Association of protein-tyrosine phosphatase PTP-BAS with the transcription-factor-inhibitory protein IκBα through interaction between the PDZ1 domain and ankyrin repeats

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PTP-BAS is a membrane-associated protein tyrosine phosphatase containing a band-4.1 homology region and five PDZ (**P**SD-95 **D**lg **Z**O-1) [discs-large homology region ('DHR')/Gly-Leu-Gly-Phe ('GLGF')] domains. The second and fourth PDZ domains were reported to associate with Fas/CD95. By using the first PDZ domain as a bait in yeast two-hybrid screening, we have identified IκBα as a binding protein. IκBα associated with PDZ1 through the stretch of the N-terminal three ankyrin repeats. The association was also confirmed in HeLa cells by co-immunoprecipitation experiments. Inhibition of PTP-BAS by expression of dominant-negative PTP-BAS mutant resulted in tyrosine-phosphorylation of IκBα. Tyrosine-phosphorylation of IκBα is a key event in activation of nuclear factor (NF)-κB during re-oxygenation. PTP-BAS may thus play a regulatory role in activation of NF-κB under high oxidative stress.

Key words: FAP-1, hPTP-1E, PTP-L1, PTP-N13, tyrosine phosphorylation

INTRODUCTION

Protein-tyrosine phosphorylation, which plays a pivotal role in the regulation of cellular activities such as growth and differentiation, is regulated by the balance between protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs) [1]. In most cases PTPs attenuate signals generated by PTKs, but some PTPs, such as SHP-2 and CD45, are directly involved in signalling pathways [2,3]. There are two types of PTPs, namely the receptor and the cytoplasmic types. Since the PTP domains usually show broad substrate specificity, correct subcellular localization and specific protein–protein interactions are considered to be important for their specific biological functions.

PTP-BAS is a cytoplasmic PTP that has been originally cloned from a human basophilic leukaemia cell line [4] and also known as PTP-L1 [5], hPTP-1E [6] or PTPN13 [7]. Notably, the apoptosis-inducing membrane protein, Fas/CD95, was found to associate with FAP-1 which is identical with PTP-BAS [8]. PTP-BAS is expressed in various human tissues, especially in the kidney and lung at high levels [4], but its biological functions in these organs remain to be addressed.

PTP-BAS contains a band-4.1 homology region, five PDZ (90-amino-acid repeat first found in **P**SD-95 **D**lg **Z**O-1), also known as DHR (discs-large homology region) or GLGF (Gly-Leu-Gly-Phe) domains and a PTP domain (Figure 1 below). The band-4.1 homology region is supposed to serve as a membrane- and/or cytoskeleton-binding module [9]. PDZ domains are found in more than 40 cytoplasmic proteins, many of which are specifically located at regions of cell–cell contact such as tight junctions, septate junctions and synaptic junctions [10]. The PDZ domain is considered to function in clustering membrane proteins and/or linking signalling molecules to a multiprotein complex at specialized membrane sites [11]. Thus PTP-BAS is associated with submembranous cytoskeleton by its band-4.1 homology region and is likely to regulate signalling events by its PTP activity through interacting with its target molecules via the PDZ domains.

Recently, the recognition motifs of some PDZ domains were identified. For example, the PDZ1 and PDZ2 of PSD-95 bound to the C-terminal Thr/Ser-Xaa-Val motifs of Shaker-type K^+ channels and of NR2 subunits of the *N*-methyl-D-aspartate receptor [12,13]. Similarly, the PDZ2 of PTP-BAS/FAP-1 was found to bind to the C-terminal Thr-Xaa-Val motif of Fas [8]. A molecular basis for C-terminal peptide recognition of the PDZ3 of PSD-95 and hDlg was uncovered by X-ray crystallography, revealing a groove structure with a conserved hydrophobic pocket and a buried arginine residue as the binding site [14,15]. In the present study we used the PDZ1 of PTP-BAS as a bait in yeast two-hybrid screening and identified IκBα as a specific association protein. In contrast with other instances, the interaction requires a stretch of the N-terminal three ankyrin repeats instead of the C-terminal region of IκBα. Thus this PDZ domain may recognize the tertiary structure of the ankyrin repeats. The association of IκBα with PTP-BAS may indicate a novel regulatory mechanism of NF-κB involving PTP-BAS.

MATERIALS AND METHODS

Yeast two-hybrid screening

A cDNA fragment encoding the PDZ1 domain (corresponding to amino acids 988–1222 of PTP-BAS type 1; [4]) was subcloned into the pBTM116 vector [16] and used as a bait to screen the human kidney MATCHMAKER cDNA library (Clontech). The yeast strain L40 harbouring *HIS3* and *lacZ* reporter genes under the control of upstream LexA-binding sites [16] was transformed, and His^+ colonies were selected. The positive colonies were further assayed for β-galactosidase activity. β-Galactosidase activity was measured as described in [17]. Briefly, overnight-cultured yeast clones in 5 ml of medium were harvested by centrifugation. The pellets were resuspended into 5 ml of Z

Abbreviations used: PTP, protein tyrosine phosphatase; PDZ, 90-amino-acid repeat first found in **P**SD-95 **D**lg **Z**O-1; PSD, postsynaptic density; PTK, protein tyrosine kinase; DHR, discs-large homology region; GLGF, Gly-Leu-Gly-Phe; NF-κB, nuclear factor-κB; GST, glutathione S-transferase; HA, haemagglutinin; IκBα, specific association protein IκBα.

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buffer (60 mM Na_2HPO_4/40 mM NaH_2PO_4/10 mM KCl/1 mM $MgSO_4$) containing 0.27% (v/v) of 2-mercaptoethanol and the A_{600} values measured. Yeast cells in resuspended solution were lysed by two repeated cycles of freezing in liquid nitrogen and thawing at 30 °C then centrifugation for 10 min. An aliquot of the supernatant (0.5 ml out of 5 ml) was preincubated at 30 °C and mixed with 0.1 ml of 4 mg/ml *o*-nitrophenyl β-D-galactopyranoside in Z buffer. After 10 minutes at 30 °C, the reaction was interrupted by adding 0.25 ml of 1 M Na_2CO_3 and the A_{420} was measured. β-Galactosidase activities were expressed as tentative enzyme units normalized by the A_{600} unit (units/A_{600}), where:

$$1 \text{ unit} = 1000 \times A_{420} \times 0.85/\text{min}.$$

Specificity of positive clones was confirmed using the PDZ1 domain with a five-amino-acid deletion (amino acids 1141–1145) in the middle as a bait.

Production and purification of GST-PDZ1 and GST-PDZ1del

A cDNA fragment encoding the PDZ1 (amino acids 998–1223) was subcloned into the *Sma*I site of the pGEX4T2 plasmid (Pharmacia) to produce GST-PDZ1 protein in *Escherichia coli* DH5α (Gibco–BRL). A pGEX-PDZ1del plasmid containing an eight-amino-acid deletion (amino acids 1141–1148) in the middle of PDZ1 was generated using PCR. The fusion proteins were purified using glutathione–Sepharose 4B (Pharmacia) according to the manufacturer's instruction manual.

In vitro binding of IκBα to GST-PDZ1

GST-PDZ1 or GST-PDZ1del protein (10 μg) was immobilized on glutathione–Sepharose 4B beads. [^{35}S]Methionine-labelled IκBα and its deletion products (Figure 3A below) were prepared using the TnT® T7-coupled reticulocyte-lysate system (Promega) and 10 μl of the lysates were individually mixed with the immobilized GST-PDZ1 or GST-PDZ1del beads in binding buffer [50 mM Tris/HCl (pH 8.0)/150 mM NaCl/5 mM dithiothreitol/1 mM EDTA/0.1% Triton X-100/protease inhibitors:complete® (Boehringer Mannheim)] overnight at 4 °C. After extensive washing with the binding buffer, beads were boiled in Laemmli buffer [18] and subjected to SDS/PAGE [18] and autoradiography.

Binding of IκBα to PDZ1 in HeLa cells

IκBα cDNA was subcloned into the pBluescript plasmid (Stratagene). Fragments of PTP-BAS cDNA encoding the PDZ1-PTP domains (amino acids 976–1222/2213–2485) or the PTP domain (amino acids 2213–2485) alone were subcloned into the pGEM-1 plasmid (Promega) under a T7 promoter with an influenza-virus haemagglutinin (HA) epitope tag sequence (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala). Transfection into HeLa cells was carried out using a recombinant vaccinia-virus technique as described [19] and the infected cells were lysed with TTE buffer [10 mM Tris/HCl (pH 7.8)/1% Triton X-100/150 mM NaCl/1 mM EDTA/protease inhibitors:complete®]. Supernatants were pretreated with Protein G–Sepharose 4B and centrifuged. Supernatants were then incubated with 1 μg of either anti-HA monoclonal antibody (12CA5; Boehringer Mannheim) or anti-IκBα polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) in the presence of 10 μl bed volume of Protein G–Sepharose 4B beads for 1 h at 4 °C. After washing six times with TTE buffer, the beads were subjected to SDS/PAGE and analysed by Western blotting using anti-HA or anti-IκBα antibodies.

Tyrosine phosphorylation of IκBα by expression of dominant-negative PTP-BAS mutant

The pGEM1HA-PDZ1-PTP(2408CS) plasmid, which expresses HA-tagged PDZ1-PTP(2408CS) domains (amino acids 976–1222/2213–2485; Cys^{2408} was converted into Ser), was generated by PCR. The mutant, in which Cys was converted into Ser in the PTPase domain, lacks phosphatase activity [20], and was expected to act as dominant-negative in the cells expressing PTP-BAS. IκBα and catalytically inactive HA-tagged PDZ1-PTP(2408CS) were expressed in HeLa cells using a recombinant-vaccinia-virus technique, and immunoprecipitated with anti-IκBα antibodies as described above. The precipitates were separated by SDS/PAGE and analysed by Western blotting using anti-phosphotyrosine monoclonal antibody (Transduction Laboratories, Lexington, KY, U.S.A.).

RESULTS

Isolation of IκBα by yeast two-hybrid screening

PTP-BAS is a cytoplasmic PTP which has a band-4.1 homology region and five PDZ domains (Figure 1). To identify molecules associating with PTP-BAS, we constructed a yeast bait vector, pBTM116G1, which encodes the PDZ1 domain fused in frame to the LexA DNA-binding domain. By screening 10^7 transformants of human kidney cDNA library in a yeast two-hybrid system, two positive clones were isolated. One of the clones, G1Y4, encoded IκBα. In the present study we examined the interaction of PTP-BAS and IκBα in detail.

G1Y4 contained a full-length coding region of IκBα, 5′-untranslated region, and a part of a 3′-untranslated region (Figure 2). Since the 5′-untranslated region contained no in-frame stop codon, the coding region of IκBα was translated as a fusion protein in the yeast. To assign the region required for binding to the PDZ1, various deletion constructs were prepared from G1Y4 and examined in the yeast two-hybrid assay. As shown in Figure 2, the stretch of the N-terminal three ankyrin repeats was necessary and sufficient for the binding. The sequence required for the association is too long to assume that this PDZ also recognizes a short stretch of peptide motif like most other PDZs, as previously reported. Thus, the PDZ1 of PTP-BAS may recognize a tertiary structure of the ankyrin repeats.

The binding of the PDZ1 of PTP-BAS to other IκB family or molecules containing multiple ankyrin repeats has not been examined yet. As mentioned above, we have also isolated an additional cDNA which encodes a fragment of unknown protein with 20 ankyrin repeats. Thus it seems possible that the PDZ1 domain of PTP-BAS associates with some other molecules containing multiple ankyrin repeats besides IκBα.

In vitro binding

To confirm the association between the PDZ1 and IκBα *in vitro*, GST-PDZ1 fusion protein and its deletion product, GST-PDZ1del, were prepared and immobilized on glutathione–Sepharose beads. *In vitro*-translated IκBα or its various deletion products (Figure 3A) were mixed with the beads, and the bound products were analysed by SDS/PAGE and autoradiography. As shown in Figure 3(B), IκBα and its deletion products, which have at least the stretch of the N-terminal three ankyrin repeats manifested binding to the PDZ1. In contrast, the PDZ1del, which lacks eight amino acids in the middle of PDZ1, failed to trap the full-length IκBα protein.

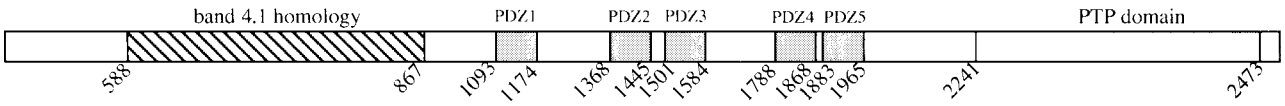

Figure 1 Schematic diagram of PTP-BAS

PTP-BAS consisted of a band-4.1-homology region, five PDZ domains and a PTPase catalytic domain. Positions of functional domains of PTP-BAS are illustrated.

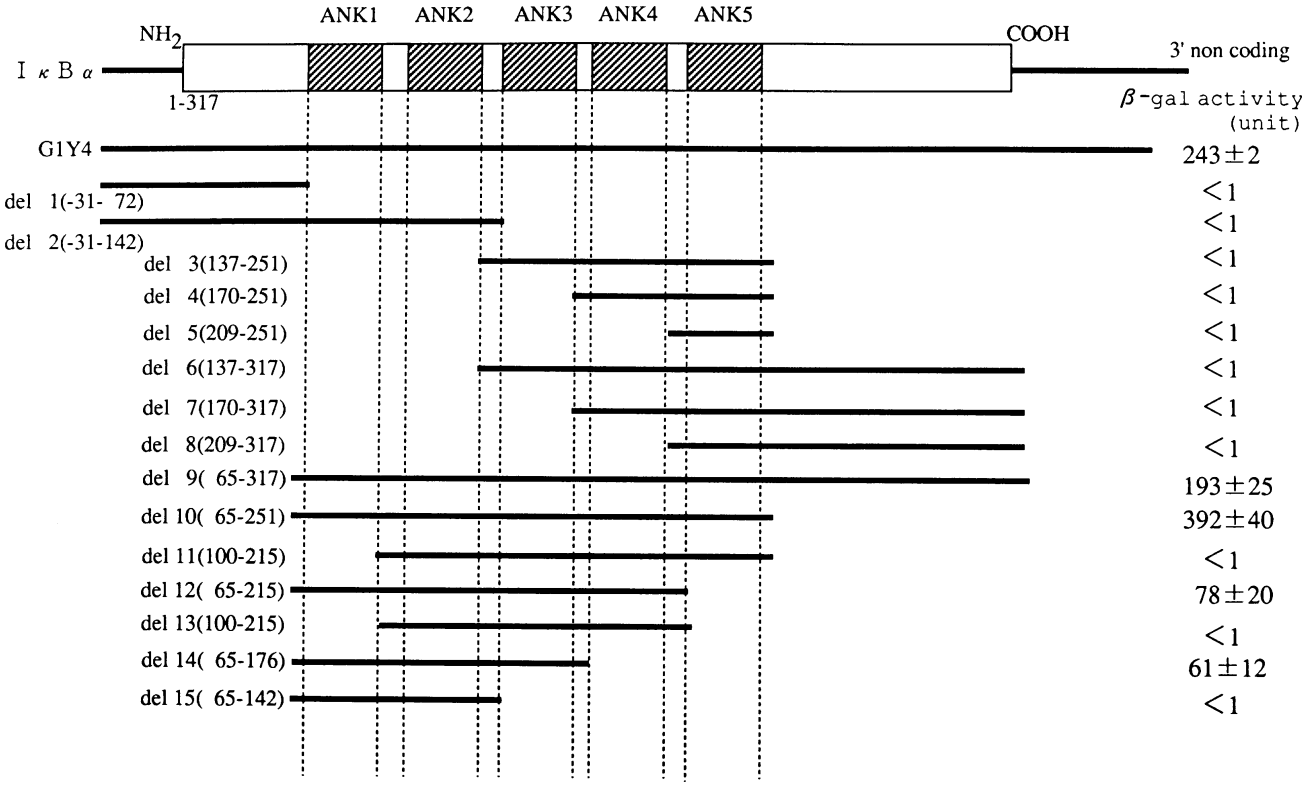

Figure 2 PTP-BAS binds ankyrin repeats of IκBα in yeast two-hybrid assay

Schematic diagrams of IκBα cDNA, G1Y4 (cDNA insert isolated by yeast two-hybrid screen) and its deletion constructs used in yeast two-hybrid assay. Positions of five ankyrin repeats of IκBα are illustrated. Constructs are depicted by **bold** lines. Amino acid positions are shown on the left side. The results of β-galactosidase (β-gal) assay are shown on the right. Results from three individual experiments are summarized.

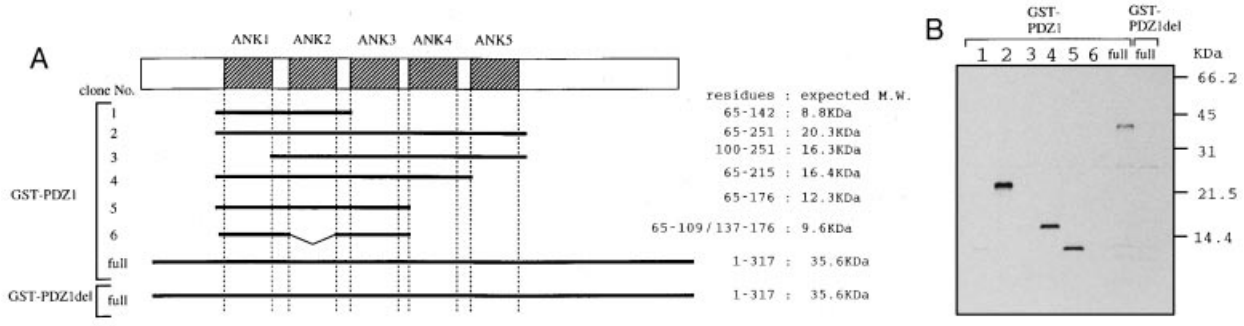

Figure 3 The PDZ1 domain of PTP-BAS interacts with IκBα *in vitro*

(**A**) Schematic diagrams of IκBα and its deletion constructs used in *in-vitro* binding assay. Constructs are depicted by bold lines. Amino acid positions and expected molecular mass ('M. W.') in kDa ('KDa') are shown on the right. (**B**) *In vitro* association of [^{35}S]methionine-labelled IκBα and its deletion products with GST-PDZ1 or GST-PDZ1del.

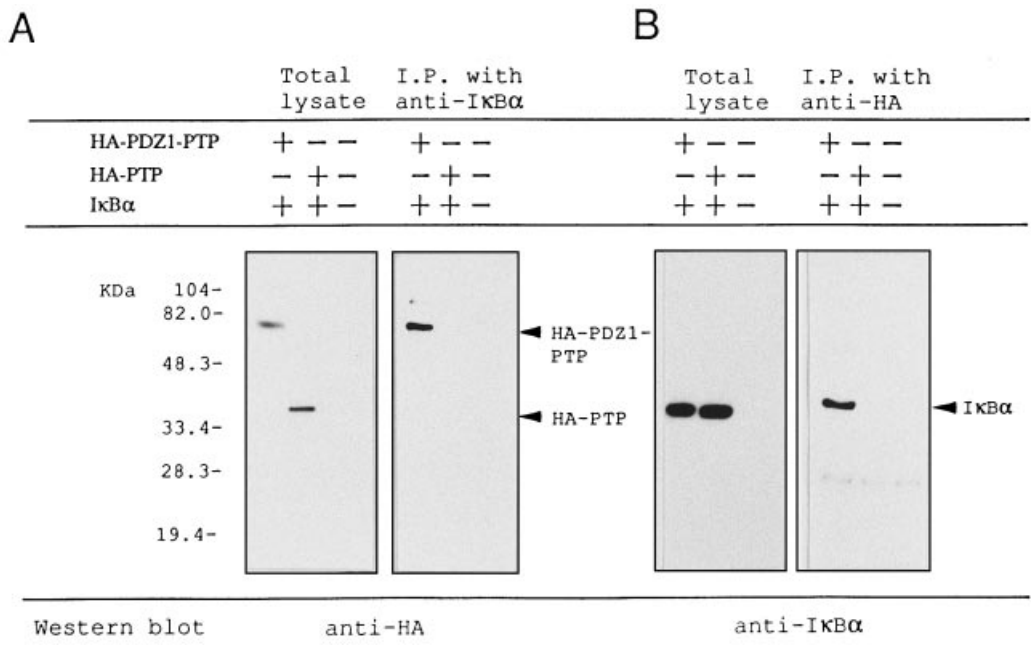

Figure 4 Association of IκBα with PDZ1 of PTP-BAS in HeLa cells

Cell lysates were analysed by Western blotting before and after immunoprecipitation. Lysates were precipitated with either anti-IκBα antibodies (**A**, right panel) or anti-HA antibody (**B**, right panel). Blotting was done using anti-HA antibody (**A**) or anti-IκBα antibodies (**B**).

In vivo binding

We next examined the association of IκBα with the PDZ1 of PTP-BAS in intact cells. Because of the tight binding of PTP-BAS to cytoskeletal structures through its band-4.1-homology region, we were incapable of solubilizing full-length PTP-BAS in lysis buffers without disrupting its associated molecules. Thus, the pGEM1-HA-PDZ1-PTP plasmid, which encodes an HA-tagged PDZ1-PTP fusion protein, was transiently co-transfected with the pGEM1-IκBα plasmid into HeLa cells using a vaccinia-virus system to achieve high expression [when β-galactosidase control plasmid was expressed via this system, about 40% of HeLa cells showed strong β-galactosidase activity with 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside ('X-Gal') staining]. Cell lysates were then subjected to immunoprecipitation and SDS/PAGE, then analysed by Western blotting. As shown in Figure 4(A), the HA-tagged PDZ1-PTP, but not the HA-tagged PTP, a PDZ1-deleted control protein, was co-precipitated by anti-IκBα antibodies in the presence of IκBα. Similarly, IκBα was co-precipitated by anti-HA monoclonal antibody only from the lysate containing HA-PDZ1-PTP and IκBα (Figure 4B). Thus IκBα is able to physically associate with the PDZ1 of PTP-BAS in intact cells.

Tyrosine phosphorylation of IκBα by co-expression of dominant-negative PTP-BAS mutant in HeLa cells

Finally, to address the question of whether PTP-BAS could affect the tyrosine phosphorylation of IκBα, a catalytically inactive mutant, HA-PDZ1-PTP(2408CS), was employed. The conversion from a conserved cysteine residue into serine in the PTP catalytic domain causes the disappearance of tyrosine-phosphatase activity, and this mutant was expected to act as the dominant-negative of PTP-BAS in HeLa cells. This mutant binds to IκBα as well as to PTP-BAS, but cannot dephosphorylate proteins, including IκBα.

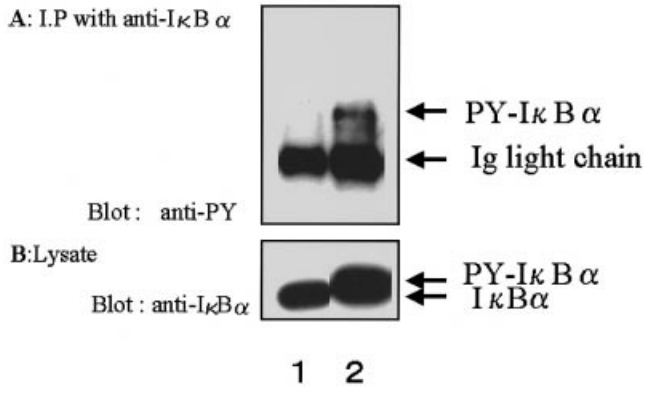

Figure 5 Tyrosine phosphorylation of IκBα is enhanced by expression of phosphatase negative mutant

Cells expressing IκBα alone (lane 1) and IκBα and HA-PDZ1-PTP(2408CS) (lane 2) were lysed and then precipitated with anti-IκBα antibody. The precipitates (**A**) or lysates (**B**) were analysed by anti-phosphotyrosine (**A**) or anti-IκBα (**B**) Western blotting. Tyrosine-phosphorylated IκBα shows a lower mobility than non-phosphorylated IκBα.

HeLa cells expressing IκBα only, or IκBα and HA-PDZ1-PTP(2408CS) together, were lysed and immunoprecipitated with anti-IκBα antibodies, followed by Western blotting with anti-phosphotyrosine monoclonal antibody. As shown in Figure 5, IκBα was tyrosine-phosphorylated by overexpression of HA-PDZ1-PTP(2408CS). The level of tyrosine phosphorylation could

be estimated from Figure 5(B). Western blotting of total cell lysate with anti-IκBα antibodies shows two molecular species of IκBα. The slightly higher band shows the tyrosine-phosphorylated form of IκBα. In Figure 5(B), tyrosine-phosphorylated and non-phosphorylated forms of IκBα in the presence of dominant-negative form of PTP-BAS exhibit almost the same band intensity, suggesting half of IκBα remains tyrosine-phosphorylated. It seems reasonable, therefore, to suppose that this phosphorylation of IκBα has biological significance.

Thus the inhibition of interaction between PTP-BAS and IκBα resulted in tyrosine-phosphorylation of IκBα. This result suggests that PTP-BAS could dephosphorylate IκBα *in vivo*.

DISCUSSION

In the present study we have demonstrated the association of IκBα with the PDZ1 of PTP-BAS. The target recognition of this PDZ, however, appears quite different from those of other PDZs previously reported. The PDZ2 of PSD-95 binds to the C-terminal Thr/Ser-Xaa-Val motifs of Shaker-type K^+ channels and of NR2 subunits of *N*-methyl-D-aspartate receptor, and the PDZ1 of PSD-95 binds weakly to the C-terminal motif of Shaker-type K^+ channels [12,13]. Similarly, the PDZ2 of PTP-BAS/FAP-1 was shown to bind to the C-terminal Thr-Xaa-Val motif of Fas ([8]; note that PDZ2 is referred to as GLGF3 in that article). Songyang et al. [21] demonstrated, by using the oriented-peptide-library technique, that nine PDZ domains they examined showed selective preferences in binding to the unique optimal motifs defined primarily by the C-terminal three to seven residues of peptides. Recently, the PDZ4 of PTP-BAS/PTPL1 was also shown to interact with the C-terminal tail of Fas [22]. The C-terminal peptide recognition of PDZ domains was supported by the X-ray crystallography of the PDZ3 of PSD-95 and human Dlg, revealing a groove structure with a conserved hydrophobic pocket and a buried arginine residue as the binding site for the C-terminal peptide [14,15]. Thus the requirement of the stretch of the N-terminal three ankyrin repeats for the association with the PDZ1 of PTP-BAS is unique. Evidently, this PDZ does not recognize the C-terminal sequence of IκBα. Since the stretch is too long to assume the recognition of a linear peptide motif, this PDZ appears to recognize a tertiary structure formed by three ankyrin repeats. The lower β-galactosidase activity of IκBα mutants containing three ankyrin repeats than that of mutants containing all ankyrin repeats in yeast two-hybrid system is consistent with this assumption. Although the tertiary structure of the ankyrin repeats of IκBα is not available yet, it seems possible that side chains of some amino acids of the three ankyrin repeats make a conformation which mimics a short stretch of peptide sequence to be recognized by the PDZ1 of PTP-BAS. Alternatively, this PDZ may recognize a more complex structure of the ankyrin repeats. It may be similar to the homotypic interaction between the PDZ of neuronal nitric oxide synthase and PDZ2 of PSD-95 in a sense that the C-terminal peptide motif is not involved in the association [23]. The homotypic interaction was, however, blocked by the C-terminal peptide of *N*-methyl-D-aspartate receptor type 2B, suggesting that the recognition site of the PDZ2 for the homotypic interaction is near to, or overlapping with, that for the C-terminal peptide. The assignment of the region of PDZs required for the homotypic interaction was not made in that study [23].

Our finding that IκBα associates with PTP-BAS evokes a notion that PTP-BAS plays a role in suppression of NF-κB activation. Inactive NF-κB is present in the cytosol associated with an inhibitory molecule of the IκB family such as IκBα [24]. Phosphorylation of IκBα results in the activation of NF-κB which translocates to the nucleus. There are two pathways of NF-κB activation. The first pathway used by a variety of stimuli involves the phosphorylation of IκBα on Ser^{32} and Ser^{36} at its regulatory N-terminus, leading to the degradation of IκBα mediated by ubiquitin proteasome system and to the release of NF-κB. The second pathway involves the phosphorylation on Tyr^{42}, leading to the release of NF-κB without degradation of IκBα. This pathway was reported in the case of PTP inhibitor (such as pervanadate) treatment and, more importantly, of reoxygenation treatment [24].

Because NFκB binds GST proteins and Protein G–Sepharose 4B under the conditions we used (results not shown), participation of NFκB in PTP-BAS–IκBα complex remains to be proved. However, it must be noted that overexpression of dominant-negative PTP-BAS mutant leads to the tyrosine-phosphorylation of IκBα. While it cannot be denied that this tyrosine-phosphorylation was due to the inhibition of another PTP working to keep IκBα dephosphorylated, PTP-BAS could have an effect on the tyrosine-phosphorylation of IκBα *in vivo*. PTP-BAS could block the tyrosine-phosphorylation-based inactivation pathway of IκBα in certain cells. The expression of dominant-negative PTP-BAS mutant prevents the association between endogenous PTP-BAS and IκBα in HeLa cells, and continuous binding also interferes in the accession of other PTPs to IκBα and causes tyrosine-phosphorylation of IκBα.

PTP-BAS is thought to be a submembranous cytoskeletal protein, and IκBα and NF-κB are cytosolic proteins. So it may therefore be thought strange that PTP-BAS works continuously to keep IκBα dephosphorylated, in spite of the different localizations of PTP-BAS and IκBα. The tyrosine kinase responsible for tyrosine-phosphorylation of IκBα is not identified yet; however, expression of membrane-anchoring src family kinase *lck* was reported to lead to tyrosine phosphorylation of IκBα [24]. Thus IκBα could be phosphorylated near the membrane and released from NF-κB complex without proteolytic degradation [24] as a substrate for PTP-BAS. Therefore, it seems possible that PTP-BAS associates with a part of IκBα–NFκB complex and dephosphorylates the phosphotyrosine of IκBα near the membrane to prevent spontaneous activation of NF-κB in cells under normal oxidative stress, although regulatory role of PTP-BAS in NF-κB activation through the serine-phosphorylation pathway might be minimal. High expression of PTP-BAS in lung, where cells are exposed to continual oxidative stress, may reflect such a protective role of PTP-BAS.

We are grateful to Ms. Yumi Kubo for technical assistance.

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Received 8 June 1998/8 September 1998; accepted 27 October 1998