

# Molecular cloning and characterization of TPP36 and its isoform TPP32, novel substrates of Abl tyrosine kinase

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**Abstract** We have molecularly cloned TPP36, a novel 36 kDa protein with 281 amino acids that was identified as a protein phosphorylated in B progenitor cells following stimulation with pervanadate/H<sub>2</sub>O<sub>2</sub>. Analysis with anti-TPP36 antiserum revealed that TPP36 was expressed ubiquitously and had an isoform with 236 amino acids, designated TPP32. TPP36/32 were localized mainly in cytoplasm despite the presence of a typical nuclear localization signal sequence. These proteins were phosphorylated preferentially by Abl among a panel of tyrosine kinases examined. Phosphorylation of tyrosine 120 in TPP36/32 led to an apparent mobility shift in sodium dodecyl sulfate–polyacrylamide gel electrophoresis, suggesting conformational change in the phosphorylated protein. Thus, TPP36/32 appear to be novel substrates of Abl tyrosine kinase.

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**Key words:** Abl; Conformational change; Isoform; Nuclear localization signal; Tyrosine phosphorylation

## 1. Introduction

B cells, precursors of antibody secreting cells, originate from pluripotent hematopoietic stem cells and differentiate in the bone marrow. Proliferation and differentiation of B precursor cells are governed by signaling through various receptors expressed on their surface, including the IL-7 receptor and the preB cell receptor [1,2]. Ample evidence suggests that an array of both tyrosine and serine/threonine protein kinases is involved in the signal transduction for the differentiation of lymphocyte precursors and the activation of mature lympho-

cytes [3,4]. However, the identities of protein kinases and their substrates involved in early B cell development have not been fully elucidated.

Treatment of living cells with pervanadate/H<sub>2</sub>O<sub>2</sub> inhibits protein tyrosine phosphatases, thereby modifying the intracellular equilibrium between dephosphorylation and phosphorylation [5,6]. Previous studies showed that treatment of mature B cell lines with pervanadate/H<sub>2</sub>O<sub>2</sub> induces the strong phosphorylation of a subset of intracellular proteins similar to that observed following antigen stimulation of the B cell receptor (BCR) [7]. In contrast, BCR-deficient B cell lines display only a weak substrate phosphorylation even following stimulation with pervanadate/H<sub>2</sub>O<sub>2</sub>. These data indicate that certain signaling components including protein tyrosine kinases (PTKs) are already associated with unligated BCR and constitute a preformed BCR-associated transducer complex [8]. Indeed, analysis of tyrosine phosphorylated proteins in pervanadate/H<sub>2</sub>O<sub>2</sub>-treated B cells led to the identification and cloning of SLP-65 (SH2 domain-containing leukocyte protein of 65, also called BLNK (B cell linker protein) and BASH (B cell adaptor containing src homology 2 domain)), an adapter protein essential for BCR signal transduction [9].

We have tried to identify possible substrates of PTKs involved in early B cell development by stimulating early B precursor cells with pervanadate/H<sub>2</sub>O<sub>2</sub>. In this process we have identified a novel protein with two isoforms, designated TPP36 and TPP32, that are phosphorylated by the tyrosine kinase Abl and evolutionarily conserved between various species. Mice deficient for c-Abl and those expressing constitutively active Abl showed altered differentiation and proliferation of progenitor B cells, suggesting the involvement of Abl in normal B cell development [10–12]. We report here the molecular cloning and biochemical characterization of TPP36/32 and discuss their possible roles in vivo.

## 2. Materials and methods

### 2.1. Cell lines and culture conditions

All media used to culture cell lines were supplemented with 10% fetal calf serum (FCS), 100 U/ml of penicillin-streptomycin, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol: IMDM medium for

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**Abbreviations:** BCR, B cell receptor; PTK, protein tyrosine kinase; FCS, fetal calf serum; EST, expressed sequence tag; CDV, carnitine deficiency-associated gene expressed in ventricle; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Btk, Bruton's tyrosine kinase; EGF, epidermal growth factor; PDGF, platelet-derived growth factor

proB cell line 63-12 [13]; RPMI 1640 medium for Cos7 cells, and DMEM medium for 293T and ST2 cells [14]. TonB.210.1 cells [15] carrying a tetracycline-inducible Bcr-Abl p210 gene were maintained in supplemented RPMI 1640 containing 10% medium conditioned by WEHI 3B cells (as an IL3 source), Bcr-Abl expression was induced by adding 1 µg/ml doxycyclin (Sigma, St. Louis, MO, USA) to the culture medium. To inhibit Bcr-Abl kinase activity, 10 µM STI-571 (Novartis Pharma, Basel, Switzerland) was added to the culture medium.

2.2. Antibodies

Antibodies specific to Syk, Btk (Bruton's tyrosine kinase), c-Abl were purchased from Santa Cruz Biotech. (Santa Cruz, CA, USA). Biotin- or agarose-conjugated anti-phosphotyrosine mAb (4G10), anti-Lyn and anti-c-Src antibodies were purchased from Upstate Biotech. (Lake Placid, NY, USA). Anti-FLAG M2 mAb was purchased from Sigma. Antiserum against TPP36 was generated by immunizing rabbits with recombinant TPP36 protein produced in *Escherichia coli*.

2.3. Purification and cDNA cloning of TPP36

63-12 cells (10<sup>12</sup>) were stimulated at 10<sup>8</sup>/ml in 5% FCS/PBS (phosphate-buffered saline) with 0.2 mM pervanadate/H<sub>2</sub>O<sub>2</sub> for 5 min at 37°C, washed with cold inhibition buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM ethylene glycol-bis(2-aminoethyl-ether)-N,N,N',N'-tetraacetic acid, 1.5 mM MgCl<sub>2</sub>, 100 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor, 1µg/ml anti-α-trypsin) and then lysed with 1% NP-40 containing inhibition buffer. Proteins with molecular weight of 30–40 kDa were enriched from the lysates by using Prep Cell (Bio-Rad Laboratories, Hercules, CA, USA) and DEAE-Sephadex. The isolated proteins were dialyzed against buffer A (20 mM Tris-HCl (pH 7.4), 10 mM ethylenediamine tetraacetic acid, 100 mM NaCl, 0.2 mM Na<sub>3</sub>VO<sub>4</sub>, 0.01% NaN<sub>3</sub>) and loaded onto 4G10-conjugated Sepharose. Tyrosine-phosphorylated proteins were eluted with buffer A containing 10 mM phenyl phosphate. Finally, TPP36 was isolated from eluted proteins with phenyl-5-PW (reverse phase chromatography), separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and stained with Coomassie brilliant blue. The TPP36 band was excised and subjected to in-gel digestion with lysyl endopeptidase, and the resulting peptides were purified by high-performance liquid chromatography and sequenced by electrospray ionization tandem mass spectrometry. Two overlapping clones in the mouse EST (expressed sequence tag) database (GenBank accession nos. L22067 and AA842053) were found to encode the three sequenced peptides from TPP36. A λgt11 cDNA library prepared from 63-12 proB cell line was screened with the cDNA fragments corresponding to these two clones, and obtained cDNAs were sequenced.

2.4. Plasmid constructs

The cDNAs coding for wild-type and mutant TPP36/32 were modified to add FLAG epitope at the N- or C-terminus of TPP36 and subcloned into BCMGS [16] for stable transfection or into pCAGGS [17] for transient transfection. pME vectors carrying Syk and Lyn cDNA [18], pcDNA3-carrying Bcr-Abl cDNA [15], pcDNA3-carrying c-Src and dominant active (DA) Src cDNA [19] and pME18S [20] carrying mouse Btk cDNA were described previously.

2.5. DNA transfection

63-12 cells (5 × 10<sup>6</sup>) were resuspended in 500 µl of K-PBS (NaCl 30.8 mM, KCl 120.7 mM, Na<sub>2</sub>HPO<sub>4</sub> 8.1 mM, KH<sub>2</sub>PO<sub>4</sub> 1.46 mM) with 5 mM MgCl<sub>2</sub> and electroporated at 330 V, 960 µF using Gene Pulsar II (Bio-Rad). To select for stably transfected cells, 2 mg/ml of hygromycin was added to culture medium 48 h after electroporation. Transient transfection of Cos7 and 293T cells was performed using a mammalian transfection kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions.

2.6. Immunoprecipitation and immunoblot analysis

Cells were collected by centrifugation and then lysed with inhibition buffer containing 1% NP-40. Cell lysates were incubated with antibodies at 4°C for 1 h followed by incubation with 20 µl of Protein A–Sepharose beads (Amersham Pharmacia, Buckinghamshire, UK) for 30 min. The immunoprecipitates were resolved on SDS–PAGE, transferred to polyvinylidene difluoride membrane, and then immunoblotted with antibodies described above. After reaction with horseradish peroxidase-conjugated second antibodies (Santa Cruz Biotech.), pro-

teins were visualized by an enhanced chemiluminescence system (Amersham Pharmacia).

2.7. Immunofluorescence

Cells were fixed with 3.7% formaldehyde/PBS for 10 min, permeabilized with 0.2% Triton X-100/PBS for 15 min, and then blocked with 3% bovine serum albumin (BSA)/PBS for 30 min. To detect exogenous or endogenous TPP36/32, cells were incubated with anti-FLAG mAb or anti-TPP36 antiserum for 30 min and then with fluorescein isothiocyanate (FITC)-conjugated anti-mouse Fcγ antibodies (Jackson Immunoresearch Laboratories, West Grove, PA, USA) or FITC-conjugated goat anti-rabbit IgG (Cappel Organon Teknika, Durham, NC, USA). Stained cells were analyzed by confocal microscopy using a 3D projection image analyzer (Leica TCS NT, Heidelberg, Germany).

3. Results

3.1. Purification and molecular cloning of TPP36

To identify possible substrates of PTKs involved in early

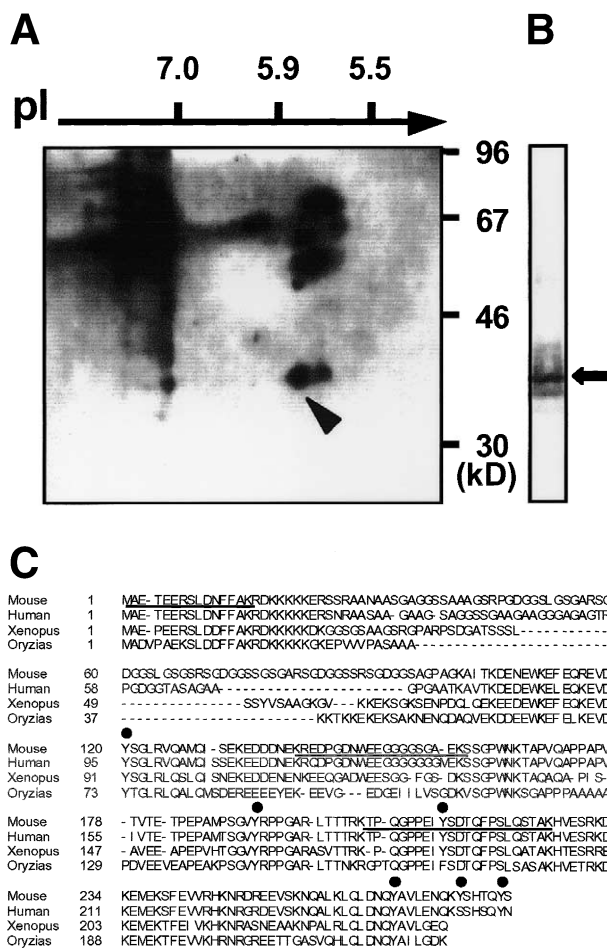


Fig. 1. Purification and cDNA cloning of TPP36. A: 63-12 cells were treated with pervanadate/H<sub>2</sub>O<sub>2</sub> at 37°C for 5 min. Cell lysates were prepared and incubated with anti-phosphotyrosine (4G10)-conjugated beads. The immunoprecipitates were analyzed by 2D (IEF/SDS–PAGE) gel electrophoresis, followed by immunoblot with 4G10. Arrowhead indicates the spots representing TPP36. B: Coomassie brilliant blue staining of the purified TPP36 in a SDS–PAGE gel as indicated by the arrow. C: The predicted amino acid sequence of mouse TPP36 has been aligned with those of its homologs in the indicated species. Three underlines indicate peptide sequences determined by tandem mass spectrum from purified TPP36 protein. The dots indicate potential tyrosine-phosphorylation sites.

B cell development, proB cell line 63-12 was stimulated with pervanadate/H<sub>2</sub>O<sub>2</sub>. Tyrosine-phosphorylated proteins were separated in 2D gel and detected with anti-phosphotyrosine antibody 4G10 (Fig. 1A). We focused our efforts on a protein with an apparent molecular weight of 36 kDa and a *pI* of 5.6–5.7. Immunoblot analysis revealed that this protein was distinct from Ig $\alpha$  and Ig $\beta$  (data not shown), both of which have been shown to be tyrosine-phosphorylated in B cell lineages following BCR or preBCR stimulation [21]. The protein, which we called tyrosine-phosphorylated protein 36 (TPP36), was purified from 10<sup>12</sup> pervanadate-stimulated 63-12 cells by a series of purification procedures including affinity chromatography with 4G10 (Fig. 1B). Three stretches of partial amino acid sequences of the purified protein were obtained from tandem mass spectrometry of lysyl endopeptidase-digested peptides (Fig. 1C, underlined sequences). Four clones in the mouse DNA database (GenBank accession nos. L22067, AA824053, AF215660 and AF320340) were found to encode all or a part of the three sequenced peptides. Polymerase chain reaction (PCR) products corresponding to these clones were used to isolate a cDNA clone from a  $\lambda$ gt11 cDNA library prepared from the 63-12 proB cell line. Sequencing of this cDNA (GenBank accession no. AB046372) revealed that it was identical to cDNA clone CDV-3B (carnitine deficiency-

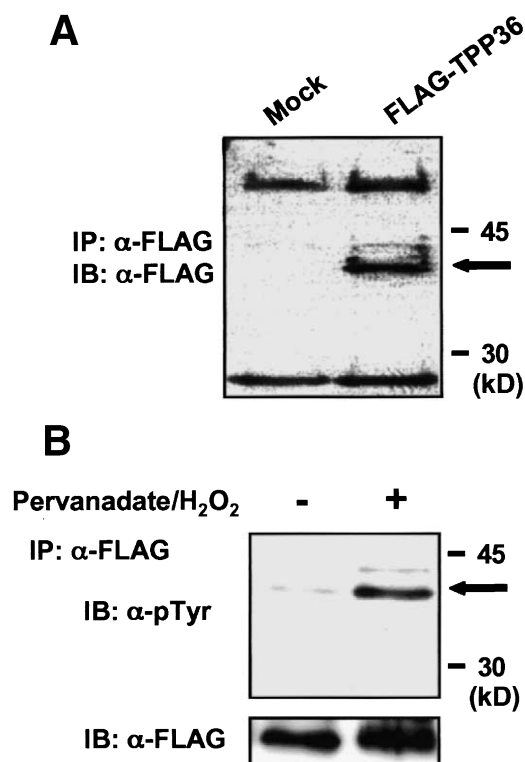


Fig. 2. FLAG-tagged TPP36 was expressed in 63-12 cells as a 37 kDa protein and tyrosine-phosphorylated upon stimulation with pervanadate/H<sub>2</sub>O<sub>2</sub>. A: 63-12 cells were stably transfected with FLAG-TPP36 cDNA or control vector. Cell lysates were incubated with anti-FLAG Ab, and the immunoprecipitates were subjected to SDS-PAGE, followed by immunoblot analysis with anti-FLAG Ab. The arrow indicates the FLAG-TPP36 protein. B: 63-12 cells stably transfected with FLAG-TPP36 cDNA were stimulated with 0.2 mM pervanadate/H<sub>2</sub>O<sub>2</sub> at 37°C for 5 min. Cell lysates were incubated with anti-FLAG Ab, and the immunoprecipitates were subjected to SDS-PAGE, followed by immunoblot analysis with 4G10 or anti-FLAG Ab.

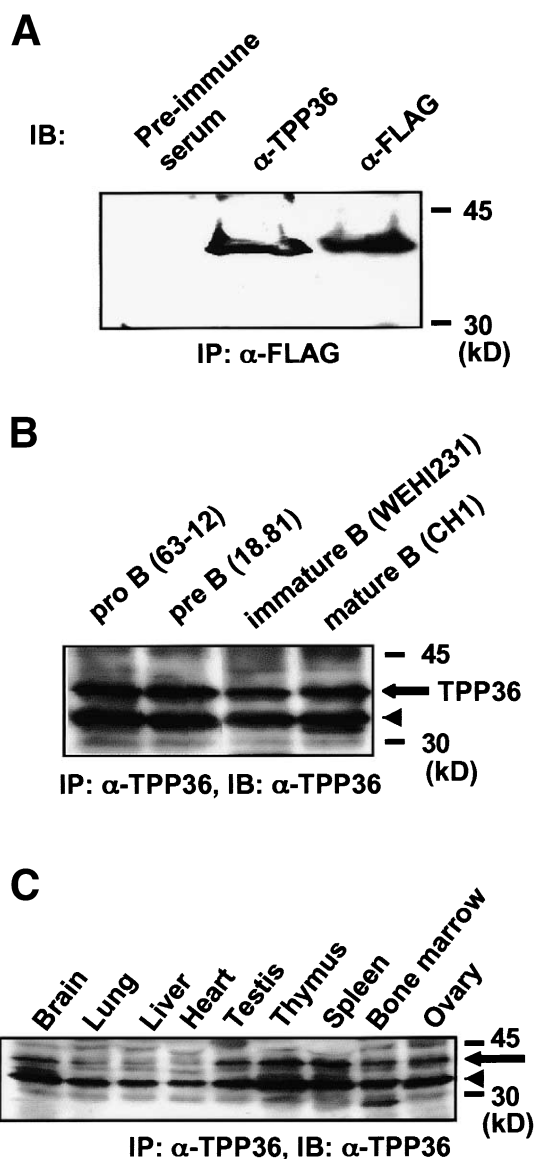


Fig. 3. Analysis with anti-TPP36 antiserum revealed the ubiquitous expression of TPP36 and the presence of its isoform TPP32. A: Cos7 cells were transiently transfected with FLAG-TPP36 cDNA. FLAG-TPP36 proteins were immunoprecipitated with anti-FLAG Ab and subjected to SDS-PAGE followed by immunoblot analysis with anti-TPP36 antiserum, pre-immune serum as a control or anti-FLAG antibody. B,C: Expression of TPP36 protein was examined in a panel of B lineage cell lines (panel B) and in various tissues (panel C) by immunoprecipitation and immunoblot with anti-TPP36 antiserum. The arrows indicate the TPP36 protein.

associated gene expressed in ventricle; GenBank accession no. AF320340), which was recently reported as a gene up-regulated in the hypertrophied ventricles of carnitine-deficient mice [22]. No biochemical characterization and functional analysis of the CDV-3B gene product has been reported.

The cDNA encoded an open reading frame corresponding to a 281 amino acid polypeptide including six tyrosine residues that are putative targets for phosphorylation. The N-terminal portion of the protein contained a short stretch of basic lysine residues (KKKKK) followed by a region rich in Gly and Ser. The central part of the protein is rich in Pro, including three PXXP motifs. The analysis of the purified protein with tandem mass spectrometry revealed that the ala-



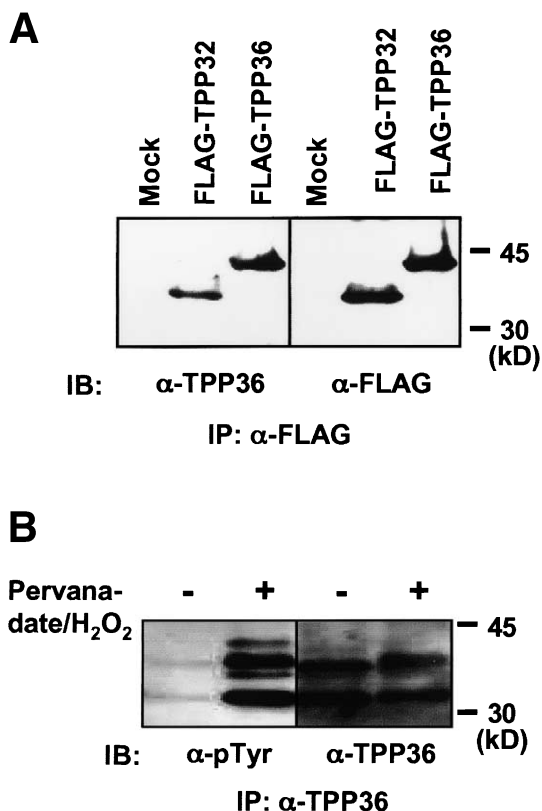


Fig. 4. Characterization of TPP32. A: Cos7 cells were transiently transfected with FLAG-TPP36 (CDV-3B) cDNA, FLAG-TPP32 (CDV-3A) cDNA or control vector. FLAG-TPP36/32 proteins in cell lysates prepared 48 h after transfection were immunoprecipitated with anti-FLAG Ab, followed by immunoblot analysis with anti-TPP36 antiserum or anti-FLAG Ab. B: 63-12 Cells were treated with or without pervanadate/H<sub>2</sub>O<sub>2</sub> at 37°C for 5 min. Cell lysates were prepared and incubated with anti-TPP36 antiserum, and the immunoprecipitates were immunoblotted with 4G10 or anti-TPP36 antiserum.

nine residue at position 2 was acetylated. A search of the GenBank database revealed that homologs of the mouse CDV-3B gene exist in many species, including mammals, birds, frogs, fishes and insects (Fig. 1C, not all data shown). The product of the CDV-3B gene has a predicted *pI* of 5.77, in good agreement with the apparent *pI* of TPP36 (*pI* 5.6–5.7) as shown in Fig. 1. In contrast, the predicted molecular weight of the protein, 29.7 kDa, is considerably smaller than the apparent molecular weight of TPP36. When the FLAG-tagged CDV-3B cDNA was expressed in 63-12 cells, the gene product was detected by anti-FLAG antibody as a protein with apparent molecular weight of 37 kDa and was tyrosine-phosphorylated upon pervanadate/H<sub>2</sub>O<sub>2</sub> stimulation, as was observed for TPP36 (Fig. 2). These results strongly suggest that the cloned cDNA corresponding to CDV-3B encodes TPP36.

### 3.2. Ubiquitous expression of TPP36 and its isoform TPP32

In order to analyze endogenous TPP36, polyclonal antibody against TPP36 was prepared by immunizing rabbits with recombinant TPP36. FLAG-tagged TPP36 transiently expressed in Cos7 cells could be detected by this antibody but not with the pre-immune serum (Fig. 3A). Immunoblot analysis of cell lysates from a panel of B lineage cell lines at

various developmental stages as well as normal tissues demonstrated that the expression of TPP36 was not restricted to the early stage of B cell development but rather that it was ubiquitous (Fig. 3B,C). This is consistent with ubiquitous expression of CDV-3B mRNA reported by Fukumaru et al. [22].

Besides TPP36, a smaller protein with an apparent molecular weight of 32 kDa was detected by the anti-TPP36 antibody in all cell lines and tissues examined. Fukumaru et al. have reported the cDNA clone CDV-3A, which is a splicing variant of CDV-3B. CDV-3A was predicted to encode a shorter protein of 236 amino acids in which the N-terminal 233 amino acids but not the C-terminal three amino acids were identical to those predicted from the nucleotide sequence of CDV-3B. A sequence analysis of the reverse-transcribed PCR products prepared from the 63-12 proB cell line confirmed the existence of transcripts corresponding to CDV-3A (data not shown). When the FLAG-tagged cDNA corresponding to CDV-3A was transiently expressed in Cos7 cells, the gene product was detected as a 33 kDa protein by anti-FLAG or anti-TPP36 antibody (Fig. 4A). Therefore, the 32 kDa protein detected by anti-TPP36 antibody in cell lines and normal tissues appeared to be encoded by the CDV-3A gene. We designated this isoform of TPP36 protein TPP32. TPP36 and TPP32 share many features, such as the KKKKK motif, the Gly/Ser-rich region, the Pro-rich region as well as the position of the first three tyrosine residues. TPP36 has six tyrosine residues while TPP32 has four. TPP32 was found to be phosphorylated on tyrosine in 63-12 cells treated with pervanadate/H<sub>2</sub>O<sub>2</sub> as was TPP36 (Fig. 4B).

### 3.3. Cytoplasmic localization of TPP36/32

Analysis of the pSORT protein database by Reinhardt's method [23] predicted with 94.1% reliability that TPP36/32 are nuclear proteins, based on the presence in these proteins of a cluster of basic amino acid residues including the repeat of five lysine residues near the N-terminus, which may be a potential nuclear localization signal. Therefore, we examined the subcellular localization of TPP36 using confocal microscopy. When expressed in Cos7 or 293T cells, FLAG-tagged TPP36 was detected by anti-FLAG antibody in cytoplasm but not in nucleus (Fig. 5A, not all data shown). The presence of

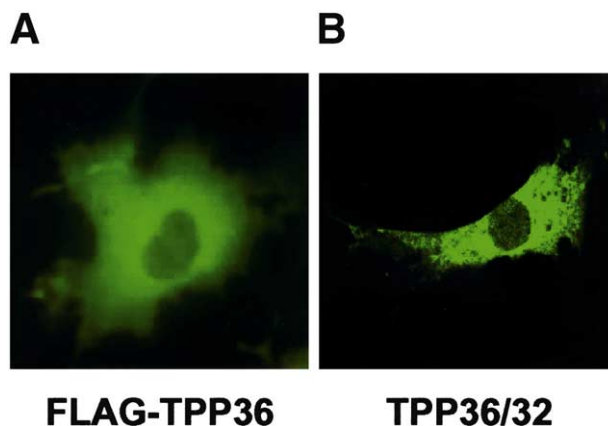


Fig. 5. Subcellular localization of TPP36/32. A: Cos7 cells transiently transfected with FLAG-TPP36 cDNA were stained with anti-FLAG antibody. B: ST2 stromal cells were stained with anti-TPP36 antiserum. The stained cells were analyzed with confocal microscopy.

the FLAG tag at either the N- or C-terminus gave the same results, and FLAG-tagged TPP32 was also shown to be cytoplasmic (data not shown). Furthermore, endogenous TPP36/32 were also detected in the cytoplasm but not in the nucleus using the anti-TPP36 antibody (Fig. 5B). Thus, TPP36/32 turned out to be not nuclear proteins.

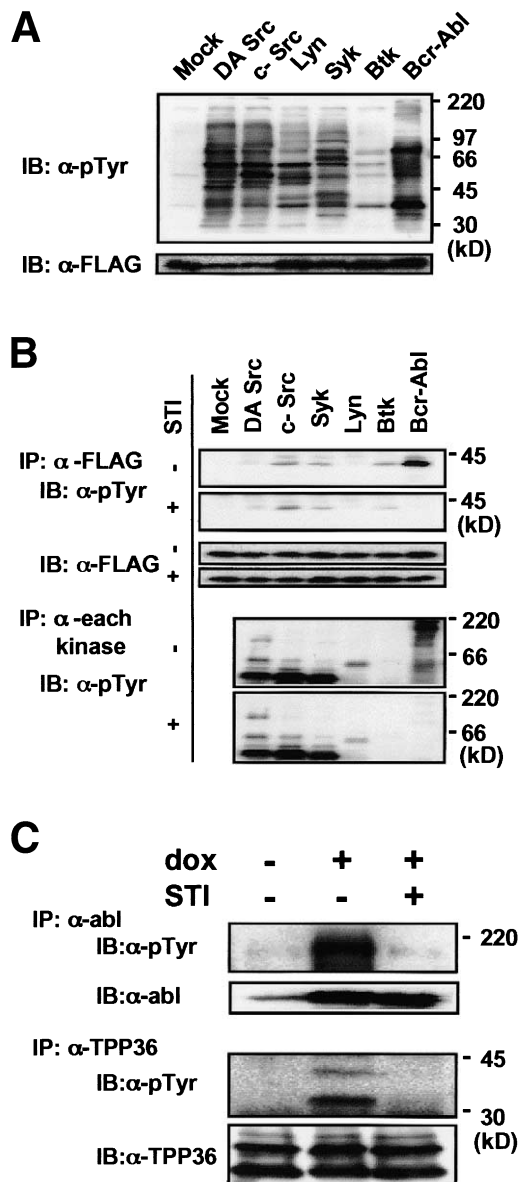


Fig. 6. TPP36/32 were tyrosine-phosphorylated by Bcr-Abl. A,B: 293T cells were co-transfected with cDNA expression vectors encoding FLAG-TPP36 and one of tyrosine kinases as indicated. In panel A, whole proteins in cell lysates prepared 48 h after transfection were subjected to SDS-PAGE, followed by immunoblot analysis with anti-phosphotyrosine Ab or anti-FLAG Ab. In panel B, 24 h after transfection, 293T cells were incubated with or without 10  $\mu$ M STI-571 at 37°C for 1 h. Cell lysates were prepared and incubated with anti-FLAG Ab or Ab specific to each kinase. The immunoprecipitates were subjected to SDS-PAGE, followed by immunoblot analysis with 4G10 or anti-FLAG Ab. C: TonB.210.1 cells were cultured at 37°C for 1 h with or without 1  $\mu$ g/ml doxycyclin in the presence or absence of 10  $\mu$ M STI-571. Cell lysates were incubated with anti-Abl Ab or anti-TPP36 antiserum, and the immunoprecipitates were subjected to SDS-PAGE, followed by immunoblot analysis with 4G10, anti-Abl Ab or anti-TPP36 antiserum.

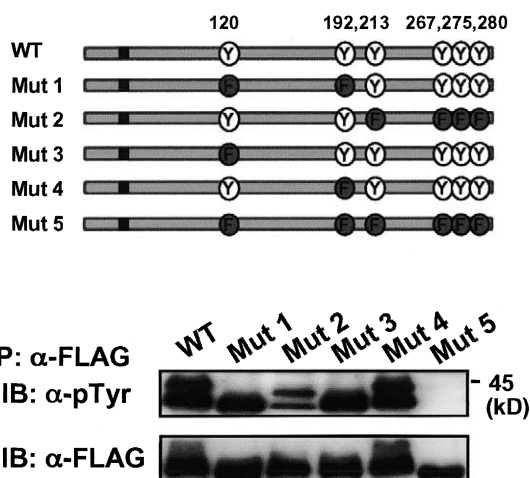


Fig. 7. Phosphorylation of tyrosine 120 of TPP36 by Bcr-Abl leads to mobility shift of TPP36 in SDS-PAGE. 293T cells were co-transfected with cDNA expression vectors encoding Bcr-Abl and one of FLAG-TPP36 YF mutants as indicated. FLAG-TPP36 proteins in cell lysates prepared 48 h after transfection were immunoprecipitated with anti-FLAG Ab and subjected to SDS-PAGE, followed by immunoblot analysis with 4G10 or anti-FLAG Ab.

#### 3.4. TPP36/32 were tyrosine-phosphorylated by Abl

In contrast to the results in 63-12 cells, we did not detect phosphorylation on tyrosine of either the endogenous TPP36/32 or the FLAG-tagged protein in Cos7 or 293T cells in response to treatment of these cells with pervanadate/ $H_2O_2$  (data not shown). We next sought to identify the tyrosine kinase responsible for phosphorylation of TPP36. FLAG-tagged TPP36 was transiently co-expressed in 293T cells with individual tyrosine kinases from a panel including c-Src, a constitutively active form of Src (DA-Src), Lyn, Syk, Btk and a constitutively active form of Abl (Bcr-Abl). Several proteins were phosphorylated on tyrosine in cells transfected with the genes encoding each kinase (Fig. 6A). Among them, a 36 kDa protein was prominently phosphorylated in cells transfected with the gene encoding Bcr-Abl. Immunoprecipitation of lysates from such cells with the anti-FLAG antibody, followed by immunoblot analysis with the 4G10 antibody, revealed that the 36 kDa protein strongly phosphorylated by Bcr-Abl was indeed TPP36 (Fig. 6B). STI-571, a specific inhibitor of Abl kinase activity [24], almost completely inhibited both the phosphorylation of TPP36 and the autophosphorylation of Bcr-Abl. Neither the weak phosphorylation of TPP36 by the other kinases nor the autophosphorylation of these other kinases was significantly influenced by STI-571 (Fig. 6B). In order to examine whether endogenous TPP36 could also be phosphorylated on tyrosine by Abl, an engineered Baf3 proB cell line was used in which expression of Bcr-Abl is induced by doxycyclin. Addition of doxycyclin to the culture induced expression and autophosphorylation of Bcr-Abl as well as phosphorylation of TPP36/32 on tyrosine (Fig. 6C). STI-571 almost completely inhibited both the phosphorylation of TPP36/32 and the autophosphorylation of Bcr-Abl.

#### 3.5. Phosphorylation of tyrosine 120 induced the mobility shift of TPP36

When FLAG-tagged TPP36 was co-expressed with Bcr-Abl in 293T cells, two distinct bands of phosphoproteins with ap-

parent molecular weights of 37 and 40 kDa were detected by immunoprecipitation with anti-FLAG antibody followed by immunoblot analysis with antibody 4G10 (Fig. 7, WT). Immunoblot analysis with the anti-FLAG antibody revealed that the 40 kDa band represented a small fraction of FLAG-tagged TPP36. We also detected phosphorylation of the 40 kDa protein in 63-12 cells treated with pervanadate/H<sub>2</sub>O<sub>2</sub> (Figs. 2B and 4B). A possible explanation for the migration of a fraction of FLAG-tagged TPP36 with an apparent molecular weight of 40 kDa is that the 40 kDa form contains an additional phosphorylated tyrosine residue compared to the 37 kDa form. To examine this directly, we created a series of TPP36 mutants with substitution of individual tyrosine residues with phenylalanine and co-expressed them with Bcr-Abl in 293T cells. The substitution of the first two tyrosine residues (Y120 and Y192) abolished the appearance of the 40 kDa phosphoprotein, but preserved the 37 kDa phosphoprotein (Fig. 7, Mut 1). In contrast, substitution of the latter four tyrosine residues did not abolish the presence of either the 37 or 40 kDa phosphoproteins (Fig. 7, Mut2), although the extent of their phosphorylation was much reduced compared to wild-type proteins (WT). Single substitution of Y120, but not Y192, abolished the appearance of the 40 kDa phosphoprotein (Fig. 7, Mut 3 versus 4), indicating that phosphorylation of Y120 induced the apparent mobility shift of TPP36. The mobility shift of TPP32 from 32 to 35 kDa was also induced by phosphorylation of tyrosine 120 (Fig. 4B, second lane; not all data shown).

#### 4. Discussion

In this study, we have molecularly cloned and biochemically characterized TPP36 and its isoform TPP32 as novel substrates of Abl kinase. c-Abl is a multidomain non-receptor tyrosine kinase that is ubiquitously expressed and highly conserved in metazoan evolution [25,26]. Mutant forms of c-Abl have been identified in murine, feline and human leukemias. Whereas the activity of wild-type Abl is tightly regulated, the Bcr-Abl fusion protein produced through chromosomal translocation and found in human leukemias shows constitutively elevated kinase activity leading to cellular transformation [27,28]. It has recently been shown that the N-terminal region of c-Abl is responsible for autoinhibition of c-Abl kinase activity, and that its loss renders c-Abl oncogenic and contributes to deregulation of Bcr-Abl [29]. Although the mechanism of the transformation by Bcr-Abl and its viral homolog v-Abl have been extensively characterized [30], the normal biological function and signaling mechanism of c-Abl are less well understood.

The expression of TPP36/32 appears to be ubiquitous, and the proteins appear to be well conserved between a wide variety of species from humans to insects, as is the case with c-Abl. In transiently transfected 293T cells, TPP36/32 were more extensively phosphorylated on tyrosine when co-expressed with Bcr-Abl than when co-expressed with the other tyrosine kinases examined. Phosphorylation of endogenous TPP36/32 was also detected in TonB.210.1 cells after induction of Bcr-Abl expression. Furthermore, the phosphorylation of TPP36/32 on tyrosine that we observed in 293T and TonB.210.1 cells was almost completely inhibited by STI-571, a specific inhibitor of Abl kinase activity. These results indicate that TPP36/32 are substrates of Abl tyrosine kinase.

It remains to be determined whether the phosphorylation of TPP36/32 on tyrosine is essential to their physiological function. In this regard, it is notable that the phosphorylation of Y120, but not the other tyrosine residues, leads to an apparent shift in the mobility of these proteins when analyzed by SDS-PAGE. This suggests that the phosphorylation of Y120 induces conformational change of TPP36/32. One might hypothesize that this phosphorylation-induced conformational change could be involved in regulation of the function of these proteins. Mice deficient for Abl showed poor viability and reduction of B cell progenitors in bone marrow [10–12], while the induced expression of Bcr-Abl in vivo led to the development of reversible preB cell leukemia [31]. Therefore, it could be possible that TPP36/32 are involved in proliferation and differentiation of B progenitor cells through phosphorylation by Abl.

The N-terminal portion of TPP36/32 is very well conserved between different species and contains a cluster of basic amino acid residues that is predicted with high likelihood to be a nuclear localization signal. However, we detected both endogenous and exogenously expressed TPP36/32 predominantly in the cytoplasm. To explore the possibility that the nuclear localization signal near the N-terminus of the protein might be masked by other parts of TPP36, the N-terminal 30 amino acids of TPP36/32 including the possible nuclear localization signal were fused to the N-terminus of GFP, and the fusion protein (N30-GFP) was expressed in Cos7 cells. N30-GFP was detected both in the cytoplasm and the nucleus (data not shown). Therefore, it was not conclusive as to whether the putative nuclear localization signal near the N-terminus was functional. In addition, we cannot formally exclude the possibility that other proteins could bind and mask a nuclear localization signal within TPP36/32. An intriguing question is whether the putative conformational change induced by phosphorylation of Y120 could affect the subcellular localization of TPP36/32. Since the fraction of TPP36/32 phosphorylated on Y120 is quite small, an antibody specific to Y120-phosphorylated TPP36/32 is being prepared to enable study of its subcellular localization.

Physiological functions of TPP36/32 remain to be determined. However, some clues exist to explore their functions. The TPP36/32 gene expression is reportedly altered in certain pathological conditions. The expression of the H41 gene, the human homolog of the TPP36/CDV-3B gene, has been shown to be up-regulated in malignant cancers of the breast and ovary that are associated with the overexpression of the HER-2/neu/c-erbB-2 proto-oncogene [32]. HER-2 encodes a transmembrane receptor tyrosine kinase with extensive homology to epidermal growth factor (EGF) [33]. c-Abl is reportedly activated by growth factors such as EGF and PDGF (platelet-derived growth factor) [34]. Therefore, TPP36 overexpression and phosphorylation by Abl might be involved in the pathogenic effects of HER-2 overexpression in human breast and ovarian cancers. Moreover, CDV-3B, which is identical to the mouse TPP36 cDNA cloned in the present study, has recently been reported to be a gene up-regulated in the hypertrophied heart of juvenile visceral steatosis (JVS) mice suffering from fatty liver, hyperammonemia, hypoglycemia and growth retardation [22]. JVS mice exhibit carnitine deficiency due to defective renal carnitine transport resulting from a point mutation in the organic cation transporter 2 (OCTN2) gene [35]. It remains unclear whether TPP36/

CDV-3B proteins are directly involved in the development of cardiac hypertrophy. Since administration of carnitine significantly lowered levels of CDV-3B transcripts within 6 h, the up-regulation of CDV-3B transcripts appeared to be linked to an alteration in the metabolic state associated with carnitine deficiency [22].

It seems too early to draw a whole picture of TPP36/32 integrating all the findings, namely the tyrosine phosphorylation of TPP36/32 by Abl, possible conformational change of phosphorylated TPP36/32 and the up-regulation of the TPP36/CDV-3B/H41 gene in hypertrophied heart of JVS mice and human breast/ovarian cancers. The ubiquitous expression of TPP36/32 and their high conservation in evolution might indicate their fundamental roles *in vivo*. In order to know physiological functions of TPP36/32, we are currently establishing mice deficient for TPP36/32.

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