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Biochemical and Biophysical Research Communications 304 (2003) 67–72
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Myosin is an in vivo substrate of the protein tyrosine phosphatase (SHP-1) after mIgM cross-linking☆

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Received 17 March 2003

Abstract

SHP-1 plays an important role in negative signaling in many cell types. For example, after BCR stimulation in apoptotic B cells, SHP-1 has been shown to be recruited to phosphorylated ITIMs present in receptors such as CD72. However, the SHP-1 substrates in the chicken B cell line, DT40, have been poorly undefined. To identify SHP-1 substrates in DT40, we used a trapping mutant SHP-1 C/S (a catalytically inactive form). BCR stimulation induced hyper-phosphorylation of 230 kDa protein in C/S transfectants. MALDI-TOF/MS analysis revealed that this was myosin carrying ITIM. SHP-1 was shown to bind to this ITIM in synthetic peptide binding experiment. Thus, myosin is a direct SHP-1 substrate in B cells. The results suggest that SHP-1 plays a critical role in the reorganization of cytoskeletal architecture mediated via BCR stimulation.

Keywords: B lymphocyte; SH2 domain containing phosphatase-1; Tyrosine phosphatase; Myosin; Signal transduction

Stimulation of the B cell antigen receptor (BCR) leads to B lymphocyte survival, proliferation, apoptosis, clonal non-responsiveness (anergy), or differentiation into memory B cells or plasma cells, balancing immunity, and tolerance [1]. Upon BCR cross-linking, several protein tyrosine kinases (PTKs) become activated and cause the signaling cascade [2–4]. Meanwhile protein tyrosine phosphatases (PTPs) are supposed to counteract these signaling pathways activated by PTKs. Thus, the balance of tyrosine phosphorylation and dephosphorylation by these enzymes is crucial for signaling events in the lymphocytes [5,6].

The Src-homology 2 (SH2)-containing PTP-1 (SHP-1) is expressed mainly in hematopoietic cells. Several reports including our own have demonstrated that SHP-1 plays a pivotal role in B cell growth, survival, and apoptosis [5,7,8]. One of these studies involved the analysis of B cells from motheaten (*me*/*me*) and viable motheaten (me^v/me^v) mice, animals in which the expression of no SHP-1 or a catalytically inactive form of SHP-1 protein is exhibited [9–11].

SHP-1 is involved in the negative regulation of several receptors such as CD72, CD22, and paired immunoglobulin-like receptor (PIR-B) containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic regions [12–17]. These molecules recruit SHP-1 upon phosphorylation. 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 BLNK are direct SHP-1 substrates, and dephosphorylation of these molecules by SHP-1 is critical in the regulation of lymphocyte activation [16,18].

SHP-1 has been much less characterized in DT40 cell line (a chicken B lymphoma line) in terms of its role in

☆ *Abbreviations:* BCR, B cell antigen receptor; IP, immunoprecipitate; ITIM, immunoreceptor tyrosine-based inhibitory motif; MALDI-TOF/MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PTP, protein tyrosine phosphatase; PY, phosphotyrosine; SHP-1, Src-homology 2-containing protein tyrosine phosphatase-1.

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0006-291X/03/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0006-291X(03)00542-4

modulating BCR signaling and substrates after mIgM cross-linking in contrast to PTKs. To identify in vivo substrates of SHP-1, we have utilized mutants of SHP-1 in SHP-1-deficient DT40 cells [19]; a trapping mutant of SHP-1 (C/S: a catalytically inactive form of SHP-1)—should retain the substrate-binding ability, or a non-trapping mutant of SHP-1 (R/M)—should be unable to bind substrates via its PTP domain [20,21]. The previous reports have proven that these mutants are extremely versatile for the identification of in vivo SHP-1 substrates [16,22]. Phosphorylated tyrosine residues, bound by the C/S mutant, are protected from dephosphorylation by cellular PTPs, exhibiting a greater tyrosine phosphorylation in the presence of the C/S mutant than in the R/M mutant.

We have found that tyrosine phosphorylation was strongly enhanced in a protein of about 230 kDa in SHP-1 C/S transfectants after the cross-linking of BCR. Peptide mass mapping of this molecule revealed that it is myosin (myosin heavy chain) carrying one ITIM domain.

Myosin is a particularly attractive candidate to participate in the spatial reorganization of cytoskeletal architecture during signal transduction, since the molecule is responsible for the motor activity of actin filaments. We showed that myosin heavy chain undergoes tyrosine phosphorylation and dephosphorylation by SHP-1 after BCR stimulation in DT40 cell. The results implicate that phosphorylation and dephosphorylation of myosin may play a role in cytoskeletal reorganization and signal transduction after BCR stimulation.

Materials and methods

Cell lines, cell culture, and stable transfection. Avian leukosis virus-transformed chicken B cell line, DT40, and its mutant were cultured in RPMI 1640 with 10% FCS, 5×10^{-5} M of 2-ME, and penicillin/streptomycin at 37 °C in a humidified atmosphere. SHP-1-deficient DT40 was provided by T. Kurosaki (Kansai Medical University, Osaka, and RIKEN Research Center for Allergy and Immunology, Yokohama, Japan). Transfection of DT40 was carried out using electroporation at 250 V and 975 μF in phosphate-buffered saline with 15 μg of expression constructs (*wt* human *SHP-1*; *wt*, Cys^{453} to Ser mutated human *SHP-1*; *C/S*, or Arg^{419} to Met mutated human *SHP-1*; *R/M*). These mutants and *wt* were obtained from B. Neel (Cancer Biology Program, Division of Hematology-Oncology, Department of Medicine, Beth Israel Deaconess Medical Center, Boston, MA) [16]. The expression vector pMIKHygB was obtained from K. Maruyama (Tokyo Medical and Dental University, Tokyo, Japan). Transfectants were selected in 2.0 mg/ml hygromycin B for 24 h after electroporation. These clones were selected based on matched surface IgM expression levels.

Antibodies. The anti-chicken IgM mAb, M1 was provided by C.-L. H. Chen (University of Alabama, Birmingham, AL). Polyclonal Abs against SHP-1 and anti-phosphotyrosine (PY) mAb (4G10) were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-myosin mAb was purchased from Progen Biotechnik GmbH (Heidelberg, Germany). Horseradish peroxidase (HRP)-labeled Protein A was purchased from Amersham Biosciences (Piscataway, NJ).

Cell stimulation and lysis. Cells (1×10^7) were suspended in PBS and treated with M1 mAb at 40 °C. Activation was stopped by addition of five volumes of ice-cold PBS. Cells in buffer containing 10 mM Tris–HCl (pH 7.7), 150 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate, and 1 μg/ml aprotinin were kept at 4 °C for 1 h with constant rotation. Lysates were centrifuged for 20 min at 4 °C to remove insoluble materials.

Immunoprecipitation and immunoblot analysis. Immunoprecipitation and immunoblot experiments were carried out as already described [17]. Briefly, lysates were immunoprecipitated with the appropriate Abs. The immunoprecipitates (IPs) were washed four times with ice-cold lysis buffer and boiled in SDS sample buffer (containing 2-ME for reducing condition unless otherwise stated) for 5 min. Proteins were separated by 9% SDS–polyacrylamide gel electrophoresis and transferred to PVDF membrane. The membranes were incubated with the appropriate Abs. The blots were visualized by incubating with HRP-labeled protein A and then developed by Enhanced Chemiluminescence (ECL) (Amersham Biosciences).

Identification of proteins by peptide mass mapping. Lysates from C/S transfectants (1×10^9) were immunoprecipitated with 4G10 mAb and protein G–Sepharose. Immune complex was washed with lysis buffer and then with PBS, and eluted by the addition of *p*-nitrophenylphosphate (final concentration, 100 mM). Then, the eluate was immunoprecipitated with anti-SHP-1 Ab. IPs were separated by SDS–PAGE and transferred to the Problot membrane (Applied Biosystems, Foster City, CA); then this membrane was stained with PONCEAU-S. The PVDF-immobilized proteins were reduced, S-carboxymethylated, and digested in situ with *Achromobacter* protease I (a Lys-C) [23]. Molecular mass analyses of Lys-C fragments were performed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry using a PerSeptive Biosystem Voyager-DE/RP. Identification of proteins was carried out by comparison between the molecular weights determined by MALDI-TOF/MS and theoretical peptide masses from the proteins registered in NCBInr (9.19.2001).

ITIM peptide and in vitro binding of SHP-1. Biotinylated and tyrosine-phosphorylated myosin ITIM peptide with the amino acid sequence TFHIFYYLLSG (Peptide 1) was synthesized by SIGMA genosys. The biotinylated ITIM peptide was coupled to avidin-coated agarose beads. These biotinylated peptides were incubated with C/S transfectant cell lysates and then bound to avidin–agarose beads (Amersham Biosciences). Bound proteins were separated by 9% SDS–PAGE and transferred to PVDF membrane. SHP-1 protein was identified by Western blotting with anti-SHP-1 Ab.

Results

p230 is hyper-phosphorylated 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 it has been shown in several studies, including our own, such catalytically inactive (but not wild-type: Wt) PTP can form stable complexes with substrates, the phenomenon termed as "substrate trapping" [16,20,21]. A substrate of SHP-1 may be specifically "trapped" by the C/S mutant of SHP-1. 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, M1. The expression levels of mIgM in these transfectants were very 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 (about 44 and 230 kDa) were markedly increased in C/S transfectants after BCR stimulation. In contrast, the phosphorylation levels of the bands in Wt and R/M transfectants were not significant (Fig. 1A). 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. The 44-kDa protein was identified with actin. The results will be published elsewhere [22]. We have concentrated on the analysis of p230 in this paper. Tyrosine phosphorylation of p230 in C/S transfectants induced by mIgM ligation did not diminish even after 5 min (Fig. 1B). The phosphorylation level was sustained for 30 min (data not shown). In contrast, tyrosine phosphorylation of p230 in SHP-1 Wt transfectants was reduced with time after cross-linking of mIgM (Fig. 1B). These results suggest that the tyrosine phosphorylation site of the p230 bound by the C/S mutant should be protected from dephosphorylation by the PTP of SHP-1, and that this phosphorylation site was dephosphorylated by SHP-1 Wt. Thus, we speculate that this protein is a direct substrate of SHP-1.

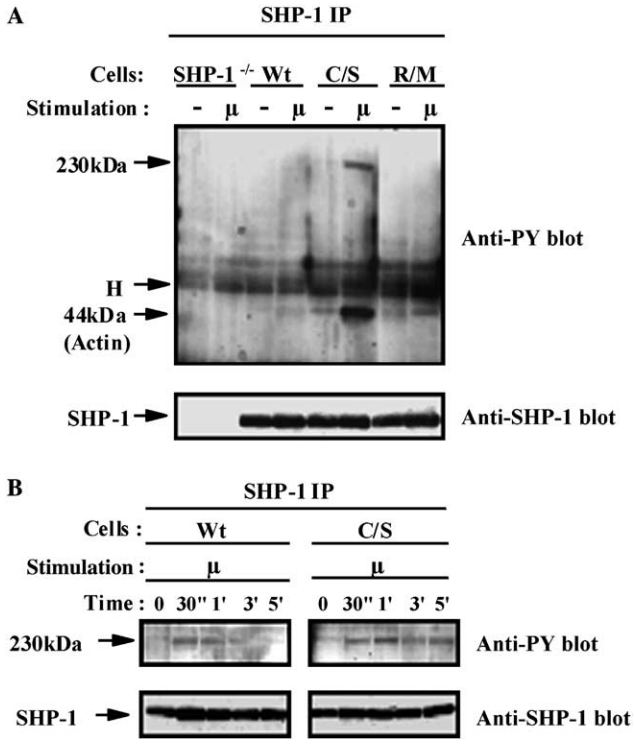

Fig. 1. p230 is an in vivo substrate of SHP-1. (A) SHP-1-deficient DT40 cells (SHP-$1^{-/-}$) were transfected with the vectors (vector alone; SHP-$1^{-/-}$, SHP-1 *wt*, and the indicated mutant forms of SHP-1 molecules). Then, the transfectants were stimulated with M1 mAb for 2 min (μ) or unstimulated (−). Lysates were prepared and immunoprecipitated with anti-SHP-1 Ab. IPs were analyzed by anti-PY blot. The blot was then reprobed with anti-SHP-1 Ab. H, Heavy chain of precipitated antibody. (B) SHP-1 Wt and C/S transfectants were stimulated with M1 mAb for varying periods of time as indicated before cell lysis. Lysates were prepared and immunoprecipitated with anti-SHP-1 Ab. IPs were analyzed by anti-PY blot. The blot was then reprobed with anti-SHP-1 Ab.

Identification of p230

p230 was partially purified as described in Materials and methods. The band representing p230 was excised from a PONCEAU-S-stained Problot membrane. An in-membrane digest was performed using Lys-C and the resulting peptide mixture was analyzed using MALDI-

Table 1
Molecular mass analysis of Lys-C peptides

Observed mass	Theoretical mass	Δ mass	Sequences	Positions
908.50	908.49	0.01	FVSELWK	607–613
1205.65	1205.64	0.01	TDLLLEPYNK	290–299
1345.73	1345.73	0.00	LRLEVNQQAM*K	1556–1566
1452.84	1452.80	0.04	VIQYLAHVASSHK	187–199
1510.81	1510.74	0.07	DLESLTQRYEEK	1393–1405
1597.93	1597.90	0.03	QISVLQQQAVEARK	1358–1371
1668.90	1668.87	0.03	NIINNPLTQADWAAK	15–29
1706.96	1706.91	0.05	ALELDSNLYRIGQSK	746–760
1960.04	1960.00	0.04	YRFLSNGHVTIPGQQDK	300–316
2141.90	2142.10	0.20	KEEELQAALARVEEEAAQK	1081–1099
2344.16	2344.13	0.03	ANLQIDQMNADLNAERSNAQK	1755–1775
2360.17	2360.13	0.04	ANLQIDQM*NADLNAERSNAQK	1755–1775
2505.27	2505.27	0.00	DVDRIVGLDQVAGM*SETALPGAFK	614–637
2554.35	2554.26	0.09	DYMRELEDTRTSREEILAQAK	1649–1669
2570.22	2570.26	0.04	DYM*RELEDTRTSREEILAQAK	1649–1669
2873.40	2873.46	0.06	IRELESQITELQEDLESERASRNK	1106–1129

p230 was partially purified and the band representing p230 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 M/S. M* indicates oxdized methionine.

TOF mass spectrometry. Thirty-eight peptide masses were determined and used to search the database. According to these data, the purified protein represented myosin (myosin heavy chain) (Table 1). Then, we confirmed by immunoprecipitation and Western blot analyses that the phosphoprotein of 230 kDa was myosin. Lysates from SHP-$1^{-/-}$ cells and all transfectants (Wt, C/S, and R/M) treated with, or without, M1 mAb were immunoprecipitated with anti-myosin 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-myosin mAb blot. The results clearly demonstrated that myosin was tyrosine-phosphorylated after the cross-linking of mIgM in C/S transfectants (Fig. 2A). Furthermore, the results shown in Fig. 2B and C indicate that SHP-1 is associated with

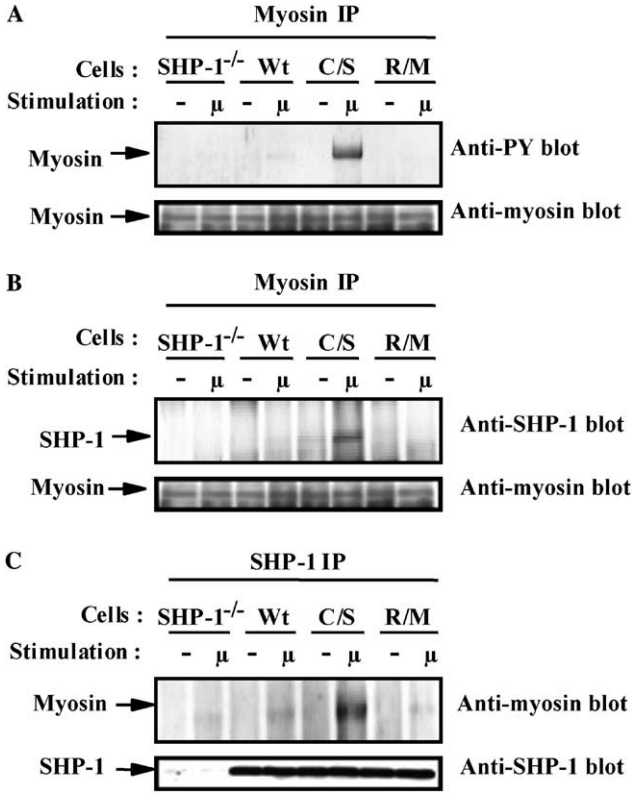

Fig. 2. Identification of p230 as myosin. (A) SHP-$1^{-/-}$ cells and all transfectants (Wt, C/S, and R/M) were stimulated with M1 mAb for 2 min (μ), or unstimulated (−). Lysates were prepared and immunoprecipitated with anti-myosin mAb. IPs were analyzed by anti-PY Ab blot. The blots were then reprobed with anti-myosin mAb. (B) SHP-$1^{-/-}$ cells and all transfectants (Wt, C/S, and R/M) were stimulated with M1 mAb for 2 min (μ) or unstimulated (−). Lysates were prepared and immunoprecipitated with anti-SHP-1 Ab. SHP-1 IPs were analyzed by anti-myosin mAb blot. The blots were then reprobed with anti-SHP-1 Ab. (C) SHP-$1^{-/-}$ cells and all transfectants (Wt, C/S, and R/M) were stimulated with M1 mAb for 2 min (μ), or unstimulated (−). Lysates were prepared and immunoprecipitated with anti-myosin mAb. Myosin IPs were analyzed by anti-SHP-1 Ab blot. The blots were then reprobed with anti-myosin mAb.

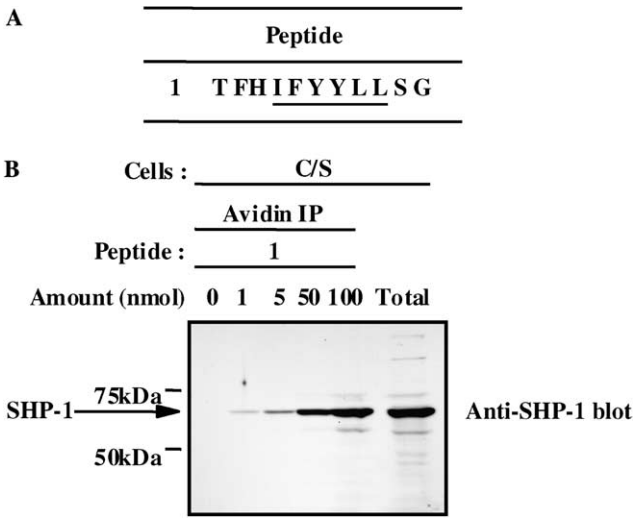

Fig. 3. SHP-1 binds to a phosphorylated ITIM domain of myosin in vitro. (A) The sequence of the biotinylated synthetic tyrosine phosphopeptide (Peptide 1) is presented. The ITIM sequence is underlined. (B) Biotinylated and tyrosine-phosphorylated myosin ITIM peptide was incubated with C/S transfectant cell lysates. Avidin IPs were subjected to Western blotting to identify SHP-1 with anti-SHP-1 Ab.

myosin upon BCR ligation in C/S transfectants. Association of myosin with SHP-1 induced by mIgM ligation was considerably weak in Wt and R/M transfectants compared with C/S transfectants (Figs. 2B and C). These results suggest that association of myosin with SHP-1 is specific. Thus, we concluded that the phosphoprotein of 230 kDa is myosin.

SHP-1 binds to a phosphorylated ITIM domain of myosin in vitro

We found one consensus amino acid sequence of the ITIM (I/V/LXpYXXL/V) in myosin. To identify a binding site for SHP-1 on myosin, we used the tyrosine-phosphorylated synthetic peptide of the ITIM sequence (Peptide 1) (Fig. 3A). This peptide was biotinylated and coupled to avidin-coated agarose beads. The peptide was incubated with C/S transfectant cell lysates and then precipitates from cell lysates were analyzed by Western blotting with anti-SHP-1 Ab. This peptide was clearly shown to bind to SHP-1 at 1 nmol and exhibited greater precipitates with the increased amount of the peptide (Fig. 3B). These results indicated that tyrosine-phosphorylated Peptide 1 was able to bind to a SH2 domain or the PTP domain of SHP-1.

Discussion

SHP-1 has been implicated as a negative regulator of BCR signaling in B cells. Upon BCR ligation, SHP-1 is recruited to CD22 and CD72, which have ITIMs in these cytoplasmic portions, and might efficiently

dephosphorylate signaling intermediaries, thereby regulating their function [12–14,16,17].

The physiological targets of SHP-1 in the DT40 B cell line have remained undefined. Thus, to identify the substrates of SHP-1 in this cell line, we used the trapping mutant; SHP-1 C/S and the non-trapping mutant; SHP-1 R/M. In C/S transfectants, a 230-kDa protein was hyper-phosphorylated compared with Wt and R/M transfectants after BCR stimulation. This protein turned out to be myosin heavy chain. In C/S transfectants, tyrosine phosphorylation of myosin induced by BCR stimulation did not diminish with time. In contrast, tyrosine phosphorylation of myosin was reduced with time after BCR ligation in Wt transfectants. These results indicate that tyrosine phosphorylation of myosin is protected by the PTP of SHP-1. The results suggest that tyrosine phosphorylation of myosin is directly controlled by SHP-1 activity. Furthermore, tyrosine-phosphorylated synthetic Peptide 1 was bound to SHP-1 (Fig. 3). The sequence of Peptide 1 is the authentic sequence of "ITIM." This could be the reason why Peptide 1 is bound to SHP-1. These results suggest that myosin is a direct substrate of SHP-1. Myosin is one of the most abundant proteins in the cell. Therefore, we carefully ruled out the possibility of non-specific detection of the myosin/SHP-1 complex (Figs. 2B and C). Experiments using synthetic peptides described above also suggest the association is specific (Fig. 3).

Goel and Dey [24] have demonstrated an increase in the tyrosine phosphorylation of myosin heavy chain during skeletal muscle differentiation in the presence of insulin. This study has shown that phosphorylation of myosin heavy chain at tyrosine residues is dependent upon the degree of differentiation. However, little is known about whether myosin heavy chain is phosphorylated at tyrosine residues in lymphocytes.

Myosin belongs to a large family of motor proteins. Myosin binds to and moves along actin filaments consuming energy supplied by ATP hydrolysis [25]. Thus, actin and myosin interaction is critical for dynamic changes in the organization of cytoskeletal architecture.

In a separate report, p44 has been shown to be actin (Fig. 1A) [22]. We have demonstrated that the rapid actin dephosphorylation by SHP-1 in lipid rafts may play a significant role in a dynamic actin reorganization following BCR stimulation. Our results clearly demonstrate, for the first time, that the cytoskeletal molecules such as myosin and actin are phosphorylated at ITIM tyrosine residue and dephosphorylated by SHP-1 after BCR stimulation. Dephosphorylation of actin/myosin by SHP-1 may play a critical role in the mechanism(s) involved in signal transduction and cytoskeletal reorganization after BCR stimulation.

Acknowledgments

We thank Dr. T. Kurosaki, Dr. C.-L.H. Chen, and Dr. B. Neel for the reagents described in this paper. This work was supported in part by grants from the Japan Health Sciences Foundation and the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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