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Targeting Src Homology 2 Domain-Containing Tyrosine Phosphatase (SHP-1) into Lipid Rafts Inhibits CD3-Induced T Cell Activation

Michael Wei-Chih Su, Chao-Lan Yu, Steven J. Burakoff, and Yong-Jiu Jin

To study the mechanism by which protein tyrosine phosphatases (PTPs) regulate CD3-induced tyrosine phosphorylation, we investigated the distribution of PTPs in subdomains of plasma membrane. We report here that the bulk PTP activity associated with T cell membrane is present outside the lipid rafts, as determined by sucrose density gradient sedimentation. In Jurkat T cells, ~5–10% of Src homology 2 domain-containing tyrosine phosphatase (SHP-1) is constitutively associated with plasma membrane, and nearly 50% of SHP-2 is translocated to plasma membrane after vanadate treatment. Similar to transmembrane PTP, CD45, the membrane-associated populations of SHP-1 and SHP-2 are essentially excluded from lipid rafts, where other signaling molecules such as Lck, linker for activation of T cells, and CD3ε are enriched. We further demonstrated that CD3-induced tyrosine phosphorylation of these substrates is largely restricted to lipid rafts, unless PTPs are inhibited. It suggests that a restricted partition of PTPs among membrane subdomains may regulate protein tyrosine phosphorylation in T cell membrane. To test this hypothesis, we targeted SHP-1 into lipid rafts by using the N-terminal region of Lck (residues 1–14). The results indicate that the expression of Lck/SHP-1 chimera inside lipid rafts profoundly inhibits CD3-induced tyrosine phosphorylation of CD3/ε, IL-2 generation, and nuclear mobilization of NF-AT. Collectively, these results suggest that the exclusion of PTPs from lipid rafts may be a mechanism that potentiates TCR/CD3 activation.


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Address correspondence and reprint request to Dr. Yong-Jiu Jin, Department of Pediatric Oncology, Room M654, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115. E-mail address: yong-jiu_jin@dfci.harvard.edu

Abbreviations used in this paper: LAT, linker for activation of T cells; PTP, protein tyrosine phosphatase; PTK, protein tyrosine kinase; anti-pTyr: anti-phosphotyrosine; SLP-76, 76 kDa (SLP-76), and murine Sons of Sevenless (mSOS) (3–7). This links TCR stimulation to the activation of downstream pathways, such as mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and phospholipase C-γ1, (8, 9).

Recent studies suggest that the compartmentalization of membrane structure imposed by lipid rafts is essential for TCR-mediated signaling (10–12). Lipid rafts have been described as a mobile and dynamic membrane structure with a diameter of 70–300 nm (13). Previous studies that used confocal microscopy have shown that stimulation by TCR, CD2, or CD3/CD28 induces reorganization and clustering of lipid raft microdomains at the site of engagement, which lead to enhanced TCR signaling and a higher and more stable protein tyrosine phosphorylation (10–12). Discrete lipid rafts are enriched in molecules involved in signal transduction, such as Src family kinases, Ras, and G proteins, as well as sphingolipid, cholesterol, and a subset of GPI-linked proteins (14–17). Studies in T cells revealed that CD3ε, coreceptor CD4 and CD8, Src family kinase Lck and Fyn, and transmembrane adaptor LAT are also concentrated in lipid rafts (18–20). In contrast, CD45, a receptor-type protein tyrosine phosphatase (PTP), is so far the only phosphatase that has been shown to be excluded from lipid rafts (21). It is known that T cell activation is controlled by the coordinated action of protein tyrosine kinases (PTKs) and PTPs (22–24). However, it is not clear whether the segregation of PTKs and PTPs on T cell membrane is important for T cell activation.

In addition to CD45, PTPs Src homology 2 domain-containing tyrosine phosphatase (SHP-1) and SHP-2 have also been implicated in the regulation of T cell activation (22–24). SHP-1 is expressed primarily in hemopoietic cells and plays a critical role in the negative regulation of signaling and development. Accordingly, thymocytes derived from motheaten (me) mice, which lack the expression of functional SHP-1, hyperproliferate in response to TCR stimulation (25–27). In contrast, SHP-2 is known for its requirement for the activation of Ras/mitogen-activated protein kinase pathways in nonhemopoietic cells and is expressed ubiquitously (28–30). In T lymphocytes, SHP-2 has also been shown to associate with TCR through its interaction with CTLA-4 and possibly to contribute to the dephosphorylation of CD3 subunits (31–33). Nevertheless, the precise functions of SHP-1 and SHP-2 as regulators in T cell membrane have yet to be determined.

Previously, we reported that when isolated T cell membranes were incubated with PTP inhibitors H2O2 or vanadate in the presence of ATP, the molecules enriched in lipid rafts, such as CD3ε and protein kinases Lck, Fyn, Syk, and ZAP70, were tyrosine phosphorylated (34). In this report, we show that PTP activity is found predominantly outside the lipid rafts and identify SHP-1 and...
SHP-2 as two important membrane-associated PTPs in T cells. The functional significance of restricted distribution of PTPs in membrane microdomains was further demonstrated by the inhibition of CD3-induced protein tyrosine phosphorylation, IL-2 generation, and NF-AT activation in T cells with SHP-1 targeted to lipid rafts. Taken together, our results suggest that restricted distribution of PTPs in membrane microdomains may play a critical role in the initiation of TCR/CD3 signaling.

Materials and Methods

Cells and Abs

Jurkat T cell line J77, a variant of clone E6–1 (American Type Culture Collection, Manassas, VA) was cultured in RPMI 1640 medium supplemented with 10% FCS at 37°C in a 5% CO₂ humidified atmosphere. JCD45− (J45.01) Jurkat T cells were obtained from ATCC. Anti-phosphotyrosine Ab (anti-pTyr; RC20) and anti-SHP-1 mAbs were purchased from BD Transduction Laboratories (Lexington, KY). Anti-CD3ε and anti-CD45 mAbs as well as anti-SHP-2 polyclonal Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-NF-AT and anti-Lck polyclonal Abs were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-CD3 Ab (OKT3) and anti-Myc Ab were prepared from a hybridoma obtained from ATCC.

T cell stimulation, immunoprecipitation, and immunoblotting

Jurkat T cells (5 × 10⁶) were washed and resuspended in 1 ml of PBS. For CD3 stimulation, cells were incubated with OKT3 (2 µg/ml) for 5 min on ice. cross-linked by rabbit anti-mouse IgG (5 µg/ml) on ice for an additional 30 s, then incubated at 37°C for 3 min. For H₂O₂ stimulation, cells were incubated with 5 mM H₂O₂, at 37°C for 3 min. After washing with PBS, cells were lysed in 1 ml of Nonidet P-40 (NP-40) lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris, pH 7.4, 0.5% sodium deoxycholate, 50 mM NaF, 1 mM PMSF, 1 µg/ml leupeptin, 1 mM antipain) at 4°C for 30 min. The NP-40 lysate was centrifuged at 12,000 × g for 15 min at 4°C. The supernatant was used as a starting point for immunoprecipitation was conducted at 4°C overnight or at room temperature for 4 h with protein A-Sepharose beads. The beads were washed twice with 0.1% Triton X-100/TBS and once with TBS. Proteins were eluted from the beads by boiling for 5 min in 50 µl of Laemmli reducing SDS sample buffer. Proteins from −10⁷ cells were subjected to SDS-PAGE and transferred to a polyvinylidene fluoride membrane. Membrane were blocked with 3% BSA in TBST and incubated with Abs in TBST for 2 h at room temperature. After 15 min washes with TBST, the membranes were incubated with HRP-conjugated Ab for 30 min. washed three times for 5 min with TBST, and developed using the ECL system (Amersham, Buckinghamshire, U.K.).

Subcellular fractionation

After washing in PBS, Jurkat T cells (2 × 10⁶) were incubated in 2 ml of hypotonic buffer (42 mM KCl, 10 mM HEPES, pH 7.4, 5 mM MgCl₂) for 15 min at 4°C. Cells were passed through a 30-gauge needle 10 times, followed by centrifugation at 250 × g for 10 min to remove the nuclei and intact cells. The supernatant was centrifuged at 150,000 × g for 30 min at 4°C to separate the cytoplasm from the membrane fraction. For sucrose density gradient centrifugation, the membrane fraction was lysised for 1 h at 4°C in 1 ml of 1% Triton X-100, 10 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, and a panel of protease inhibitors (Sigma, St. Louis, MO). The lysate was incubated with 1 ml of 85% sucrose by TNE buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA). In a SW41 centrifuge tube, the lysate was overlaid with 7 ml of 30% and 3.5 ml of 5% sucrose in TNE buffer. The samples were centrifuged for 16–17 h at 38,000 rpm. After equilibrium centrifugation, the gradients were fractionated from sucrose in TNE buffer. The samples were centrifuged for 16–17 h at 38,000 rpm. The supernatants were measured for IL-2 by standard ELISA techniques with paired capture and detection anti-IL-2 Abs (OptEIA; BD PharMingen, San Diego, CA).

Phosphatase activity assays

For assays that used p-nitrophenyl phosphate (pNPP) as the substrate, 50 µl of membrane suspension (OD₅₅₀ = 6.0) was mixed with 50 µl of 2× assay buffer containing 100 mM pNPP, 100 mM 2-(-N-morpholino)ethanesulfonic acid at pH 5.5, 10 mM DTT, 150 mM NaCl, and 2 mM EDTA and incubated at 25°C for 20 min. After centrifugation, the supernatant was transferred to cuvettes containing 0.9 ml of 1% NaOH for OD reading at 450 nm. For assays with T cell membrane as the substrate, the membrane fraction was incubated with 1.0 mM vanadate at 4°C for 10 min to inhibit PTP activity. The membrane was then pelleted by centrifugation at 13,000 rpm for 10 min and washed twice with 1.5 ml of cold hypotonic buffer, and then adjusted to concentration of 6.0 OD reading at 600 nm wavelength. Fifty microliters of vanadate-treated membrane suspension was used as substrate in 100 µl of reaction mixture, which was incubated at 4°C for 10 min. One millimolar ATP was then added to the mixture for 3 min at 37°C. The membrane was precipitated by centrifugation at 13,000 rpm for 10 min and then solubilized in NP-40 lysis buffer. The tyrosine phosphorylation of membrane proteins was determined by anti-pTyr (4G10) immunoprecipitation followed by anti-pTyr (RC20) immunoblotting. For assays with purified phosphatases, the Escherichia coli-overexpressed GST fusion proteins were purified by glutathione-Sepharose affinity column, and the activity was determined by pNPP assay with the enzyme unit of T cell PTP (New England Biolabs, Beverly, MA) as standard.

Construction, transfection, and stable expression of Lck/SHP-1 plasmid in Jurkat T cells

To construct Lck/SHP-1 fusion plasmid, PCR was performed with human SHP-1 template with PCR primers 5’-ATGGCGTGTGCTGACAGCTCA CACCCCGGAAAGATGCTGATGAGGACCTGGCTCAGAAGGGCGGAGG TGTC (underlined 42 nucleotides are from the N terminus of Lck) and 5’-CTCCCTCAGGAGGACCTGGCTC TCTC. The PCR product was subcloned into mammalian expression vector pEF/-Myc-His (Invitrogen, Carlsbad, CA). To generate Lck/SHP-1 (ΔP), Lck/SHP-1 was digested by BglII and EagI and religated by blunt-end ligation, which removed a DNA fragment of 144 bp. Lck/SHP-1 (ΔP) cDNA encodes a mutant SHP-1 protein with a 48-aa truncation from residues 409–457. The sequence was confirmed by DNA sequencing. Five micrograms of plasmids were transfected into Jurkat T cells (1 × 10⁶/500 µl) by electroporation with the settings of 800 µF, low ohm, and 250 V. The clones with stable expression of Lck/SHP-1 or Lck/SHP-1 (ΔP) were selected in medium containing neomycin (G418) at 2.5 mg/ml.

Luciferase assays

Luciferase assay was conducted with dual-luciferase reporter assay system (Promega, Madison, WI). Jurkat T cells (2 × 10⁶) with stable expression of Lck/SHP-1 or Lck/SHP-1 (ΔP) were transiently transfected with 5 µg of pNP-AT-Luciferase together with 1 µg of pRenilla-luciferase, and then cultured in 25 ml of medium for 16–20 h. Cells were aliquoted into a 24-well plate to a density of 2 × 10⁶ per well in 500 µl of culture medium for subsequent stimulation. Cells were either left unstimulated, stimulated with plate-bound CD3 plus PMA (50 ng/ml), or stimulated with PMA plus 1 µM ionomycin. After 6–8 h, cells were harvested and washed with 1 ml of PBS. Harvested cells were lysed in 100 µl of lysis buffer provided by the manufacturer and dual-luciferase activity was quantitated according to the manufacturer’s instruction with a monolight luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI). Luciferase activity was determined in triplicate for each experimental condition.

ELISA of IL-2 in supernatants

Anti-CD3 mAb (OKT3, 100 µg of 10 µg/ml) was immobilized on an enzime immunosassay RIA 96-well Costar microtiter plate (Cambridge, MA) in sterile PBS overnight at 4°C. Wells were washed three times with PBS followed by the addition of 10⁴ Jurkat T cells to wells containing PMA (Calbiochem, La Jolla, CA) or ionomycin (Calbiochem). After 24 h, supernatants were measured for IL-2 by standard ELISA techniques with paired capture and detection anti-IL-2 Abs (OptEIA; BD Pharmingen, San Diego, CA).

Results

SHP-1 and SHP-2 phosphatases are predominantly localized in Triton X-100 soluble, nonlipid raft membrane fractions

To date, the distribution of phosphatases in the cytoplasmic membrane of T lymphocytes has not been fully characterized. Given our knowledge of the importance of lipid rafts in the propagation of signal transduction, we sought to identify the location of phosphatases in relation to lipid microdomains. To this end, we segregated the plasma membrane of Jurkat T cells into nine fractions with sucrose gradient sedimentation, of which fractions 3 and 4 are enriched in lipid rafts (insoluble in Triton X-100) and fractions 8 and 9 are derived from outside the lipid rafts (soluble in Triton X-100; Fig. 1). We first identified the position of phosphatases by measuring PTP activities in each gradient fraction (Fig. 1, middle and right). The results show that PTP activity is essentially located in fractions 8 and 9, or outside the lipid rafts. A likely candidate of
membrane-associated phosphatase would be CD45, a transmembrane PTP known to be excluded from lipid rafts (21). To address this possibility, we compared the total PTP activity in the plasma membrane of Jurkat T cells to its mutant cell line (J45.01) lacking the expression of CD45. We found that the membrane of J45.01 T cells still contained ∼40% of the PTPs activity as compared with its parental cell line (Fig. 1, left). The phosphatases that remain in J45.01 T cells shared a similar distribution of PTP activity as the parental Jurkat T cells (Fig. 1, right). We conclude that the majority of CD45 and other membrane-resident phosphatases are basically excluded from lipid microdomains.

SHP-1 is a phosphatase described to associate with a number of substrates, of which some are associated with plasma membrane, such as ZAP70, CD3ε, CD5, leukocyte-associated Ig-like receptor-1, and signal-regulatory proteins (SIRP or SHPS-1; Refs. 26, 35–39). In additional to CTLA-4, SHP-2 also has been shown to associate with membrane anchor proteins such as SIRP or SHPS-1 and Protein Zero Related (PZR) (40, 41). Given their importance in regulating TCR signaling, we attempted to examine whether SHP-1 and SHP-2 could be associated with cytoplasmic membrane. To address this issue, Jurkat T cells were fractionated into cytosolic or membrane fractions with or without vanadate, an inhibitor of PTPs known to induce potent tyrosine phosphorylation (Fig. 2A, top). Our results indicate that ∼5–10% of SHP-1 was associated with T cell membrane, and this level increased after vanadate treatment. SHP-2 did not associate with T cell membrane in basal state. However, after vanadate treatment, nearly half of SHP-2 was recruited to the cell membrane. This population of SHP-2 appeared to migrate at a higher position on SDS-PAGE, which is probably because of tyrosine phosphorylation (Fig. 2A, bottom). Consistent with the distribution of PTP activity in lipid microdomains, Fig. 2B shows that, after vanadate treatment, the majority of SHP-1 and SHP-2 were present in Triton X-100-soluble fractions 8 and 9 or outside the lipid rafts. Of note, a small amount of SHP-2 was also detected in fraction 3.

The distribution of PTPs also was compared with that of other signaling molecules, such as CD3ζ, Lck, and LAT, which are enriched in lipid rafts. As shown in Fig. 2C, we found that SHP-1, SHP-2, and CD45 PTPs were highly concentrated in fractions 8 and 9 or outside the lipid rafts, whereas CD3ζ, Lck and LAT were enriched in Triton X-100-insoluble fractions 3 and 4. Stimulation by anti-CD3 Ab did not alter the distribution of SHP-1. Likewise, SHP-2 was not found in the plasma membrane until the cells were

FIGURE 1. Distribution of PTP activity in membrane fractions of sucrose density gradient. T cell membranes were prepared from CD45+ Jurkat T cells or CD45− (J45.01) T cells. The PTP activity present in either the total membrane fraction (left) or individual sucrose density gradient fraction (middle and right) was analyzed by using pNpp as a substrate. The PTP activity was determined by the release of nitrophenyl phosphate, which was measured at 450 nm.

FIGURE 2. Distribution of membrane-associated SHP-1 and SHP-2 in microdomains. A, Localization of SHP-1 and SHP-2 in T cell membrane. Jurkat T cells untreated (−) or treated (+) with 1 mM vanadate were fractionated into cytosolic (C) and membrane (M) fractions. Proteins in each fraction were immunoprecipitated with anti-SHP-1 Ab (top) or anti-SHP-2 Ab (bottom), resolved by 10% SDS-PAGE, followed by immunoblotting with anti-SHP-1 (top) or anti-SHP-2 (bottom), respectively. B, Detection of membrane-associated SHP-1 and SHP-2 in microdomains. Vanadate-treated Jurkat T cell membrane was segregated into nine fractions by sucrose gradient centrifugation. Twenty microliters of each fraction was boiled in 3× SDS sample buffer, resolved by 10% SDS-PAGE, and then immunoblotted with anti-SHP-1 (top) or anti-SHP-2 (bottom) Ab. C, Comparison of microdomain distribution of PTPs with other T cell signaling molecules. T cell membranes were isolated from Jurkat T cells unstimulated (CD3−) or stimulated (CD3+) with OKT3 and segregated by sucrose gradient centrifugation. Twenty microliters of each gradient fraction was subjected to SDS-PAGE as described above and then analyzed by immunoblotting with anti-CD45, anti-SHP-1, anti-SHP-2, anti-CD3ζ, anti-Lck, and anti-LAT Abs.
stimulated with anti-CD3 Ab. Although the distribution of Lck and LAT appeared unchanged after stimulation, the amount of CD3ζ in lipid rafts (fractions 3 and 4) appeared to have increased slightly. All together, these results indicate that SHP-1 and SHP-2 as well as CD45 phosphatases are excluded from lipid rafts, and their distribution is consistent with the location of bulk PTP activity (Fig. 1).

**CD3-induced tyrosine phosphorylation of CD3ζ and CD3ε was detected predominantly in Triton X-100-insoluble lipid raft membrane fractions**

To investigate whether the restricted distribution of PTPs in membrane subdomains could affect CD3-induced tyrosine phosphorylation (pTyr), we analyzed the protein tyrosine phosphorylation in membrane subdomains. As shown in Fig. 3A, CD3-induced tyrosine phosphorylation of CD3ζ and CD3ε was observed mainly in fractions 3, 4, and 5, which corresponded to materials derived from lipid rafts, and their distribution is consistent with the location of bulk PTP activity (Fig. 1).

**FIGURE 3.** Restriction of CD3-induced tyrosine phosphorylation in membrane microdomains. A, Jurkat T cells were either unstimulated (CD3<sup>−</sup>) or stimulated by CD3 cross-linking (CD3<sup>+</sup>). Isolated membranes were then subjected to sucrose gradient centrifugation. Proteins in each gradient fraction were lysed in 1% NP-40 lysis buffer at 4°C for 4 h. Tyrosine phosphorylated proteins were immunoprecipitated by anti-pTyr mAb (4G10) and analyzed by immunoblotting with HRP-conjugated anti-pTyr Ab (RC20). B, Membranes isolated from Jurkat T cells unstimulated (pVa<sup>−</sup>) or stimulated with 0.1 mM pervanadate (pVa<sup>+</sup>) were analyzed as described in A.

**FIGURE 4.** Inhibition of protein tyrosine phosphorylation in isolated T cell membranes by purified SHP-1 and SHP-2. A, SHP-1 and SHP-2 quantitatively inhibited tyrosine phosphorylation in T cell membranes. GST-fusion proteins of SHP-1 and SHP-2 containing PTP activity ranging from 0.2 to 20 U were incubated with vanadate (Va)-pretreated T cell membrane as a substrate. After incubation with ATP, tyrosine phosphorylation of membrane proteins was determined by anti-pTyr (4G10) immunoprecipitation and anti-pTyr (RC20) immunoblotting. Lanes 1 and 2 are membrane controls with or without vanadate treatment, respectively. B, Time course of tyrosine dephosphorylation of membrane proteins with purified SHP-1, SHP-2, and CD45. Vanadate-pretreated T cell membranes were first incubated with ATP for tyrosine phosphorylation. Tyrosine-phosphorylated membranes were then incubated with either buffer control or 3 U of SHP-1, SHP-2, or CD45 for 1–10 min. Tyrosine phosphorylation of membrane proteins was determined as described in A. Molecular mass standards are shown on the left in kDa.
SHP-1 and SHP-2 inhibits membrane protein tyrosine phosphorylation in vitro

To investigate whether SHP-1 and SHP-2 are involved in the regulation of tyrosine phosphorylation in T cell membrane, we compared the relative efficiencies of SHP-1, SHP-2, and CD45 phosphatases in inhibiting protein tyrosine phosphorylation. As shown in Fig. 4A, both purified SHP-1 and SHP-2 could quantitatively block the overall protein tyrosine phosphorylation in T cell membrane. The effect of purified SHP-1, SHP-2, and CD45 on substrate dephosphorylation were further compared in a time course (Fig. 4B). We observed that both SHP-1 and SHP-2 dephosphorylated proteins within 5 min of incubation. In comparison, CD45 was less effective at the same catalytic units, as the dephosphorylation of proteins was less apparent even after 10 min.

N-terminal region of Lck is sufficient to confer localization of SHP-1 into lipid rafts

If perpetual exclusion of PTPs from lipid microdomains is necessary to protect phosphorylated molecules from phosphatases, the presence of PTPs in lipid rafts may diminish CD3-induced tyrosine phosphorylation and T cell activation. To test this hypothesis, we targeted SHP-1 phosphatase into lipid microdomains by using the N-terminal region of Lck, which contains sites of palmitoylation known for anchoring proteins to lipid rafts (42, 43). Lck/SHP-1 chimeric molecule was constructed by fusion of the 14-aa N-terminal of Lck to a full-length SHP-1 phosphatase (Fig. 5A). The control fusion molecule was made with a catalytically inactive SHP-1 (ΔP), which has a 48-aa deletion in the PTP domain. These constructs were stably expressed as C-terminal Myc-tagged proteins in Jurkat T cells. To control for the expression of Lck/SHP-1 and Lck/SHP-1 (ΔP), cell lines were selected based on equivalent levels of anti-Myc immunoblotting. Fig. 5B shows the expression and subcellular distribution of Lck/SHP-1 and Lck/SHP-1 (ΔP) molecules in Jurkat T cell lines. The top band detected by both anti-SHP-1 and anti-Myc Ab is Lck/SHP-1, which is 24 aa longer than the endogenous SHP-1 (middle band). The bottom band is Lck/SHP-1 (ΔP), which is 24 aa shorter than the endogenous SHP-1. In contrast to endogenous SHP-1, most of which were cytosolic, more of the overexpressed Lck/SHP-1 and Lck/SHP-1 (ΔP) were detected in the membrane. More importantly, both Lck/SHP-1 and Lck/SHP-1 (ΔP) were enriched in lipid rafts whereas the patterns of membrane distribution of CD3ζ, Lck, and LAT were

FIGURE 5. Targeting SHP-1 into lipid rafts by the construction of chimeric Lck/SHP-1. A, Schematic map of chimeric Lck/SHP-1 and Lck/SHP-1 (ΔP). B, Expression and subcellular localization of Lck/SHP-1 and Lck/SHP-1 (ΔP) in Jurkat T cells. Wild-type Jurkat T cells (WT) and Jurkat T cells stably expressing Lck/SHP-1 or Lck/SHP-1 (ΔP) were fractionated into cytosolic (C) and membrane (M) fractions. Proteins in each fraction were immunoprecipitated with anti-SHP-1 Ab, subjected to SDS-PAGE, and then analyzed by immunoblotting with anti-SHP-1 (left) or anti-Myc (right) Ab. C, Microdomain distribution of membrane-associated Lck/SHP-1, Lck/SHP-1 (ΔP) CD3ζ, Lck, and LAT. Membranes were isolated from Jurkat T cells stably expressing Lck/SHP-1 (top) or Lck/SHP-1 (ΔP) (bottom) and segregated by sucrose gradient centrifugation. Proteins in each fraction were solubilized in NP-40 lysis buffer and immunoprecipitated with anti-SHP-1 Ab followed by immunoblotting analysis with anti-Myc Ab; or 40 μl of each fraction was immunoblotted with anti-CD3ζ, anti-Lck, and anti-LAT.
basically not altered and resemble that as seen in wild-type Jurkat T cells (Figs. 3 and 5C). Therefore, the 14-aa N-terminal of Lck is sufficient for targeting SHP-1 into lipid microdomains.

**Targeting of Lck/SHP-1 into lipid rafts inhibited CD3-induced tyrosine phosphorylation in lipid rafts, IL-2 generation, and NF-AT activation**

To examine the effects of targeting SHP-1 into lipid microdomains, Jurkat T cells expressing Lck/SHP-1 were stimulated with anti-CD3 Ab to induce tyrosine phosphorylation. As shown in Fig. 6, Jurkat T cells that expressed Lck/SHP-1 chimeric molecules were refractory to CD3-induced tyrosine phosphorylation of CD3ζ and CD3ε in the lipid raft fraction. However, in Jurkat T cells expressing the mutant Lck/SHP-1(ΔP) construct, tyrosine phosphorylation of CD3ζ and CD3ε appeared similar to that detected in wild-type Jurkat T cells. The inhibition of CD3-induced phosphorylation is dependent on the catalytic domain of SHP-1 phosphatase, and the mechanism of inhibition is not attributable to simple displacement of endogenous Lck from lipid rafts.

The functional consequence of targeting Lck/SHP-1 into lipid rafts was analyzed for its effect on the activation of NF-AT, a T cell-specific transcriptional factor necessary for IL-2 gene expression and IL-2 generation. Fig. 7 demonstrated that the presence of Lck/SHP-1 in lipid rafts inhibited profoundly the mobilization of NF-AT and IL-2 generation after stimulation with OKT3 plus PMA (left panel). This inhibition is again dependent on an intact catalytic domain of SHP-1, as cells expressing Lck/SHP-1 (ΔP) were largely not inhibited from activating NF-AT-mediated transcription and IL-2 generation (Fig. 7, left). The proximal events of TCR-induced activation can be bypassed with PMA plus ionomycin. Accordingly, we showed that PMA plus ionomycin could circumvent the defect seen in Jurkat T cells transfected with Lck/SHP-1; thus, the inhibition imposed by Lck/SHP-1 lies upstream of Ras/mitogen-activated protein kinase pathways. Taken together, by allowing phosphatases to enter lipid rafts, the physiological boundary imposed by lipid microdomains is disrupted, and the cells are no longer capable of responding to agonist ligands.

**Discussion**

To study the mechanism by which PTPs down-regulate CD3-induced tyrosine phosphorylation, we analyzed the subdomain distribution of PTPs in T cell membrane. Our results indicate that PTPs are not distributed homogeneously in the plasma membrane. After sucrose density gradient segregation, PTP activity was detected preferentially in Triton X-100 soluble membrane fractions.
suggesting that PTPs are excluded from lipid rafts (Fig. 1). Previously, CD45, an abundant transmembrane receptor-like PTP, was shown to be excluded from lipid rafts (21). Here, we provide evidence showing that membrane-associated population of SHP-1 and SHP-2 are also excluded from lipid rafts (Fig. 2). Because SHP-1 and SHP-2 are more potent than CD45 in the inhibition of protein tyrosine phosphorylation (Fig. 4), this observation suggests that exclusion of SHP-1 and SHP-2 from lipid rafts may be important for the initiation of TCR activation.

Aggregated lipid rafts may provide the molecular basis for segregating PTPs from PTKs. In resting state, various molecules involved in T cell activation are sequestered among the small and discrete clusters of lipid rafts. In this physical state, phosphatases like SHP-1 and CD45 can readily dominate and interfere with the initiation of protein tyrosine phosphorylation. However, when lipid rafts are aggregated by receptor cross-linking or when concentrated in a specific contact area between T cells and APCs, a large patch of the membrane formed by the coalescence of smaller clusters of lipid rafts may bring together molecules like CD3ζ, Lck, and LAT and expel PTPs from the contact area. Aggregated lipid rafts may impose a physical barrier that further distances phosphatases from their potential substrates, thus creating an environment within lipid rafts that would favor unregulated kinase activity. Therefore, our finding reinforced the notion that exclusion of PTPs from T cell activation domain or segregation of PTPs from receptor/PTK complexes by lipid rafts may potentiate the initiation of receptor tyrosine phosphorylation. This is supported by our results demonstrating that CD3-induced tyrosine phosphorylation of CD3ζ is largely restricted to lipid rafts (Fig. 3).

Our observations indicate that a fraction of SHP-1 was constitutively associated with T cell membrane. The molecular basis for this association is currently unclear. A plausible explanation would be the association between Src homology 2 domains of SHP-1 and tyrosine-phosphorylated residues of transmembrane receptors or anchors, such as the recently identified leukocyte-associated Ig-like receptor-1 and SHPS-1 (38, 39). This would be consistent with the increased association of SHP-1 with plasma membrane of vanadate-treated cells (Fig. 2A). SHP-1 in untreated cells could be associated with transmembrane receptors that have a low level of basal phosphorylation.

The association of SHP-2 with T cell membrane is dependent on vanadate or anti-CD3 stimulation. Several transmembrane proteins, such as CTLA-4, SIRP (SHPS-1), and PZR contain the immunoreceptor tyrosine-based inhibitory motifs that are potential membrane anchors for SHP-2 (31–33, 40, 41). It is interesting, though, that a small but significant amount of SHP-2 was detected in lipid rafts on T cell stimulation (Fig. 2). This could be mediated by the association of Grb2 with SHP-2, because Grb2 has been shown to enter lipid rafts upon phosphorylation (20). SHP-2 is so far the only PTPs shown to translocate into lipid rafts on T cell activation, and the biological significance of this observation warrants further studies.

To address the biological significance of PTP exclusion from lipid rafts, we targeted SHP-1 into lipid rafts by using the 14-aa N-terminal of Lck, which contains a lipid rafts targeting motif (Fig. 5). Our results indicate that the presence of Lck/SHP-1 in lipid rafts inhibited CD3-induced tyrosine phosphorylation of CD3ζ, as well as IL-2 generation and NF-AT-mediated transcriptional activation (Figs. 6 and 7). As reported previously, overexpression of the wild-type SHP-1 in Jurkat T cells did not affect T cell function; thus, it is unlikely that the increased phosphatase activity of Lck/SHP-1 alone is responsible for the inhibition (35). Also, by using the 14-aa N-terminal of Lck as a targeting strategy did not result in the displacement of endogenous Lck from lipid rafts, as the control Lck/SHP-1 (ΔP) chimera did not inhibit CD3-induced signaling. Therefore, we surmise that the Lck/SHP-1 chimeric molecule was able to breach the physical barrier imposed by lipid rafts, gaining access to the potential substrates that otherwise would not be available. Collectively, our results strongly support the notion that phosphatases, such as SHP-1, SHP-2, and CD45, are normally excluded from lipid rafts, and their restricted distribution may serve a unique function in regulating T cell activation.

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References


