Lck through direct dephosphorylation of this PTK. Consistent with this notion, overexpression of SAP-1 markedly reduced the activation of MAP kinase and the subsequent surface expression of CD69 induced by TCR stimulation. Moreover, we also found that the TCR-induced tyrosine phosphorylation of ZAP-70 and that of LAT were reduced by SAP-1 overexpression. In contrast, the tyrosine phosphorylation of TCRζ, induced by TCR stimulation was not affected by overexpression of SAP-1. Although Lck plays a primary role in the tyrosine phosphorylation of TCRζ induced by TCR stimulation, Fyn might thus be responsible for this effect of CD2 stimulation. Overexpression of SAP-1 inhibited both the basal and CD2-induced tyrosine phosphorylation of p62akt. Given that Lck is thought to be responsible for this effect of CD2 stimulation, it is possible that SAP-1 overexpression inhibited the TCR-induced activation of MAP kinase and surface expression of CD69 through the dephosphorylation of LAT. CD148 (DEP-1) negatively regulates TCR-mediated T cell responses (22–24). This transmembrane-type PTP contains 8–10 fibronectin type III-like domains in its extracellular region and a single PTP domain in its cytoplasmic region (20, 21). The physiological relevance of tyrosine phosphorylation of Dok/H9256 induced by TCR stimulation (9), Fyn also contributes to the down-regulation of Lck activity. However, whereas this PTP directly dephosphorylates CD148 in T cells is up-regulated in response to cell activation (22, 24). Therefore, whereas this PTP directly dephosphorylates LAT, its overexpression does not inhibit the activation of Lck in response to TCR stimulation (24). Thus, despite their structural similarity and the fact that they both negatively regulate TCR-mediated T cell responses, SAP-1 and CD148 dephosphorylate distinct signaling molecules in the TCR signaling pathway. Overexpression of SAP-1 inhibited both the basal and CD2-induced tyrosine phosphorylation of p62akt. Given that Lck is thought to be responsible for this effect of CD2 stimulation, it is possible that SAP-1 overexpression inhibited the TCR-induced activation of MAP kinase and surface expression of CD69 through the dephosphorylation of LAT. CD148 (DEP-1) negatively regulates TCR-mediated T cell responses (22–24). 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Regulation by SAP-1 of Lck and T Cell Function

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Interaction of SAP-1, a Transmembrane-type Protein-tyrosine Phosphatase, with the Tyrosine Kinase Lck: ROLES IN REGULATION OF T CELL FUNCTION
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