Cross-Linking Depolymerization After Membrane IgM Phosphatase Is Essential for Actin Tyrosine Dephosphorylation by the Src Homology 1-Containing Protein Tyrosine Phosphatase

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Actin Tyrosine Dephosphorylation by the Src Homology 1-Containing Protein Tyrosine Phosphatase Is Essential for Actin Depolymerization After Membrane IgM Cross-Linking

Takehisa Baba,* Noemi Fusaki,* Nobuko Shinya,† Akihiro Iwamatsu,† and Nobumichi Hozumi2*†

Src homology protein 1 (SHP-1) plays an important role in B cell Ag receptor (BCR) differentiation, proliferation, survival, and apoptosis. After BCR stimulation in apoptotic cells, SHP-1 has been shown to be recruited to phosphorylated immunoreceptor tyrosine-based inhibitory motifs present in receptors such as CD22 and CD72. However, the substrates of SHP-1 in the chicken B cell line, DT40, have remained undefined. To identify SHP-1 substrates in DT40, we used a trapping mutant, SHP-1 C/S (a catalytically inactive form). Cross-linking of BCR induced hyperphosphorylation of a ~44-kDa protein in C/S transfectants. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis revealed that this was actin (cytoplasmic type 5) carrying three immunoreceptor tyrosine-based inhibitory motif-like sequences. SHP-1 was shown to bind to one of these sequences in synthetic peptide binding experiment. Thus, actin is a direct SHP-1 substrate. Furthermore, more SHP-1 molecules translocate into lipid rafts, and their association with actin was increased after BCR stimulation. In C/S transfectants, actin polymerization induced by membrane IgM ligation was sustained to a greater extent for a longer time compared with wild-type transfectants. Therefore, actin dephosphorylation by SHP-1 is essential for actin depolymerization after BCR stimulation. Our data suggest that SHP-1 plays a pivotal role in reorganization of cytoskeletal architecture inducing actin dephosphorylation. These results clearly demonstrate the direct interaction of SHP-1 with actin. The Journal of Immunology, 2003, 170: 3762–3768.

The B cell Ag receptor (BCR) plays a central role in the development, activation, survival, and apoptosis of B lymphocytes. Cross-linking of BCR results in the activation of several cytoplasmic protein tyrosine kinases (PTKs), starting the signaling cascade (1–3). In contrast, protein tyrosine phosphatases (PTPs) control the activation signaling by PTKs. Thus, the balance of tyrosine phosphorylation and dephosphorylation by these enzymes is crucial for signaling events (4, 5).

The SH2-containing PTP-1 (SHP-1) is expressed mainly in hematopoietic cells. Studies of B cells from SHP-1-deficient (moteheaten (me/me) and motetheaten viable (me+/m+)) mice have shown that SHP-1 plays an important role in B cell differentiation, proliferation, and survival (6). Point mutations in the SHP-1 gene lead to aberrant splicing and no protein expression in the me/me mice and production of the protein at a normal amount (but with very low PTP activity) in the me+/m+ mice (7, 8). The mutant phenotypes are characterized by abnormalities in multiple hematopoietic lineages, especially in enhanced myeloid cell growth and function, causing inflammation and systemic autoimmune diseases (9).

SHP-1 has been shown to be involved in the regulation of several BCR-mediated signaling pathways. SHP-1 is recruited to phosphorylated immunoreceptor tyrosine-based inhibitory motifs (ITIMs) present in cytoplasmic regions of inhibitory receptors such as CD22, CD72, and paired Ig-like receptor (PIR-B) (10–15). Identification of substrates of SHP-1 is important for a better understanding of the molecular mechanisms involved in B cell signaling. We and others have demonstrated that CD72, Syk, and B cell linker protein are direct SHP-1 substrates, and dephosphorylation of these molecules by SHP-1 is critical in the regulation of lymphocyte activation (14, 16).

To date, detailed studies on B cell signaling using mutated DT40 cells have accumulated significant information on the functions of PTKs. In contrast to PTKs, SHP-1 has been much less characterized in DT40 cells in terms of its role in modulating BCR signaling and substrates after membrane IgM (mIgM) cross-linking. To identify in vivo substrates of SHP-1, we have expressed mutants of SHP-1 in SHP-1-deficient DT40 cells (17): a trapping mutant of SHP-1 (C/S) that should be involved in mediating inhibitory signaling (9–11), a nontrapping mutant of SHP-1 (R/M) that should be unable to bind substrates via its PTP domain (18, 19), a catalytically inactive form of SHP-1 that should retain substrate-binding ability; or a nontrapping mutant of SHP-1 (R/M) that should be unable to bind substrates via its PTP domain (18, 19). Tyrosine-phosphorylated sites, bound by the C/S mutant, should be protected from dephosphorylation by cellular PTPs. Therefore, substrates of SHP-1 should exhibit greater tyrosine phosphorylation in the presence of the C/S mutant than in the R/M mutant. Tyrosine phosphorylation was strongly enhanced in a ~44-kDa protein in SHP-1 C/S transfectants harbors. Therefore, after cross-linking of BCR, peptide mass mapping of this protein revealed that the protein is actin (cytoplasmic type 5), carrying three ITIM-like domains. The microfilament component of the cytoskeleton, composed primarily of actin, undergoes rapid and substantial alterations in conformation and arrangement in response to cell stimulation, and the BCR appears to be

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3 Abbreviations used in this paper: BCR, B cell Ag receptor; PTK, protein tyrosine kinase; IP, immunoprecipitate; ITIM, immunoreceptor tyrosine-based inhibitory motif; PIR-B, paired Ig-like receptor; mIgM, membrane IgM; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; PTP, protein tyrosine phosphatase; PY, phosphoryroside; SHP-1, Src homology 2-containing protein tyrosine phosphatase-1; PVDF, polyvinylidene difluoride; EGFP, enhanced green fluorescent protein; WT, wild-type.

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associated with the actin cytoskeleton following receptor cross-linking (20). Thus, the reorganization of the actin cytoskeleton may play a pivotal role in BCR signaling. In SHP-1 Wt transfectants, actin was polymerized after BCR stimulation and then rapidly depolymerized. In contrast, more cells exhibited actin polymerization induced by mlgM ligation, and actin polymerization was more prolonged in SHP-1 C/S transfectants. Therefore, actin dephosphorylation by SHP-1 is essential for actin depolymerization. We suggest that the rapid actin dephosphorylation by SHP-1 may play a significant role in a dynamic actin reorganization following BCR stimulation.

Recent reports demonstrate that BCR translocates into detergent-insoluble lipid rafts consisting of enriched sphingolipids and cholesterol upon receptor cross-linking (21). Translocation of BCR into lipid rafts is a critical mechanism in signaling. Our results suggest that BCR cross-linking recruits more SHP-1 molecules bound to actin in the lipid rafts. These results for the first time provide direct evidence linking SHP-1 to the actin cytoskeleton.

Materials and Methods

Cell lines, cell culture, and stable transfection

Avian leukaosis virus-transformed chicken B cell lines, DT40, and its mutant were cultured in RPMI 1640 with 10% FCS, 5 x 10^{-5} M 2-ME, and penicillin/streptomycin at 37°C in a humidified atmosphere. SHP-1-deficient DT40 was provided by T. Kurosaki (Kansai Medical School, Osaka, Japan). SHP-1 was inserted into the RIKEN Research Center for Allergy and Immunology (Yokohama, Japan). Transfection of DT40 was conducted using electroporation at 250 V and 975 μF in phosphate-buffered saline with 15 μg of expression constructs (wt human SHP-1; wt, Cys530 to Ser mutated human SHP-1; C/S, or Arg151 to Met mutated human SHP-1; R/M). These mutants and wt cDNA were obtained from B. Neel (Cancer Biology Program, Division of Hematology-Oncology, University of Medicine, Bethesda Research Center for Allergy and Immunology, Yokohama, Japan). Transfection from C/S transfectants (1 x 10^7) were suspended in PBS and treated with M1 mAb at 40°C for 2 min. Lysates from these cells were mixed with an equal volume of 80% sucrose in lysis buffer containing 25 mM Tris-HCl (pH 7.7), 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 1 mM sodium orthovanadate, and 1 μg/ml aprotinin. The resulting solution was loaded onto a 10-30% sucrose density gradient (6.5 ml of 30% sucrose, then 3.5 ml of 5% sucrose) and centrifuged at 39,000 rpm for 16 h. Fractions of 1 ml were collected from the top to the bottom of the gradient. Proteins were separated by 9% SDS-PAGE and transferred to PVDF membrane. To confirm the location of lipid rafts on the gradients, the blots were probed with anti-Lyn Ab, because Lyn was known to reside in the lipid rafts, and were reprobed with anti-SHP-1 Ab and anti-actin mAb.

Transient enhanced green fluorescent protein (EGFP)-actin expression and fluorescence microscopy

Plasmid containing the EGFP-actin fusion construct (pEGFP-actin; Clontech Laboratories, Palo Alto, CA) was transfected with electroporation at 250 V and 975 μF into DT40 expressing SHP-1 Wt or C/S. After 24 h, cells were suspended in PBS and treated with M1 mAb at 40°C for varying periods of time, and then expression of the fusion construct was evaluated by fluorescence microscopy using a FITC filter.

Results

p44 is hyperphosphorylated in C/S transfectants after BCR stimulation

To identify a new substrate of SHP-1 in DT40 cells we used a catalytically inactive form of SHP-1 (trapping mutant; C/S). As has been shown in several studies, including our own, such catalytically inactive (but not Wt) PTP can form stable complexes with substrates, the phenomenon termed as substrate trapping (14, 18, 19). A substrate of SHP-1 may be specifically trapped by the C/S mutant of SHP-1. To test this, we transfected the expression vectors (SHP-1 wt, C/S, or R/M) into SHP-1-deficient DT40 cells. Then, these transfectants were treated with, or without, the anti-chicken IgM mAb. The expression levels of mlGm in these transfectants were similar (data not shown). The lysates were immunoprecipitated with anti-SHP-1 Ab, and the precipitates were analyzed by anti-PY immunoblotting using 4G10 mAb. Two tyrosine-phosphorylated proteins (~44 and 230 kDa) were identified in C/S transfectants after cross-linking of mlGm. In particular, the degree of phosphorylation of p44 was markedly increased in C/S transfectants. In contrast, the phosphorylation levels of the bands in Wt and R/M transfectants were marginal. Reprobing of the blot for SHP-1 revealed that the expression levels of SHP-1 in Wt, C/S, and R/M transfectants were very similar. In this paper, we have concentrated on the analysis of p44. Tyrosine phosphorylation of
and this phosphorylation site was dephosphorylated by SHP-1 Wt. Thus, we speculate that this protein is a direct substrate of SHP-1.

Identification of p44

p44 was partially purified as described in Materials and Methods. The band representing p44 was excised from a Ponceau S-stained Problot membrane (Applied Biosystems, Foster City, CA). An in-membrane digest was performed using Lys-C, and the resulting peptide mixture was analyzed using MALDI-TOF mass spectrometry. Twenty-three peptide masses were determined and used to search the database. According to these data, the purified protein represented actin (cytoplasmic type 5; Table I). Actin accounts for 5–10% of total cellular protein (24). Therefore, we conducted experiments to confirm in all transfectants that the 44-kDa phosphoprotein was actin. Lysates from SHP-1-/- cells and all transfectants (Wt, C/S, and R/M) treated with or without M1 mAb were immunoprecipitated with anti-actin mAb, and the precipitates were investigated by anti-PY or anti-SHP-1 Ab blot experiments. As well, these lysates were immunoprecipitated with anti-SHP-1 Ab, and the precipitates were examined by anti-actin mAb blot. The results demonstrated that actin was tyrosine phosphorylated after cross-linking of mIgM in C/S transfectants (Fig. 2A). Furthermore, the results shown in Fig. 2, B and C, indicate that SHP-1 is associated with actin upon BCR ligation in C/S transfectants. Association of actin with SHP-1 induced by mIgM ligation was considerably weak in Wt and R/M transfectants compared with C/S transfectants (Fig. 2, B and C). These results suggest that association of actin with SHP-1 is specific. Thus, we concluded that the 44-kDa phosphoprotein is actin.

SHP-1 binds to a phosphorylated ITIM-like domain of actin

We found three consensus amino acid sequences of the ITIM (I/V/LXpYXXL/V) in actin. To identify a binding site for SHP-1 on actin, we used the tyrosine-phosphorylated synthetic peptides of the three ITIM-like sequences (peptides 1, 2, and 3; Fig. 3A). These peptides were biotinylated and coupled to avidin-coated agarose beads. Each peptide was incubated with C/S transfectant cell lysates; then precipitates from cell lysates were analyzed by Western blotting with anti-SHP-1 Ab. Peptide 2 was shown to bind to SHP-1 at 5 nmol and exhibited greater precipitates with the increased amount of the peptide (Fig. 3B). In contrast, SHP-1 was barely detectable with 100 nmol of peptide 1. No binding of SHP-1 was found with 100 nmol of peptide 3 (Fig. 3B). These results indicated that tyrosine-phosphorylated peptide 2 was able to bind to a SH2 domain or the PTP domain of SHP-1. We mutated Tyr219

### Table I. Molecular mass analysis of Lys-C peptides

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<tr>
<th>Observed Mass (Da)</th>
<th>Theoretical Mass (Da)</th>
<th>Δ Mass (Da)</th>
<th>Sequences</th>
<th>Positions</th>
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<tr>
<td>800.54</td>
<td>800.53</td>
<td>0.01</td>
<td>RGILTLK</td>
<td>63–69</td>
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<tr>
<td>923.59</td>
<td>923.57</td>
<td>0.02</td>
<td>ITAPPERK</td>
<td>330–337</td>
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<tr>
<td>1161.61</td>
<td>1161.62</td>
<td>0.01</td>
<td>EITALAPSTMK</td>
<td>317–327</td>
</tr>
<tr>
<td>1177.68</td>
<td>1177.62</td>
<td>0.06</td>
<td>EITALAPSTMK*K</td>
<td>317–327</td>
</tr>
<tr>
<td>1644.81</td>
<td>1644.80</td>
<td>0.01</td>
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<td>361–374</td>
</tr>
<tr>
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<tr>
<td>2602.27</td>
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<td>293–316</td>
</tr>
<tr>
<td>2618.32</td>
<td>2618.26</td>
<td>0.06</td>
<td>DLYANTVLSGGTM*YPGIADRKM**QK</td>
<td>293–316</td>
</tr>
<tr>
<td>3308.73</td>
<td>3308.69</td>
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<td>3324.68</td>
<td>3324.69</td>
<td>0.01</td>
<td>AGFAGDAPRAVFSSIVGRPRHCGVM*VGM**QK</td>
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<tr>
<td>3340.72</td>
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<td>0.09</td>
<td>IWHHETYNELRVAPEEHPVLITTEAPLNPK</td>
<td>86–114</td>
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</table>

*p44 was partially purified, and the band representing p44 was excised from a Ponceau-stained Problot membrane. An in-membrane digest was performed using Lys-C, and the resulting peptide mixture was analyzed using MALDI-TOF MS. M indicates oxidized methionine. M** indicates that one of the two residues in the sequence is oxidized.
of the second ITIM of actin to phenylalanine to prove that SHP-1 binds to the ITIM sequence of native actin protein. Tyrosine phosphorylation of the Y/F EGFP-actin mutant diminished considerably compared with that of EGFP-actin after mIgM stimulation (Fig. 3C). Furthermore, this mutant did not associate with SHP-1 after cross-linking of BCR. These results strongly suggest that the second ITIM of actin is phosphorylated, and then this phosphorylated ITIM is essential for association with SHP-1 after mIgM stimulation. Because all experiments described above were performed in the transfectants overexpressing SHP-1 or SHP-1 mutants, we confirmed association of SHP-1 with actin in physiological conditions using untransfected DT40 cells (Fig. 3D). The results demonstrated that SHP-1 is associated with actin upon BCR ligation in untransfected DT40 cells as well.

**SHP-1 translocates into lipid rafts after cross-linking of BCR**

Lipid rafts are enriched in sphingolipids and cholesterol. This domain was proposed to function as platforms for signal transduction and actin cytoskeletal reorganization (25–27). We investigated whether SHP-1 binding to actin after cross-linking of BCR translocates into lipid rafts. We used sucrose gradient ultracentrifugation to identify detergent-insoluble raft proteins in DT40 cells, and then proteins were separated by SDS-PAGE and transferred to
PVDF membrane. The blots were probed with anti-Lyn Ab, which was a standard marker of the lipid rafts fraction (23), and then reprobed with anti-SHP-1 Ab and anti-actin mAb. Lyn and actin are tightly anchored into lipid rafts regardless of BCR stimulation. Lyn was exclusively detected in fraction 4 (Fig. 4A). More SHP-1 molecules translocated into the lipid rafts after cross-linking of BCR in C/S transfectants than in R/M transfectants. Raft proteins in fraction 4 were immunoprecipitated with anti-SHP-1 Ab, and then the precipitates were analyzed by anti-actin immunoblotting. From this result, recruitment of SHP-1 to lipid rafts and its association with actin were demonstrated to be increased after cross-linking of BCR (Fig. 4B). Moreover, actin in lipid rafts was phosphorylated after BCR stimulation (Fig. 4C). The results suggest that SHP-1 bound to phosphorylated actin translocated into the lipid rafts may play a role in B cell signaling and actin cytoskeletal reorganization.

**FIGURE 4.** SHP-1 translocates into lipid rafts after cross-linking of BCR. A, C/S or R/M transfectants were stimulated with M1 mAb for 2 min (μ) or unstimulated (−). Detergent-insoluble raft proteins purified by sucrose gradient ultracentrifugation were separated by SDS-PAGE and transferred to PVDF membrane. The blots were probed with anti-(α)-Lyn Ab and then reprobed with anti-SHP-1 Ab and anti-actin mAb. B, Detergent-insoluble raft proteins (fraction 4) were immunoprecipitated with anti-SHP-1 Ab. SHP-1 IPs were analyzed by anti-actin mAb blot. The blots were then reprobed with anti-SHP-1 Ab. Saturated amount of anti-SHP-1 Ab was used to obtain the similar quantities of SHP-1 IPs from unstimulated and stimulated cells. Thus, the difference in the actin signal bound to SHP-1 is evident. H, Heavy chain of precipitated Ab.

**FIGURE 5.** Visualization of polymerized actin by transfection of EGFP-actin. A, DT40 SHP-1 Wt and C/S cells transfected with plasmids containing the EGFP-actin fusion construct either were left untreated (1–3) or were stimulated with M1 mAb for 5 h. The EGFP-actin expression was observed by fluorescence microscopy. Arrow, Polymerized actin. B, Actin polymerization in EGFP-actin-expressed SHP-1 Wt and C/S transfectants. Values are the number of cells with polymerized actin per 1 × 10⁵ EGFP-positive cells.

Actin dephosphorylation is essential for actin depolymerization

To investigate the relationship between phosphorylation status of actin and polymerization of actin, we transfected a plasmid containing the EGFP-actin fusion construct into DT40 SHP-1 Wt and C/S transfectants. A greater extent of actin polymerization after mlgM ligation was demonstrated in C/S transfectants than in Wt transfectants. In SHP-1 Wt transfectants, polymerized actin was rapidly depolymerized, and the number of cells exhibiting actin polymerization returned to the original level within 5 h. In contrast, polymerization of actin was sustained without a significant sign of depolymerization even after 5 h in SHP-1 C/S transfectants (Fig. 5), suggesting that dephosphorylation of actin by SHP-1 is
essential for actin depolymerization. The EGFP-actin was phosphorylated after BCR cross-linking in C/S transfectants (data not shown).

**Discussion**

SHP-1 has been shown to be involved in the regulation of BCR mediated signaling events. SHP-1 is recruited to CD22 and CD72, which have ITIMs in these cytoplasmic regions and dephosphorylate signaling intermediaries, thereby regulating their function (10–12, 14, 15).

The physiological targets of SHP-1 in the DT40 B cell line have remained undefined. Thus, to identify the substrates of SHP-1 in the B cells, we used the trapping mutant; SHP-1 C/S and the non-trapping mutant, SHP-1 R/M. In C/S transfectants, a 44-kDa protein was hyperphosphorylated compared with Wt and R/M transfectants after BCR stimulation. This protein turned out to be actin. Tyrosine phosphorylation of actin in C/S transfectants induced by BCR ligation did not diminish with time. In contrast, tyrosine phosphorylation of actin was reduced with time after BCR ligation in Wt transfectants. These results indicate that increased tyrosine phosphorylation of actin is due to protection by the PTP of SHP-1. The fact that a prolonged and stable interaction was observed in inactive C/S transfectants further supports the notion that SHP-1 activity negatively regulates actin tyrosine phosphorylation. The results suggest that tyrosine phosphorylation of actin is directly controlled by SHP-1 activity. Furthermore, tyrosine-phosphorylated synthetic peptide 2 was bound to SHP-1. The sequences of peptides 1 and 3 were ITIM-like, but the sequence of peptide 2 was the authentic sequence of ITIM. Thus, we speculate that this is why only peptide 2 is bound to SHP-1. These results suggest that actin is a direct substrate of SHP-1. Actin is one of the most abundant proteins in the cell. Therefore, we ruled out the possibility of nonspecific detection of the actin/SHP-1 complex (Fig. 2, B and C). Experiments using synthetic peptides described above (Fig. 3) also suggest the association is specific.

In T cells, the remodeling of actin cytoskeleton is an essential event for their activation (28, 29). TCR signaling and reorganization of the actin cytoskeleton are linked by the intracellular molecules such as Vav, SLP-76, and the Rho family GTPases (28). These molecules regulate the remodeling of the actin cytoskeleton. Also, cross-linking of BCR increases the filamentous actin content in B cells (20). Thus, the reorganization of the actin cytoskeleton is crucial for AgR signaling cascades in lymphocytes. However, the requirement for tyrosine phosphorylation in actin reorganization is not known.

To investigate the relationship between phosphorylation status of actin and polymerization of actin, we transfected a plasmid containing the EGFP-actin fusion construct into DT40 SHP-1 Wt and C/S transfectants. In SHP-1 Wt transfectants, polymerized actin should be rapidly depolymerized. In contrast, polymerization of actin was sustained at least for 5 h in SHP-1 C/S transfectants. These results indicate that actin dephosphorylation by SHP-1 in lipid rafts is essential for actin depolymerization. We suggested that actin phosphorylation induces actin polymerization; furthermore, actin dephosphorylation by SHP-1 results in actin depolymerization, and then cells return to the original state before BCR stimulation. Thus, phosphorylation/dephosphorylation of actin may be crucial for cycling of actin reorganization, and even for signaling. We have constructed several actin molecules with the tyrosine residues mutated to phenylalanine. Currently, we are working on a series of experiments using these mutants to elucidate the potential physiological relevance of phosphorylation/dephosphorylation and polymerization/depolymerization of actin. These results will be published elsewhere.

Plasma membranes of many cell types, including B cells, contain microdomains commonly referred to as lipid rafts. These domains are enriched in sphingolipids and cholesterol. Accumulating data support a role for lipid rafts as platforms for signal transduction involved in receptor-induced recruitment and activation of PTKs (25). A role for lipid rafts in actin reorganization has been, in addition, supported by their function in membrane trafficking (30, 31). BCR activation leads to the induction of PTKs, mitogen-activated protein kinases, and Ca2+ signals, as well as actin polymerization and cytoskeletal reorganization (32). Lipid rafts are important in inducing BCR-mediated signal transduction and cytoskeletal changes. Recent reports suggest that SHP-1 may be associated with LAT in lipid rafts in T cells, and the results argue that SHP-1 is involved in the regulation of TCR signaling in rafts (33). Another paper suggests that LAT is required for TCR-induced spreading and actin rearrangement (34). We demonstrate that SHP-1 translocates into the lipid rafts also in B cells and that more SHP-1 molecules are bound to actin in the lipid rafts after cross-linking of BCR. These results suggest that SHP-1 is recruited to phosphorylated actin and that SHP-1 translocates into the lipid rafts. A report describes a relationship between SHP-1 and actin polymerization (35). This paper suggests that the ITIM sequence of ILT2, an inhibitory receptor, binds to SHP-1 and negatively affects cytoskeletal changes triggered by TCR. They speculate that the mechanism by which ILT2 inhibits TCR-triggered actin polymerization is most likely based on dephosphorylation and subsequent lack of recruitment/activation of proteins involved in this event.

We suggest that tyrosine phosphorylation of actin and its dephosphorylation by SHP-1 in lipid rafts may play a key role in a dynamic actin reorganization. These results demonstrate for the first time the direct interaction of SHP-1 with actin.

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**References**


