Molecular cloning and characterization of Byp, a murine receptor-type tyrosine phosphatase similar to human DEP-1

Satomi Kuramochi[a], Satoru Matsuda[a], Yoichi Matsuda[b], Toshiyuki Saitoh[b], Miho Ohsugi[a], Tadashi Yamamoto[a,*]

[a]*The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan*
[b]*Division of Genetics, National Institute of Radiological Sciences, 4-9-1, Anagawa, Inage-ku, Chiba 263, Japan*

Received 8 November 1995; revised version received 22 November 1995

Abstract **Novel murine cDNAs encoding a receptor-like protein tyrosine phosphatase, termed Byp (HPTP *b*eta-like *t*yrosine *p*hosphatase) were cloned. The putative Byp protein consists of 1238 amino acids, which possesses a single catalytic domain in the cytoplasmic region. The extracellular region comprises eight repeats of a fibronectin type III module and contains multiple *N*-glycosylation sites. The *byp* mRNA was 7.7-kb long and expressed in every tissue examined, its level being high in the brain and kidney. Transfection of the *byp* cDNA expression plasmid into COS7 cells resulted in the expression of a 220-kDa tyrosine phosphorylated protein. Furthermore, co-expression of Byp and the Src family kinase Fyn increased the level of tyrosine phosphorylation of Byp, suggesting that Byp was tyrosine-phosphorylated by Fyn. Finally, the *byp* gene was located to mouse chromosome 2E1-2 and rat chromosome 3q32-33.**

Key words: Protein tyrosine phosphatase; Tyrosine phosphorylation; Fyn tyrosine kinase

1. Introduction

Accumulating data show that protein tyrosine phosphorylation is an important event in cell growth and differentiation, malignant transformation, and cytoskeletal function [1]. The level of tyrosine phosphorylation is determined by the opposing actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). In contrast with the extensive analysis and information on the role of PTKs, studies on the physiological importance of PTPs are limited. A cytosolic PTP was first purified by Tonks et al. [2]. Since then, the number of PTP characterized has been increased steadily. Consequently, PTPs can now be divided into three subfamilies: (1) transmembrane, receptor-like PTPs (RPTPs); (2) soluble intracellular PTPs; and (3) dual-specificity PTPs [3]. All PTPs have at least one conserved catalytic domain containing an 11-amino acid consensus motif (I/V)HCXAGXGR(S/T)G (X can be any amino acid). The cysteinyl residue within the consensus motif is essential for the PTP activity [4].

The RPTPs have highly-conserved intracellular phosphatase domains and extracellular domains with diverse amino acid sequences. The sequences of the extracellular domains share sequence similarity with the cell adhesion molecules, such as fibronectin, N-CAM and chondroitin sulfate. Namely RPTPs are characterized by having fibronectin type-III (FN-III)-like motif, immunogloblin (Ig)-like motif, or other motifs unique to transmembrane proteins. Based on the amino acid sequences of the extracellular domains, the RPTPs can be divided into five classes [5,6]. Among these, type III RPTPs are characterized by having only FN-III-like repeats in the extracellular region. This motif, which was originally described in fibronectin, may mediate heterophilic interactions with ligands located on the adjacent cells or the extracellular matrix [5]. Type III RPTPs include HPTPβ [7], *Drosophila* DPTP10D [8,9], SAP-1 [10], DEP-1 [11] or HPTPη [12], rabbit GLEPP1 [13], OST-PTP [14], and PTP-U2 [15]. It has been reported that DEP-1 expression was increased in dense cell culture relative to sparse cell culture, and *PTP-U2* expression was enhanced by phorbol ester, dihydroxyvitamin D_3, retinoic acid, and dimethyl-sulfoxide. The other biological function of these RPTPs remains to the characterized.

We report here the molecular cloning, gene expression, and chromosomal location of a new member, termed Byp (HPTP *b*eta-like *t*yrosine *p*hosphatase), of mouse type III RPTP family. The cDNA for this RPTP was identified in the RT-PCR products prepared from mRNAs of lymph nodes of *lpr* (lymphoproliferation) mice. *Lpr* mice have hypertrophied lymph nodes comprising $CD3^+CD4^-$ $CD8^-$ T cells with high expression of the Fyn tyrosine kinase [16]. Interestingly, the level of tyrosine phosphorylation of the Byp protein expressed in COS7 cells was increased by the co-expressed Fyn kinase. Therefore, Byp might be regulated by Fyn-mediated tyrosine phosphorylation.

2. Materials and methods

2.1. Cell culture

COS7 cells and NIH3T3 cells were cultured in DMEM supplemented with 10% calf serum. Murine pre-B cell line WEHI-231, T cell hybridoma HBC21.7.31, erythroid cell line F5-5 were cultured in RPMI 1640 supplemented with 10% fetal calf serum. Myeloid cell line FDC-P2 were cultured in RPMI1640 supplemented with 10% fetal calf serum and 10% of WEHI-3 culture supernatant.

2.2. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was prepared from hypertrophied lymph nodes of a 5-month-old *lpr* mouse by the AGPC (guanidium cid/phenol/chloroform) method [17]. The first-strand cDNA was synthesized with SuperScript reverse transcriptase (Gibco BRL) using a primer which is designed from an amino acid sequence within the relatively conserved C-terminus of the PTP domain QTX(A/E/D)Q: 5′-(A/T)(A/G)(C/T)TG(C/G/T)(G/T)CN(G/T)(A/C/G)(C/G/T)GT(C/T)TG-3′. The cDNA made was used as a template for PCR. A pair of degenerate oligonucleotide primers for PCR was designed from the conserved

*Corresponding author. Fax: (81) (3) 5449-5413.

The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence database with the following accession number: D45212.

SSDI 0014-5793(95)01415-2

amino acid sequences in the PTP domains DYINA and HCSAG: 5′-ATGAAGCTTGA(C/T)TA(C/T)AT(C/T)AA(C/T)GC-3′ and 5′-CA-TGAATTCC(A/G/T)GCACTGCA(A/G)TG-3′, respectively, having an *Hin*dIII or *Eco*RI site at the 5′ end (underlined). PCRs were performed for 40 cycles with denaturation at 94°C for 30 s, annealing at 45°C for 30 s, and extension at 72°C for 1.5 min. The products of the expected size (about 500 bp) were cloned between the *Eco*RI and *Hin*dIII sites of pUC119 and subjected to sequence analysis by the dideoxynucleotide chain termination method using the BcaBest sequencing kit (Takara).

2.3. Screening of the cDNA library

A λgt11 cDNA library was constructed from poly(A)$^+$ RNA of lymph node of *lpr* mice using random hexamer primer. One of the RT-PCR products (Fig. 1, probe 1) was labeled by random priming and used as a screening probe. Filters representing 1×10^6 plaques were hybridized overnight at 42°C in 4 × SSC (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate)/10 × Denhardt's solution/50 mM HEPES (pH 7.0)/50% (v/v) formamide/100 μg/ml denatured salmon sperm DNA. Hybridized filters were washed in 0.1 × SSC/0.1% SDS at 50°C and autoradiographed. Complementary DNA fragments isolated from positive clones were subcloned into the pBluescript II SK(+) vector and sequenced. Initial screening yielded incomplete cDNAs without ATG and polyA tail. Thus, the second round screening was carried out using the *Xba*I-*Pst*I fragment of the clone 1–14 (probe 2) and the *Xho*I-*Bst*EII fragment of the clone 1-2 (probe 3) as probes.

2.4. Northern blotting

Total RNA was extracted from normal and *lpr* mice tissues and murine cell lines by the AGPC method. Total RNA (20 μg) was electrophoresed on a 1% formaldehyde-agarose gel and transferred to a nylon membrane. After UV cross-linking, the blots were hybridized at 42°C with ^{32}P-labeled *byp* probe (2.6 kbp cDNA fragment from clone 1-14) in 50% formamide/5 × SSC/4 × Denhardt's solution/50 mM sodium phosphate (pH 6.5)/0.2% SDS, washed at 50°C in 0.1 × SSC/0.1% SDS and autoradiographed. The blots were then re-hybridized with β-actin probe to verify equivalent RNA loading.

2.5. Bacterial protein expression

GST-Byp(p) was constructed by inserting the *Pvu*II fragment of the *byp* cDNA encoding the PTP domain (from Ala-961 to Ala-1238) into the pGEX-2T expression vector (Pharmacia LKB). The Cys-1140 to Ser mutant, GST-Byp(p)CS, was made from GST-Byp(p) by oligonucleotide-directed mutagenesis [18]. GST-Byp(c) and GST-Byp(ex) were constructed by inserting the *Pvu*I and *Pvu*II fragment corresponding to the C-terminal region (Arg-1155 to Ala-1238), and the *Bam*HI and *Eco*O109 fragment corresponding to the extracellular domain located just upstream of the transmembrane portion (Gly-721 to Asp-870), respectively, into the pGEX-2T vector. The GST-fusion proteins were expressed in *Escherichia coli* DH5α at 25°C overnight in the presence of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The bacterial culture was centrifuged and the pellets were resuspended in L buffer (50 mM Tris-HCl (pH 7.5)/25% sucrose/5 mM $MgCl_2$/0.5% Nonidet P-40) and sonicated on ice. After centrifugation at 10,000 × *g* for 15 min, the GST-fusion proteins were purified with glutathione-agarose (Sigma) as described [19].

2.6. Analysis of the protein tyrosine phosphatase activity

PTP assay was performed as described [20] using *p*-nitrophenyl phosphate (pNPP) as a substrate. Varying amounts of the GST, GST-Byp(p), or GST-Byp(p)CS protein bound to glutathione-agarose beads were incubated at room temperature for 10 min in 200 μl reaction mixture containing 50 mM imidazole (pH 7.5), 0.1% β-mercaptoethanol, and 10 mM pNPP. The reaction was stopped with 800 μl of 0.25 N NaOH, and the absorbance at 440 nm was measured.

2.7. Polyclonal antibodies against Byp

Rabbits were first immunized with 1 mg of the GST-Byp(c) or GST-Byp(ex) protein in Freund's complete adjuvant and boosted every 2 weeks with 0.5 mg of the antigens in Freund's incomplete adjuvant. Polyclonal antibodies against Byp were affinity purified using the GST and GST-Byp proteins bound to CNBr-activated Sepharose 4B (Pharmacia). Affinity purified antibodies against GST-Byp(c) and GST-Byp(ex) were named anti-BypC and anti-BypEx, respectively.

2.8. Transient expression, immunoblotting, and immunoprecipitation

A *byp* expression vector, pME-*byp*, was constructed by cloning a cDNA fragment encoding the entire open reading frame of *byp* (combined the cDNA sequences of clones 2–21 and 1–14) into an eukaryotic expression vector, pME18S [21]. Each plasmid DNA was transfected into COS7 cells (1×10^6 per 10 cm dish) by the calcium phosphate method. After 48 h of transfection, the cells were lysed in TNE buffer (50 mM Tris-HCl (pH 8.0)/1% NP-40/20 mM EDTA) containing sodium orthovanadate (1 mM) and aprotinin (50 units/ml). The proteins in the lysates from 1×10^5 cells or the precipitates by the anti-BypC antibody were fractionated by 7.5% SDS-PAGE under reducing conditions and transferred to Immobilon-P membrane (Millipore). The proteins reacted with the probe were visualized with ECL detection kit (Amersham) or ProtBlot Western AP system (Promega). For metabolic labeling, the cells were cultured for 12 h in methionine-free DMEM supplemented with 10% (v/v) dialyzed calf serum and 20 μCi/ml [^{35}S]methionine (ICN). The cells were then washed in serum-free DMEM and lysed in TNE buffer containing aprotinin at 4°C. Cell lysates were precleared with protein A-Sepharose 4B (Pharmacia LKB) for 30 min and incubated with anti-BypC for 2 h at 4°C. The immune complexes were precipitated with protein A-Sepharose, washed five times with TNE buffer, and analyzed by 7.5% SDS-PAGE and autoradiography.

2.9. In situ hybridization

The direct R-banding FISH (*f*luorescence *i*n *s*itu *h*ybridization) method was used for chromosomal assignment of the *byp* gene on mouse and rat chromosomes. Preparation of R-banded chromosomes and FISH were performed as described [22]. Briefly, the chromosome slides were hardened at 65°C for 2 h, and then denatured at 70°C in 70% formamide in 2 × SSC, and dehydrated in 70–85–100% ethanol at 4°C. The 4.2 kbp mouse *byp* cDNA fragment was labeled by nick translation with biotin 16-dUTP (Boehringer-Mannheim) following the manufacture's protocol. The fluorescence signals of hybridized probes were amplified with anti-biotin antibody (Vector), fluorescein anti-goat IgG (Nordic Immunology) and propidium iodide. Excitation at wavelength 450–490 nm (Nikon filter set B-2A) and near 365 nm (UV-2A) were used for observation.

3. Results

3.1. Isolation and sequence analysis of byp cDNA

Lpr mice have hypertrophied lymph nodes comprising CD4$^-$ CD8$^-$ T cells which express high level of the Fyn protein-tyrosine kinase [16]. Assuming that expression of some PTPs may be induced in *lpr* mice to counteract the overexpressed Fyn tyrosine kinase, we searched for a novel PTP expressed in the lymph nodes of *lpr* mice. For this purpose, we employed the RT-PCR method with the primers for the conserved PTP do-

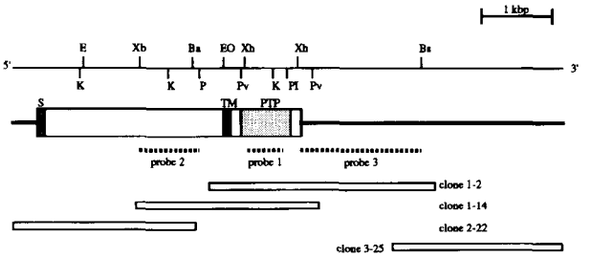


Fig. 1. Overlapping *byp* cDNA clones and a diagram of the complete cDNA structure. Untranslated sequences are represented by a line and coding sequences are boxed; shaded boxes represent sequences for the putative signal peptide (S), the transmembrane domain (TM), and the PTP domain (PTP). The dotted lines indicate the cDNA fragments used as probes for library screening and the open bars represent the four overlapping clones. Restriction map of the *byp* cDNA is shown at the top: E, *Eco*RI; K, *Kpn*I; Xb, *Xba*I; Ba, *Bam*HI; P, *Pst*I; EO, *Eco*O109; Xh, *Xho*I; PI, *Pvu*I; Pv, *Pvu*II; Bs, *Bst*EII.

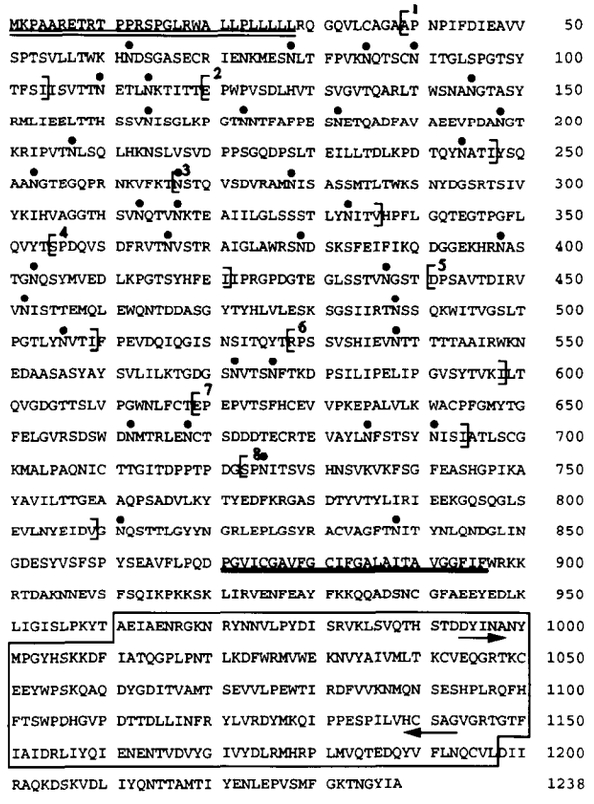


Fig. 2. Amino acid sequence of Byp. The putative signal peptide is double-underlined and the transmembrane region is single-underlined. A segment of each predicted FN-III motif is bracketed with an outlined number designating each domain. Potential *N*-glycosylation sites are indicated by dots. The sequences used to design the PCR primers are indicated by arrows. The phosphatase domain is boxed, and predicted amino acid sequence is numbered on the right.

main and mRNAs from the T cells of *lpr* mice. PCR amplification yielded about 500-bp fragments that were subsequently cloned into pUC119. Nucleotide sequencing of the cDNA inserts from 52 clones resulted in the identification of 9 clones containing cDNAs for a novel PTP. Using an insert from one of the clones as a probe (Fig. 1, probe 1), we screened a λgt11 cDNA library from lymph nodes of *lpr* mice at high stringency and obtained 10 positive clones. Restriction mapping analysis of the 10 clones revealed that clone 1-2 and clone 1-14 included the sequences of the other 8 clones. The nucleotide sequences of clones 1-2 and 1-14 showed that the cDNA inserts of these two clones did not contain a translation initiation codon or a poly(A) tail. In order to obtain further upstream and downstream sequences, the same cDNA library was screened with an *Xba*I-*Pst*I fragment of clone 1-14 (Fig. 1, probe 2) and an *Xho*I-*Bst*EII fragment of clone 1-2 (Fig. 1, probe 3). Several overlapping clones including clone 2-22 and clone 3-25 were identified as positives (Fig. 1). After determination of the nucleotide sequences of these clones (clones 1-2, 1-14, 2-22, and 3-25), a putative open reading frame of *byp* was pieced together from the four overlapping cDNA fragments (Fig. 1). The Byp protein was then predicted to consist of 1238 amino acids, calculated molecular weight being 134,000. The deduced amino acid sequence is shown in Fig. 2. The Byp protein comprises a stretch of 28-hydrophobic amino acids, which may serve as a signal peptide, at the extreme amino-terminus, followed by an 842-amino acid extracellular domain, a 26-amino acid hydrophobic transmembrane domain, and a 342-amino acid cytoplasmic region containing a single PTP domain. The PTP domain of Byp shows high homology to that of type III RPTPs such as DEP-1 (97% amino acid identity), HPTPβ (59%), DPTP10D (52%), SAP-1 or GLEPP1 (50%) (Fig. 8A). These data suggest that Byp belongs to type III RPTP family. Indeed, like other members of type III RPTPs, Byp contained multiple (eight) fibronectin type III (FN-III)-like repeats and no immunoglobulin (Ig)-like domains in the extracellular region. It also had a single PTP domain in the cytoplasmic region. Note that other types of RPTPs all contain tandemly repeated PTP domains.

3.2. Expression of byp in tissues and cell lines

To analyze the expression level of the *byp* mRNA in mouse tissues and cell lines, we performed Northern blotting experiments using the ^{32}P-labeled DNA insert of clone 1-14 as a probe. As shown in Fig. 3A, a 7.7-kb *byp* mRNA was expressed at higher level in the brain, kidney and spleen and at lower levels in the other tissues. Though *byp* cDNA was originally isolated from lymph nodes of *lpr* mice, *byp* expression was not high in this tissue. We also examined the expression of *byp* in several murine cell lines, and showed that the *byp* mRNA was not detectable in fibroblasts (NIH3T3) and erythroid (F5-5) cells, but was expressed at relatively elevated level in myeloid (FDC-P2) cells and lower level in pre-B lymphoid (WEHI-231) and T hybridoma (HBC21.7.31) cells (Fig. 3B).

3.3. Demonstration of the PTP activity of Byp

To demonstrate the PTP activity of Byp, the cDNA fragment encoding the putative PTP domain was subcloned into the pGEX-2T vector. The fusion protein encoded by the resulted plasmid was termed GST-Byp(p) and was purified from bacterial lysates. The purified protein were incubated with *p*-nitrophenyl phosphate (pNPP) and assayed for the PTP activity (Fig. 4). The GST-Byp(p) protein but not GST was able to dephosphorylate pNPP. Hydrolysis of pNPP by GST-Byp(p) was inhibited in the presence of 1 mM sodium orthovanadate, a specific inhibitor of PTPs, suggesting that Byp carried the PTP activity. In addition, we showed that a CS mutant, GST-Byp(p)CS, in which a catalytically obligatory cysteine residue at position 1140 was mutated to serine, did not exhibit the PTP activity (Fig. 4).

3.4. Transient expression of Byp in COS7 cells

To analyze the protein product of *byp*, a rabbit polyclonal antibodies were raised against the GST-Byp(c) protein containing carboxy-terminal tail of Byp or the GST-Byp(ex) protein containing the extreme amino-terminal sequence of Byp (see section 2). As shown in Fig. 5A, anti-Byp(c) could specifically detect a 220 kDa protein in the lysates of COS7 cells transfected with the *byp* expression plasmid, pME-*byp* (lane 3). The 220-kDa Byp protein was also detected by immunoprecipitation with anti-Byp(c) from the *byp*-transfected COS7 cells metabolically labeled with [^{35}S]methionine (Fig. 5B). Byp is thought to be a glycoprotein, since it contains 37 potential sites for N-linked glycosylation in the extracellular region (Fig. 2). Treatment of *byp*-transfected cells with tunicamycin, which inhibits

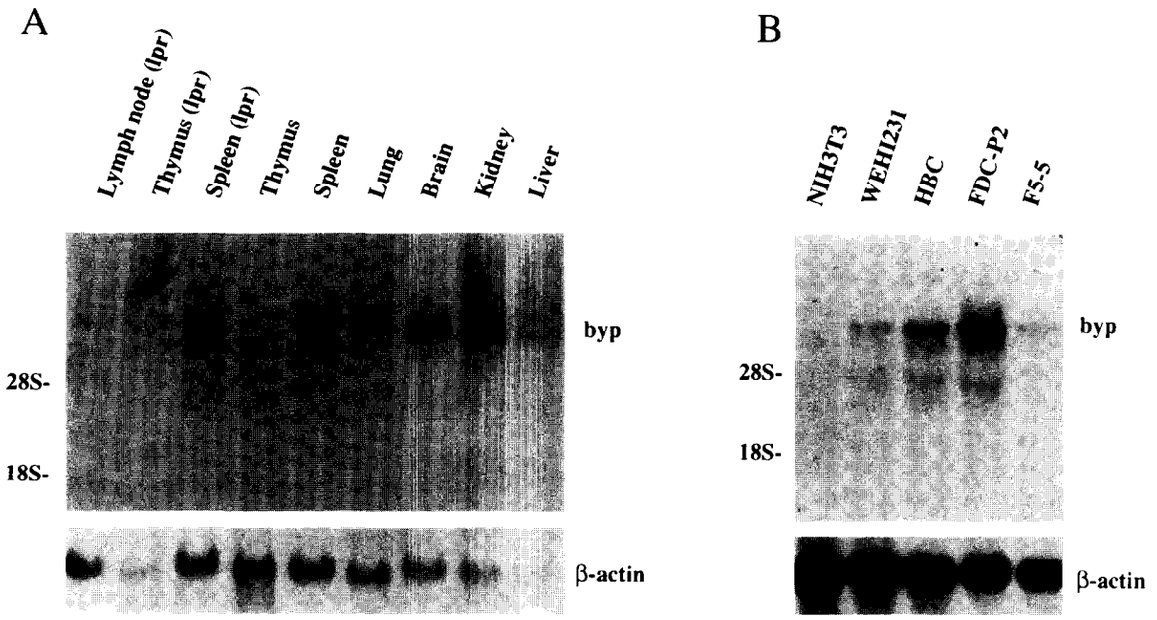


Fig. 3. Expression of the *byp* mRNA in normal and *lpr* mouse tissues and in murine cell lines. Twenty μg of total RNA prepared from the indicated tissues (A) and from different murine cell lines (B) were analyzed by Northern blotting. Filters were sequentially hybridized with the ^{32}P-labeled cDNA insert of clone 1–14 (*byp* probe) (top) and with a β-actin probe (bottom). Positions of 28S and 18S rRNA are indicated on the left.

addition of N-linked oligosaccharides at asparagine residues, reduced the apparent molecular mass of Byp to about 130 kDa (Fig. 5A, lane 4). Since the calculated molecular weight of Byp is 134,000, these data indicate that Byp is highly N-linked glycosylated. O-Linked glycosylations may be poorly occurring. Western blotting analysis showed that the size of Byp expressed in different tissues or cells slightly differed from each other (200 kDa in the brain, 250 kDa in the spleen, 220 kDa in HBC21.7.31 and 250 kDa in FDC-P2) (data not shown), which may be due to the different degree of glycosylation.

3.5. *Tyrosine phosphorylation of Byp*

Several lines of evidence suggest that PTPs may be regulated by tyrosine phosphorylation [23,24]. Tyrosine-phosphorylated PTPs may play a role in assembling multiprotein complexes through phosphotyrosine-SH2 (Src homology 2) interaction [23,25]. To examine whether the Byp protein is tyrosine phosphorylated or not, immunoblot analysis with anti-phosphotyrosine antibody was performed. As shown in Fig. 6, tyrosine phosphorylation of Byp was detected only after treatment of cells with a potent phosphatase inhibitor, pervanadate (1 mM hydrogen peroxide and 1 mM sodium orthovanadate) (lanes 2, 3). Since Byp was first identified in lymph node of *lpr* mice where the expression of Fyn tyrosine kinase is highly enhanced, we speculated that Byp might be tyrosine phosphorylated by Fyn. To examine this possibility, we co-transfected the *byp* and *fyn* expression plasmids into COS7 cells, and found that expression of Byp together with Fyn dramatically increased the level of Byp tyrosine phosphorylation (Fig. 6). Co-expression of Byp and Fak, another member of PTKs, had no effect on tyrosine phosphorylation of Byp (data not shown), suggesting that Byp can be phosphorylated by only a subset of PTKs.

3.6. *Chromosomal localization of the* byp *gene*

The chromosomal localization of the mouse and rat *byp* gene was determined by direct R-band FISH using the mouse *byp* cDNA fragment as a probe. As shown in Fig. 7, the signals were located on the E1-2 band of mouse chromosome 2 and on the

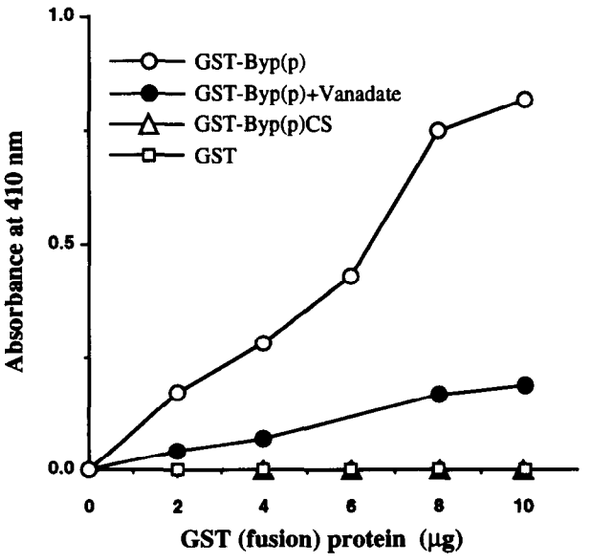


Fig. 4. PTP activity of the GST-Byp(p) fusion protein. The PTP activities of GST-Byp(p), GST-Byp(p) CS, and GST were assayed using *p*-nitrophenyl phosphate (pNPP) as a substrate. Dephosphorylation of pNPP was measured by the absorbance at 410 nm. The phosphatase activity of GST (□), GST-Byp(p)CS (△), or GST-Byp(p) in the presence (●) or absence (○) of 1 mM sodium orthovanadate is shown. The result shown is a representative of the three independent experiments.

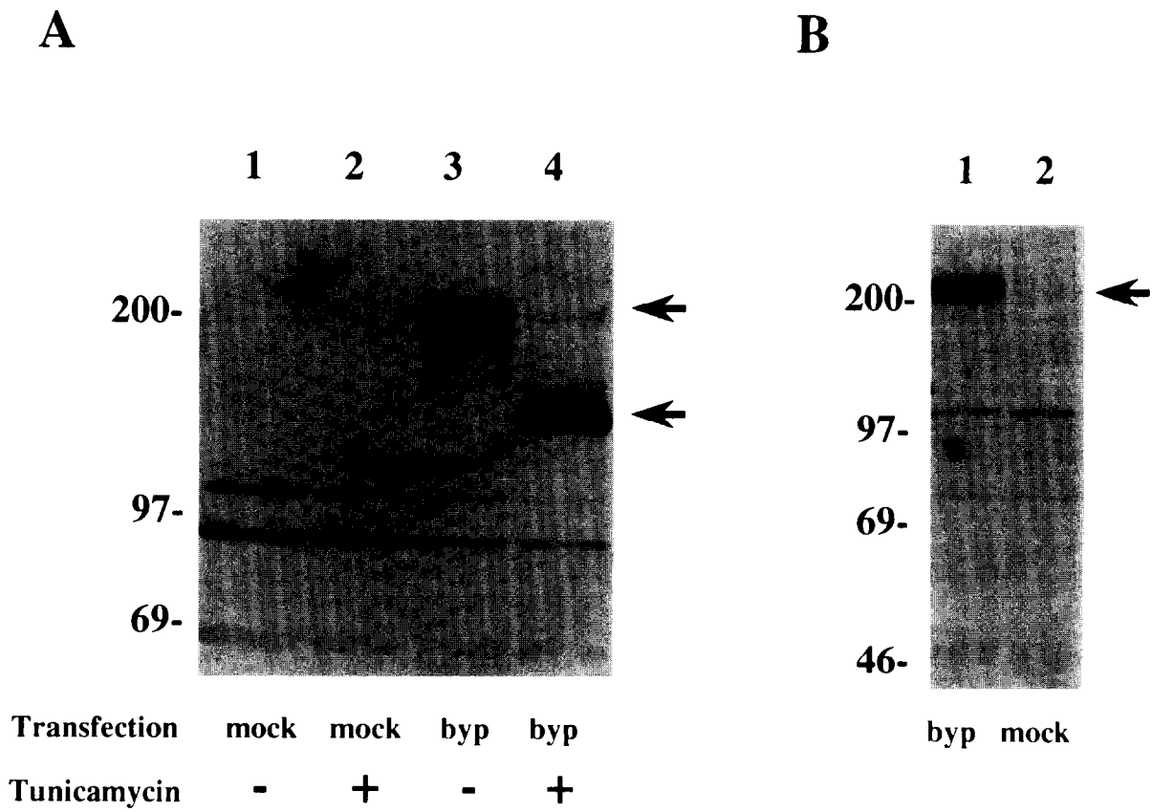


Fig. 5. Identification of the Byp protein. (A) Detection of Byp expressed in COS7 cells by immunoblotting. COS7 cells were transfected with pME-*byp* expression vector (lanes 3 and 4; byp) or empty vector (lanes 1 and 2; mock). After 24 h of transfection, the transfectants were cultured in the presence (lanes 2 and 4; +) or absence (lanes 1 and 3; −) of tunicamycin (1 mg/ml) for additional 24 h, and lysed. The proteins in the lysates were fractionated by 7.5% SDS-PAGE and immunoblotting with anti-BypC antibody. (B) Immunoprecipitaion of Byp expressed in COS7 cells. COS7 cells were transfected with pME-*byp* (lane 1; byp) or empty vector (lane 2; mock). The metabolically labeled protein in the lysates were immunoprecipitated with anti-BypC and the immunoprecipitates were analyzed by 7.5% SDS-PAGE followed by autoradiography. Molecular mass markers are indicated in kilodaltons on the left. Arrows indicate the positions of the Byp protein.

q32-33 band of rat chromosome 3. These regions are included in a large segment in which conserved linkage homology has been identified between the two species [22]. They correspond to the p11-13 region of human chromosome 11, where deletion is frequently observed in human cancers such as breast, liver, and bladder carcinomas [26–28].

4. Discussion

We have cloned a mouse transmembrane-type PTP termed Byp that contains a single PTP domain in the cytoplasmic region and eight FN-III domains and no Ig-like domains in the extracellular region. From the structural characteristics, Byp was classified as a member of type III RPTPs, which include DEP-1 (HPTPη), HPTPβ, SAP-1, GLEPP1, PTP-U2 and DPTP10D. Comparison of amino acid sequences of these proteins reveals that Byp is most homologous to human DEP-1 showing 95% amino acid identity in the cytoplasmic region and 63% identity in the extracellular region. Like murine Byp and human DEP-1, human SAP-1 and PTP-U2 that belong to type III RPTP subfamily also carry eight FN-III domains. However, the extracellular domains of these PTPs show much less homology to Byp than DEP-1 (Fig. 8B). It is noteworthy that the sequences at the extracellular domains of some receptor-like proteins significantly vary between species [29]. The *byp* gene was mapped to the region E1-2 of mouse chromosome 2 and q32-33 of rat chromosome 3 (Fig. 7). This region corresponds to the region p11-13 of human chromosome 11. Recently, the HPTPη gene was also mapped to p11.2 of human chromosome 11 [12]. The chromosome mapping data together with high degree of sequence identity suggest that Byp is a mouse homologue of DEP-1 (HPTPη).

Immunoblotting and immunoprecipitation analyses showed that the apparent molecular mass of Byp is about 220 kDa (Fig. 5), though its calculated molecular weight is 134,000. The apparent difference is largely due to the glycosylation of the Byp protein, because tunicamycin treatment entirely reduces the size of Byp to 130 kDa (Fig. 5). The degree of modification of Byp by sugar chains differs among organs and cell lines, the size of Byp being between 220 kDa and 250 kDa (data not shown). Whether the difference in the degree of glycosylation has any effect on its PTP activity and/or its ligand specificity remains to be seen.

All PTPs contain the conserved amino acid motif (I/V)HCX-AGXGR(S/T)G located near the carboxyl-terminus of the phosphatase domain. Biochemical studies have established that

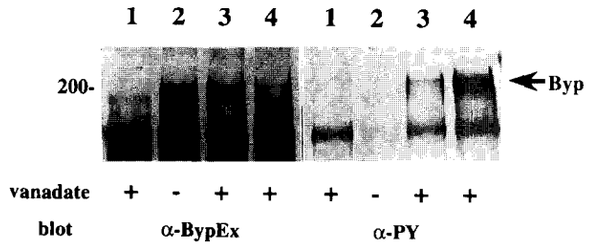


Fig. 6. Tyrosine phosphorylation of the Byp protein. COS7 cells were transfected with 7 μg of empty vector (lane 1), 7 μg of pME-*byp* (lanes 2 and 3), 7 μg of pME-*byp* and 5 μg of pME-*fyn* [21] (lane 4). Two days after transfection, cells were treated with 1 mM sodium orthovanadate and 1 mM hydrogen peroxide for 10 min (lanes 1, 3 and 4) and lysed in TNE buffer. The proteins immunoprecipitated with anti-BypC were analyzed by immunoblotting with anti-BypEx antibody (left) or anti-phosphotyrosine antibody, PY-20 (ICN) (right). The arrow indicates the position of the Byp protein.

the cysteine residue within this consensus motif is essential for the PTP activity and forms a covalent thiol-phosphate bond with the phosphorylated tyrosine residue in the substrate [4,30]. Mutation of this cysteine results in complete loss of the PTP activity [31]. Indeed, the GST-Byp(p)CS protein containing a Cys to Ser mutation at position 1140 of Byp shows no PTP activity (Fig. 4). PTPs with the Cys to Ser mutation have been reported to associate stably with their substrates. For example, the Cys to Ser mutants of 3CH134 and CD45 well associate with $p44^{MAPK}$ [32] and the CD3ζ chain [33], respectively. Therefore, the Cys to Ser mutant could help identify the substrate of each PTP. The tight association between the Cys to Ser mutant of Byp and the Fyn kinase was observed in COS7 cells (unpublished data), suggesting their functional interaction. We also demonstrated that the Byp protein expressed in COS7 cells was tyrosine phosphorylated, and co-expression of the Fyn tyrosine kinase dramatically increased tyrosine phosphorylation of Byp. The Fyn kinase is highly expressed in the unusual $CD4^-$ $CD8^-$ T-cells of lymph node of *lpr* mice [16]. Thus, it is likely that Byp is highly tyrosine phosphorylated in the lymphocytes of *lpr* mice and thereby the activity of Byp may be modulated in these cells.

Recent reports demonstrate that tyrosine phosphorylation of PTPs is involved in regulation of their activity. PTP1C (also known as SH-PTP1, HCP, or SHP) is directly phosphorylated on two tyrosine residues by Lck [24]. CD45 is tyrosine phosphorylated by CSK and tyrosine phosphorylation of CD45 causes an increased PTP activity and its association with the SH2 domain of Lck [23]. It is also known that SH-PTP2 (also known as PTP1D, PTP2C, SH-PTP3, or Syp) binds via its SH2 domain to phosphotyrosines on the platelet-derived growth factor receptor, epidermal growth factor receptor and IRS-1 in response to the extracellular stimuli. In addition, ligand stimulation of these receptors induces tyrosine phosphorylation of SH-PTP2 [34–37]. Therefore, it is likely that tyrosine phosphorylation of Byp alters its PTP activity and/or cause association with various SH2- or PTB (phosphotyrosine binding domain)-containing molecule(s) [38,39], leading to activation of downstream signaling.

Type III RPTPs show high degree of sequence similarity with each other in their cytoplasmic regions compared with other types of RPTPs. They may have evolutionary conserved function in intracellular signaling. We show here that Byp is phosphorylated on tyrosine residues. There are twenty tyrosine residues within the intracellular region of Byp. The intracellular domains of DEP-1, HPTPβ, and GLEPP-1 also contain many tyrosine residues which might be tyrosine phosphorylated. An YEN(L/V) amino acid motif is conserved in the carboxyl-terminus of these RPTPs. This motif is a potential binding site of the SH2 domain of the 3BP2 protein [40]. In addition to the SH2 domain, 3BP2 contains a proline-rich domain and a plekstrin-homology (PH) domain, which implies its participation in signal transduction [41]. Thus, the 3BP2 protein might mediate signaling downstream of type III RPTPs. Although the role of Byp in cellular signaling is yet to be established, the present data should provide clues to unravel the biological significance of RPTPs including Byp.

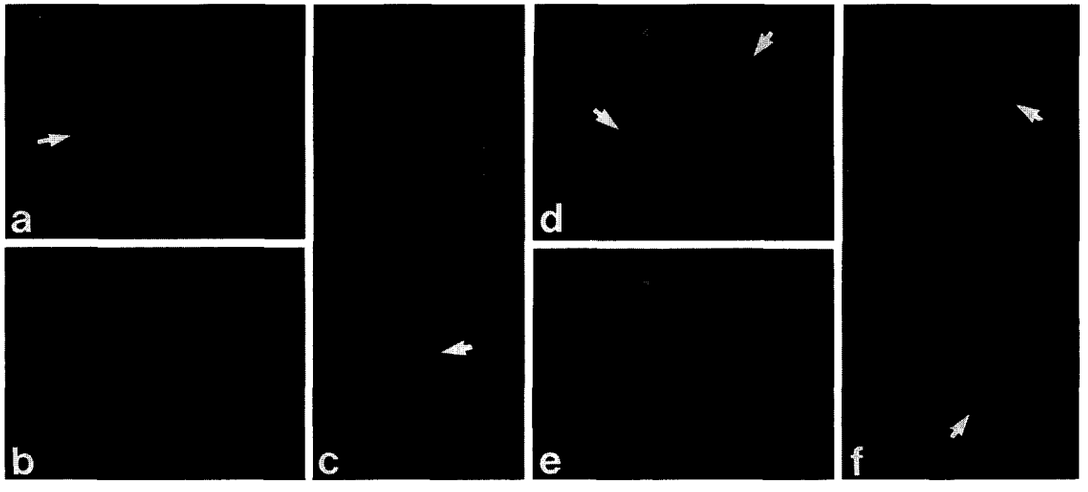


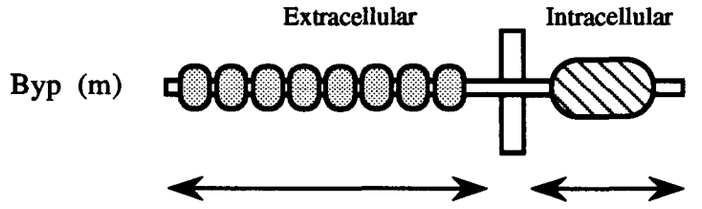
Fig. 7. Chromosomal localization of the *byp* gene. A 4.2 kbp mouse *byp* cDNA fragment was used as a biotinylated probe for in situ hybridization on mouse and rat R-banded chromosomes. The hybridization signals are indicated by arrows. The signals are located on 2E1-2 band of mouse chromosome 2 (a,c) and q32-33 band of rat chromosome 3 (d,f). The metaphase spreads were photographed with Nikon B-2A (a, b and d–f) and UV-2A (c) filters. R-band and G-band patterns are demonstrated in (a, c, d and f) and (b, e), respectively.

```
Byp      (961) AEIAENRGKNRYNNVLPYDISRVKL SVQTHSTDDYINANYMPGYHSKKDFIATQGPLPNTLKDFWRMVWEKNVYAIVMLT
DEP-1   (1060) --L---------------------- ----------------------------------------------------I---
HPTPβ   (1672) -LLP----------I----AT----SN-DDDPCS-----S-I--NNFRREY-V------G-KD---K----Q--HN---V-
SAP-1    (841) -SAS--NA----R------W---P-KPIHEEPGS-----SF---LW-PQE---------Q-VG----L---QQSHTL----
PTP-U2   (957) -DLPL--C----T-I----F---R-V-MNEEEGA-------I---N-PQEY--------E-RN---K--LQQKSQI-----
GLEPP1   (928) -DLPL--C----T-I----F---R-L-MNEEEGA-------I---N-PQEY--------E-RN---K--LQQKSQM-----
DPTP10D(1291) -DLPC--P---FT-I----H--F--QP-DDDEGS-------V--HN-PRE--V-----HS-RD-----C--S-SR------
OST-PTP(1169) --HPD-II----PH-----H---R-TQLPGEPHS------FI---SHTQEI-------KK--E----L---QQ-HV-I---

Byp      (1041) KCVEQGRTKCEEYWP SKQAQDYGDITVAMTSEVVLPEWTIRDFVVKNMQNSESHPL RQFHFTSWPDHGVP  DTTDLLI
DEP-1    (1140) --------------- -----------------I----------T---I-T------ --------------  -------
HPTPβ    (1781) Q---K--V--DH---ADQDSLY---LILQ-L--S--------E-KICGEEQLDA---I-H--Y-V-------  E--QS--
SAP-1     (877) N-M-A--V---H---LDS-PCTH-HLRVTLVG-E-MEN--V-ELLLLQVEEQKTLS V----YQA-------  SSP-T-L
PTP-U2   (1038) Q-N-KR-V--DH---FTEEPIA------E-I--EEQDD-AC-H-RINYADEMQD   VMH-NY-A-------TANAAESIL
GLEPP1   (1009) Q-N-KR-V--DH---FTEEPIA------E-I--EEQDD-AH-H-RINYADEMQD   VMH-NY-A-------TANAAESIL
DPTP10D(1371) R-F-K--E--DQ---NDTVPVF----K-QILNDSHYAD-VMTE-MLCRGSEQ   RIL-H----T---F---  NPPQT-V
OST-PTP(1250) VGM-N--VL--H---ANSTPVTH-H--IHLLA-EPED---R-E-QLQHGTEQKQRR VK-LQ--T----S--  EAPSS-L

Byp      (1120) NFRYLVRDYMKQIPPESPILVHCSAGVGRTGTFIAIDRLIYQIENENTVDVYGIVYDLRMHRPLMVQTEDQYVFLNQCVL
DEP-1    (1219) ------------S-------------------------------------------------------------------
HPTPβ    (1862) Q-VRT----INRS-GAG-TV---------------L--ILQ-LDSKDS--I--A-H---L--VH-----C---Y-H---R
SAP-1     (957) A-WRML-QWLD-TMEGG-PI------------L--L-V-LR-LQS-GLLGPFSF-RKM-ES--------A-----H--IC
PTP-U2   (1118) Q-VHM--Q  QATKSKG-MII--------------L---LQH-RDHEF--IL-L-SEM-SY-MS-----E--I-IH---Q
GLEPP1   (1089) Q-VHM--Q  QATKSKG-MII--------------L---LQH-RDHEF--IL-L-SEM-SY-MS-----E--I-IH---Q
DPTP10D(1449) R-VRAF--RI  GAEQR--V---------S----TL--ILQ--NTSDY--IF----AM-KE-VW-----Q--ICIH--L-
OST-PTP(1360) A-VE--QEQVQATQGKG----------------V-LL--LR-L-E-KVA--FNT--I--L-----I--LS--I--HS-L-
```

A



B

DEP-1 (h)	63 %	95 %
SAP-1 (h)	27	43
PTP-U2 (h)	20	46

Fig. 8. Comparison of Byp with other type III RPTPs. (A) Sequences of the catalytic domain of the following PTPs are aligned: Byp, DEP-1 [11], SAP-1 [10], PTP-U2 [15], GLEPP1 [13], DPTP10D [8,9], and OST-PTP [14]. The numbers in parentheses indicate the amino acid positions. Dashes indicate amino acid residues identical to those of Byp. (B) Schematic representation of the predicted Byp protein showing the FN-III motifs (dotted) and the PTP domain (hatched). The numbers indicate the percent amino acid identity at the extracellular domain and cytoplasmic domain between Byp and each PTP that carries eight FN-III motifs. m = mouse; h = human.

Acknowledgments: We thank J. Tsuzuku for technical support, Y. Shiio for valuable discussions, T. Katagiri for providing us with *lpr* mice, and A. Tojo for providing us with FDC-P2 cells. This work was supported in part by Human Frontier Science Program grant and Grants-in-Aid for Advanced Cancer Research from the Ministry of Education, Science, Sports, and Culture of Japan. S.K. is a fellow of the Japan Society for the Promotion of Science.

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