Activation of Protein-tyrosine Phosphatase SH-PTP2 by a Tyrosine-based Activation Motif of a Novel Brain Molecule*

(Received for publication, May 14, 1996, and in revised form, June 24, 1996)

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BIT (a brain immunoglobulin-like molecule with tyrosine-based activation motifs) is a brain-specific membrane protein which has two cytoplasmic TAMs (tyrosine-based activation motifs). Using the Far Western blotting technique, we detected association of a 70-kDa protein with the tyrosine-phosphorylated TAMs of BIT. A mouse brain cDNA library in λgt11 was screened for this association, and two positive clones encoding tyrosine phosphatase SH-PTP2 were isolated. SH-PTP2 has two SH2 domains and is believed to function as a positive mediator in receptor tyrosine kinase signaling. SH-PTP2 and BIT were coimmunoprecipitated from phosphorylated rat brain lysate, and BIT was a major tyrosine-phosphorylated protein associated with SH-PTP2 in this lysate. This interaction was also observed in Jurkat T cells transfected with BIT cDNA depending on tyrosine phosphorylation of BIT. Biphosphotyrosyl peptides corresponding to BIT-TAMs stimulated SH-PTP2 activity 33–35-fold in vitro, indicating that two SH2 domains of SH-PTP2 simultaneously interact with two phosphotyrosines of BIT-TAM. Our findings suggest that the tyrosine phosphorylation of BIT results in stimulation of the signal transduction pathway promoted by SH-PTP2 and that BIT is probably a major receptor molecule in the brain located just upstream of SH-PTP2.

Protein tyrosine phosphorylation plays an important role in signal transduction and regulates a wide range of cellular processes. Protein-tyrosine kinases and protein-tyrosine phosphatases are highly expressed in the central nervous system, consistent with the importance of tyrosine phosphorylation in neural function (1).

BIT* (a brain immunoglobulin-like molecule with tyrosine-based activation motifs) is a novel immune antigen receptor-like molecule of the brain.2 This molecule is composed of an antigen receptor-like extracellular domain, a transmembrane domain, and a cytoplasmic region containing two variants of TAM (tyrosine-based activation motif) that was recently designated ITAM (immunoreceptor TAM). This cytoplasmic motif contains two tyrosine phosphorylation sites. TAM was originally described in the immune system where it plays a crucial role in the activation responses of B and T cells (2–5). BIT is one of major substrates of protein-tyrosine kinase(s) in crude brain suspensions2 and is widely distributed in the brain in synapse-rich regions and in some nerve fibers.3 These findings suggest that the tyrosine phosphorylation of TAMs in BIT may be involved in neural signal transduction. Recent studies in the immune system have demonstrated that the oligomerization of TAMs allows the phosphorylation of two tyrosine residues found in this motif and these phosphotyrosine residues act as a bidentate docking site for the paired Src homology 2 (SH2) domains present in the cytoplasmic tyrosine kinases, Syk and ZAP-70, believed to be indispensable for initiation of the signaling cascade (6–8). From these investigations, we predicted that TAMs of BIT may recruit tyrosine kinases containing paired SH2 domains to the inner face of the plasma membrane and thus initiate neural signaling. To ascertain the role of BIT in signal transduction, we isolated and characterized cDNA clones encoding the BIT-TAMs-binding protein by Far Western blotting. In contrast to our expectations, the cDNA clones obtained encoded the cytoplasmic protein-tyrosine phosphatase containing paired SH2 domains, SH-PTP2 (also termed PTP1D, SH-PTP3, PTP2C, PTPL1, or Syp). We demonstrated here that tyrosine-phosphorylated BIT and SH-PTP2 can interact both in vitro and in living cells, and that phosphotyrosyl peptides derived from BIT-TAMs are capable of evoking catalytic activity of SH-PTP2. BIT is probably a major receptor molecule located just upstream of SH-PTP2 in the signal transduction pathway in the brain.

EXPERIMENTAL PROCEDURES

Antibodies—The anti-BIT monoclonal antibody, named 1D4, and anti-BIT polyclonal antibodies which we used have been described (9, 10). Polyclonal antibodies against SH-PTP2 and Grb2, and a monoclonal antibody against SH-PTP2 was obtained from Transduction Laboratories. The anti-phosphotyrosine monoclonal antibody, 4G10, was from Upstate Biotechnology, Inc. Peroxidase-conjugated goat anti-rabbit IgG and anti-mouse IgG, and a monoclonal mouse peroxidase anti-phosphoantigen with a bidentate docking site for the paired Src homology 2 (SH2) domains present in the cytoplasmic tyrosine kinases, Syk and ZAP-70, believed to be indispensable for initiation of the signaling cascade (6–8). From these investigations, we predicted that TAMs of BIT may recruit tyrosine kinases containing paired SH2 domains to the inner face of the plasma membrane and thus initiate neural signaling. To ascertain the role of BIT in signal transduction, we isolated and characterized cDNA clones encoding the BIT-TAMs-binding protein by Far Western blotting. In contrast to our expectations, the cDNA clones obtained encoded the cytoplasmic protein-tyrosine phosphatase containing paired SH2 domains, SH-PTP2 (also termed PTP1D, SH-PTP3, PTP2C, PTPL1, or Syp). We demonstrated here that tyrosine-phosphorylated BIT and SH-PTP2 can interact both in vitro and in living cells, and that phosphotyrosyl peptides derived from BIT-TAMs are capable of evoking catalytic activity of SH-PTP2. BIT is probably a major receptor molecule located just upstream of SH-PTP2 in the signal transduction pathway in the brain.

Bacterial Expression of the Cytoplasmic Domain of BIT as a GST Fusion Protein—A DNA fragment corresponding to the cytoplasmic domain of BIT (amino acids 397–509) was amplified by a polymerase chain reaction from rat BIT cDNA.2 BamHI and EcoRI sites were included at the ends of the forward primer (5′-ACCGGATCCGGAATCAACACAGAAGAAGCC-3′) and the reverse primer (5′-ACAGAATTCTACCCCTTGGACTTG-3′), respectively, to facilitate cloning.

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Tandem stop codons were also introduced in the reverse primer. The amplicons were purified, digested with BamHI and EcoRI, and cloned into the pGEX-2X expression vector (Pharmacia). The cloned sequence was confirmed by DNA sequence analysis. The GST fusion protein (GST-BIT) expression vector was used to transform Escherichia coli JM109 to ampicillin resistance, and the expressed fusion protein was purified on a glutathione-Sepharose 4B column (Pharmacia) according to the instruction manual.

**Tyrosine Phosphorylation of GST-BIT Fusion Protein**—The purified GST-BIT fusion protein was tyrosine-phosphorylated with recombinant c-Src kinase (Upstate Biotechnology, Inc.). GST-BIT fusion protein, bound to glutathione-Sepharose 4B, was washed with and suspended in phosphorylation buffer containing 20 mM Tris (pH 7.4), 0.1% Triton X-100, 10 mM MgCl₂, and 1 mM 1,4-dithiothreitol. To start the reaction, ATP (25 μM) and GST-c-Src kinase were added to final concentrations of 3 μM and 30 units/ml, respectively, and then the solution was incubated at 30 °C with gentle shaking. After 24-h incubation, the resin was collected and rinsed with phosphorylation buffer. The eluted protein was incubated with 10 μM reduced glutathione in 50 mM Tris-HCl buffer (pH 8.0). Tyrosine phosphorylation of the fusion proteins was checked by Western blotting with the anti-phosphotyrosine antibody 4G10.

**Function of BIT**—Rat brain homogenate (10%) in 15 mM HEPES (pH 7.5) buffer containing 1 mM Na₃VO₄, 1 μM leupeptin, 0.7 μg/ml pepstatin, 0.1 mM phenylmethylsulfonyl fluoride, and 200 μg/ml 1,10-phenanthroline monohydrate was centrifuged at 1,000 × g for 10 min at 4 °C, and the supernatant was discarded. After addition of CHAPS to a final concentration of 1%, the supernatant was diluted, and the supernatant was added to final concentrations of 3 μM, 30 units/ml, respectively, and then the solution was incubated at 30 °C with gentle shaking. After 24-h incubation, the resin was collected and rinsed with phosphorylation buffer. The eluted protein was incubated with 10 μM reduced glutathione in 50 mM Tris-HCl buffer (pH 8.0). Tyrosine phosphorylation of the fusion proteins was checked by Western blotting with the anti-phosphotyrosine antibody 4G10.

**Screening of the cDNA Library**—A λgt11 cDNA expression library constructed from the BALB/c mouse brain (ML1042b) was obtained from Clontech. Recombinant clones expressing GST-BIT binding proteins were identified by Far Western blotting using tyrosine-phosphorylated GST-BIT and anti-GST monoclonal antibody. Positive plaques were developed by the PAP method as described previously (10). The cDNA inserts isolated from positive recombinant phages were subcloned into Bluescript II KS(-) (Stratagene), and the sequences were determined. DNA and deduced amino acid sequences were analyzed using an application software, GENETYX-SV/R and GENETYX-SVDB (Software Development).

**Tyrosine Phosphorylation of BIT in Vitro**—Rat brain homogenate (8%) in 15 mM Tris-HEPES buffer (pH 7.5) containing 1 mM Na₃VO₄, 1 μM leupeptin, 0.7 μg/ml pepstatin, 0.1 mM phenylmethylsulfonyl fluoride, and 200 μg/ml 1,10-phenanthroline monohydrate was centrifuged at 1,000 × g for 10 min at 4 °C. After centrifugation, the sediment was discarded, and the supernatant was used as the protein source. To immunoprecipitate BIT, the lysate samples were mixed with mono- or dual-column affinity chromatography. The final purification and determination were carried out using the ECL system according to the manufacturer’s protocol or the PAP method as described previously (6).

**Tyrosine Phosphorylation of BIT in Living Cells**—Jurkat T cells transfected with GST-BIT were incubated with 10 μM of monoclonal anti-BIT antibody (1D4) (1 mg/ml) in 200 μl of RPMI 1640 containing 10% fetal calf serum at 37 °C for 20 min, then washed with 5 ml of RPMI 1640 at 4 °C and suspended in 60 μl of RPMI 1640. After preincubation at 37 °C for 5 min, cellular BIT was cross-linked by applying 30 μl of polyvalent anti-mouse IgG (1 mg/ml) at 97 °C for 0, 2, 5, and 15 min. The reactions were stopped by adding NP-lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 1 mM Na₃VO₄, 1 mM EDTA, 500 units/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The solubilized materials were centrifuged, and the supernatants were incubated with anti-BIT serum and protein A-agarose at 4 °C for 1 h. The washed immunoprecipitates were immunoblotted with 4G10, 1D4, and anti-SH-PTP2 monoclonal antibodies.

**Preparation of Recombinant SH-PTP2—** A 2.2-kilobase pair KpnI/ ApaI cDNA fragment containing the entire coding region of SH-PTP2 was isolated, and the ApaI site was polished. The cDNA fragment was cloned into KpnI/NcoI-digested plBluescript II SK(-) (Stratagene) whose NcoI site was blunted. A 2.2-kilobase pair AccI/AccII fragment encoding amino acid residues 4–597 of SH-PTP2 was isolated from this plasmid and inserted into blunt-ended EcoRI site of pGEX-2T (Pharmacia) to yield a GST-SH-PTP2 fusion protein expression plasmid, pGEX-2T-SH-PTP2. Bacterial expression and purification of SH-PTP2 were carried out as described elsewhere (12), except that we used E. coli strain JM109 as a host strain.

**Assay for Protein-tyrosine Phosphatase Activity**—With n-propenyl phosphonate (pNPP) as a substrate, typically 20 μl pNPP and the effecter phosphotyrosine (varying concentrations) were incubated with 25 μg/ml recombinant SH-PTP2 at 24 °C in 100 μl of 50 mM sodium acetate buffer (pH 5.25) containing 50 mM NaCl and 10 mM dithiothreitol. At 30-min intervals, 30 μl of the reaction mixture were quenched with 40 μl of 5 M NaOH, and after 15 min at room temperature, the absorbance of p-nitrophenol (pNP) at 405 nm was determined. The amount of pNP was calculated by comparison with a standard curve obtained with pNP (Nalcaci Tesque).

To assay the dephosphorylation of phosphopeptides, the release of inorganic phosphate (P) was measured with a tyrosine phosphatase assay kit (Upstate Biotechnology, Inc.). Typically, 370 μl substrate phosphopeptide and 27.7 μl effecter phosphopeptide were incubated with 37 μM/ml SH-PTP2 at 24 °C in 27 μl of 50 mM HEPES buffer (pH 7.1) containing 150 mM NaCl, 10 mM dithiothreitol, and 2 mM EDTA. At 5-min intervals, 8 μl of the reaction mixture were quenched with 100 μl of 3 M NaOH after green solution, and after 15 min at room temperature the absorbance at 620 nm was measured. The amount of released P was calculated from a standard curve.

**Peptides**—The sequences of the synthesized phosphopeptides used in this study were as follows (pY indicates phosphorylated tyrosine): 1DNTAM2P, NDNDITYpYADLNFKEPKARPVPNPHETTEpYAS- IETGKL-NH₂, 1D4CTAM2P, RPEDELTpYAYDLMDVHNRAPQTPK- EPPSFpEYASVQVQKR, 1DPTPS, RPEDELTpYAYDLMDVHNR-NH₂, 1D1D3P, FKKpPEpSFpEYASVQVQKR. A nonphosphorylated peptide, 1DCTAM, with the sequence RPEDLT, was used to determine the reaction rate of SH-PTP2. A nonphosphorylated peptide, 1DCTAM, with the sequence RPEDLT, was used to determine the reaction rate of SH-PTP2. A nonphosphorylated peptide, 1DCTAM, with the sequence RPEDLT, was used to determine the reaction rate of SH-PTP2.
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RESULTS

Far Western Blotting—Proteins from rat brain were separated by SDS-PAGE, transferred onto a nitrocellulose filter, and overlaid with GST fusion proteins. Binding of the fusion protein was revealed with anti-GST antibody. Only tyrosine-phosphorylated GST-BIT fusion protein could bind to the 70-kDa protein on the filter (Fig. 1). GST alone or the unphosphorylated GST fusion protein did not exhibit any binding.

These results demonstrate that the observed interaction is specific and is dependent on tyrosine phosphorylation of BIT-TAMs where all four cytoplasmic tyrosine residues are located. Essentially the same results were obtained when mouse brain proteins were used instead of those from rat brain (data not shown).

Isolation of cDNA Clones Encoding a BIT-associated Protein—The tyrosine-phosphorylated GST-BIT fusion protein was used as a probe to screen expression libraries from BALB/c mouse brain. 400,000 plaques were screened and four positive clones (nos. 8, 20, 25, and 33) were identified. The positive signals of clones 25 and 33 were more intense than those of clones 8 and 20. The DNA sequences from the insert of clone 8 and 20 were used to search the DNA sequence data bases, but no significant homologies were detected (data not shown). The DNA sequences from the inserts of clones 25 and 33 were almost identical. The insert of clone 25 was slightly longer than that of clone 33, with an additional 20 and 8 nucleotides at 5’ - and 3’-ends, respectively. A homology search of the DNA data bases revealed that these inserts are parts of the cDNA of a protein-tyrosine phosphatase, SH-PTP2. These clones encoded only two SH2 domains and lacked its phosphatase domain. These results suggest that the tyrosine-phosphorylated BIT-TAM associates directly with the SH2 domain(s) of SH-PTP2.

To obtain the remaining coding region for SH-PTP2, the library was rescreened using the cloned fragment of clone 33 as a probe, and three overlapping clones were isolated. Two of them were used for reconstruction of a cDNA containing the complete open reading frame. The deduced amino acid sequence of BALB/c mouse SH-PTP2 was highly homologous to the human SH-PTP2 (15, 16) sharing a 98.8% identity. The calculated molecular mass of the cloned mouse SH-PTP2 is 68.4 kDa, consistent with the results of Far Western blotting.

SH-PTP2 Associates with Tyrosine-phosphorylated BIT in Vitro—To assess the potential association of SH-PTP2 with BIT, we initially examined the ability of anti-BIT monoclonal antibody (1D4) to coimmunoprecipitate SH-PTP2. The 1D4 monoclonal antibody recognizes a extracellular region of BIT (10). Treatment of rat brain crude suspension with ATP resulted in tyrosine phosphorylation of BIT, and SH-PTP2 was communoprecipitated in response to ATP incubation (Fig. 2). The amounts of BIT in the immunoprecipitates with or without ATP treatment were equivalent.

The reverse was also examined. When SH-PTP2 was immunoprecipitated with BIT, we initially examined the ability of anti-BIT monoclonal antibody (1D4) to coimmunoprecipitate SH-PTP2. The 1D4 monoclonal antibody recognizes an extracellular region of BIT (10). Treatment of rat brain crude suspension with ATP resulted in tyrosine phosphorylation of BIT, and SH-PTP2 was communoprecipitated in response to ATP incubation (Fig. 2). The amounts of BIT in the immunoprecipitates with or without ATP treatment were equivalent.

SH-PTP2 Associates with Tyrosine-phosphorylated BIT in Living Cells—The interaction between BIT and SH-PTP2 was also observed in Jurkat T cells transfected with rat BIT cDNA. Immunoprecipitates of BIT from the Jurkat cells contained trace amounts of SH-PTP2, indicating the possibility of slight phosphorylation of BIT in Jurkat cells. When BIT on the cells was cross-linked with monoclonal anti-BIT antibody (1D4) and polyclonal anti-mouse IgG, the BIT was tyrosine-phosphorylated, and the amount of communoprecipitated SH-PTP2 increased in proportion to the level of tyrosine phosphorylation of BIT (Fig. 4). Tyrosine phosphorylation of BIT increased for 2–5 min and then decreased. Phosphorylation of BIT occurred a little faster than when induced by cross-linking with polyclonal anti-BIT antibody, possibly due to the different efficiency of cross-linking. Polyclonal anti-mouse IgG or monoclonal anti-BIT alone (data not shown) were unable to induce tyrosine phosphorylation of BIT. Our results demonstrated that the association between BIT and SH-PTP2 can also take place in living Jurkat T cells and is dependent on tyrosine phosphorylation of BIT.

Activation of Recombinant SH-PTP2 Activity by Tyrosine-phosphorylated BIT-TAM—To elucidate the effect of tyrosine-phosphorylated BIT-TAM binding on SH-PTP2, we examined whether addition of a phosphotyrosyl peptide corresponding to BIT-TAM affected the phosphatase activity of SH-PTP2. Recombinant SH-PTP2 was prepared as described under “Experimental Procedures” and was used to assay the enzyme activ...
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**Fig. 3.** BIT is a major tyrosine-phosphorylated protein associated with SH-PTP2 in vitro. Rat brain samples were incubated with (lane 2 and 4) or without (lanes 1 and 3) 10 mM ATP for 10 min at 37°C. Detergent-lysed extracts were prepared and SH-PTP2 was immunoprecipitated with polyclonal anti-SH-PTP2 C terminus antibody (lanes 3 and 4). Rabbit normal antibody was used as a control (lanes 1 and 2). The resulting immunoprecipitates were subjected to Western blotting using monoclonal anti-BIT antibody. The resulting immunoprecipitates were subjected to Western blotting using monoclonal anti-BIT, monoclonal anti-BIT (1D4), or monoclonal anti-phosphotyrosine (4G10) antibody. Positions of molecular mass markers are indicated on the left. IB, immunoblot.

**Fig. 4.** Association of SH-PTP2 with tyrosine-phosphorylated BIT in living cells. Jurkat T cells transfected with rat BIT cDNA were pretreated with (lanes 1–4) or without (lane 5–8) monoclonal anti-BIT (1D4) antibody, and then BIT on the cells was cross-linked with polyclonal anti-mouse IgG for 0 min (lanes 1 and 5), 2 min (lanes 2 and 6), 5 min (lane 3 and 7) or 15 min (lanes 4 and 8) at 37°C. Cell detergent lysates were prepared and BIT was immunoprecipitated with polyclonal anti-BIT antibody. The resulting immunoprecipitates were subjected to Western blotting using monoclonal anti-BIT (1D4), monoclonal anti-phosphotyrosine (4G10) or monoclonal anti-SH-PTP2 antibody. Positions of molecular mass markers are indicated on the left. IB, immunoblot.

**Fig. 5.** Activation of recombinant SH-PTP2 with tyrosine-phosphorylated BIT-TAMs. A, phosphatase activities were determined using 20 mM pNPP as a substrate. Reaction rates were plotted versus peptide concentrations for 1D4NTAM2P (open circles), 1D4CTAM2P (closed circles), 1D4CTAM (open squares), 1D4PTP3 (open triangles), 1D4PTP4 (closed triangles), 1D4PTP3 + 1D4PTP4 (closed diamonds). B, phosphatase activities were determined using 370 μM Src-C phosphotyrosylpeptide as a substrate. Effector peptides (1D4NTAM2P, 1D4CTAM2P, or 1D4CTAM) were added at the concentration of 27.7 μM.

The stimulation effects of BIT-TAMs on SH-PTP2 were more marked when a phosphopeptide was used as a substrate (Fig. 5B). Phosphatase activity was assayed at a fixed Src-C phosphopeptide concentration of 370 μM. In the absence of the effector phosphopeptide (1D4NTAM2P or 1D4CTAM2P), the Src-C peptide was dephosphorylated very slowly (k(2.2 × 10^-3 s^-1)). When Src-C was incubated with the effector peptide, P_i release was markedly stimulated (Fig. 5B). The reaction rates of P_i release in the presence of the effector peptides 1D4NTAM2P and 1D4CTAM2P were k(6.55 × 10^-3 s^-1) and k(7.98 × 10^-3 s^-1), respectively. In the reaction mixture containing the substrate peptide (Src-C) and the effector peptide, P_i could be released from both peptides. In practice, when only the effector peptide (27.8 μM) was incubated with SH-PTP2, it was dephosphorylated, and thus behaved as a substrate (k(8.4 × 10^-3 s^-1) for 1D4NTAM2P and k(7.2 × 10^-3 s^-1) for 1D4CTAM2P). In this case, the peptide was considered to be a substrate for SH-PTP2.

The maximal rate of pNP release was increased 7–8-fold (k(0.87 s^-1)) with 1D4CTAM2P and k(0.79 s^-1) with 1D4NTAM2P at 100 μM peptide) (Fig. 5A). This potent activation was dependent on the phosphotyrosine residues of the peptides since an unphosphorylated peptide, 1D4CTAM, had no effect.

**Fig. 6.** Phosphatase activity of SH-PTP2 in vitro. 1D4PTP3 and 1D4CTAM2P, corresponding to two tyrosine phosphorylation sites of C-TAM were also used as effector peptides. 1D4PTP3 stimulated SH-PTP2 catalytic activity 4–5-fold (k(0.50 s^-1)) but 1D4PTP4 had no effect. An equimolar mixture of the monophosphopeptides 1D4PTP3 and 1D4PTP4 yielded a velocity of 0.52 s^-1 at 340 μM total peptide concentration. This effect was nearly equivalent to that elicited by 1D4PTP3 alone. The activation effects by the bisphosphopeptides were greater than those produced by the two monophosphorylated peptides.
both an activator (effector) and a substrate. Consequently, it was not clear which peptide produced the observed activation. However, when pNPP was used as a substrate, Src-C had no effect on the catalytic activity of SH-PTP2 (data not shown), thus suggesting that the 1D4NTAM2P or 1D4CTAM2P (effector peptide) stimulated catalytic activity of SH-PTP2 for Src-C (substrate peptide). Assuming simply that the difference between two reaction rates for P_i release observed under the two conditions (± Src-C peptide in the presence of the effector peptide) is the actual rate for P_i release from the Src-C peptide, 1D4NTAM2P and 1D4CTAM2P stimulated the dephosphorylation rate by 35-fold ($k(77.1 \times 10^{-3} \text{ s}^{-1})$) and 33-fold ($k(72.6 \times 10^{-3} \text{ s}^{-1})$, respectively.

**DISCUSSION**

This study demonstrated a direct interaction between tyrosine-phosphorylated BIT-TAMs and the protein-tyrosine phosphatase SH-PTP2, and an activation effect of this interaction on the phosphatase activity. SH-PTP2 is a cytosolic protein-tyrosine phosphatase that possesses two SH2 domains (15, 16, 19–23) and is the mammalian homologue of the *Drosophila* csw gene product which is required for signaling downstream of the receptor protein-tyrosine kinase Torso (24). SH-PTP2 has been reported to bind directly to receptor tyrosine kinases such as platelet-derived growth factor receptor (25, 26), epidermal growth factor receptor (16, 22), and c-Kit (27) in response to stimulation by their ligands. Insulin receptor substrate 1 (IRS-1) also associates with SH-PTP2 in response to insulin (28). Several studies have suggested additional details concerning the contribution of SH-PTP2 to signal transduction, and SH-PTP2 appears to serve an essential positive role in mediating tyrosine kinase signaling (17, 18, 29–33). Thus, there have been multiple investigations into the functions of SH-PTP2, but little is known about its role in the brain in which the highest level of SH-PTP2 expression occurs (34). BIT, a novel antigen receptor-like molecule containing two TAMs is a novel antigen receptor-like molecule containing two TAMs is a major endogenous substrate of protein-tyrosine phosphatase SH-PTP2, and an activation effect of this interaction on the phosphatase activity. SH-PTP2 is a cytosolic protein-tyrosine phosphatase that possesses two SH2 domains (15, 16, 19–23) and is the mammalian homologue of the *Drosophila* csw gene product which is required for signaling downstream of the receptor protein-tyrosine kinase Torso (24). SH-PTP2 has been reported to bind directly to receptor tyrosine kinases such as platelet-derived growth factor receptor (25, 26), epidermal growth factor receptor (16, 22), and c-Kit (27) in response to stimulation by their ligands. Insulin receptor substrate 1 (IRS-1) also associates with SH-PTP2 in response to insulin (28). Several studies have suggested additional details concerning the contribution of SH-PTP2 to signal transduction, and SH-PTP2 appears to serve an essential positive role in mediating tyrosine kinase signaling (17, 18, 29–33). Thus, there have been multiple investigations into the functions of SH-PTP2, but little is known about its role in the brain in which the highest level of SH-PTP2 expression occurs (34). BIT, a novel antigen receptor-like molecule containing two TAMs is a major endogenous substrate of protein-tyrosine kinase(s) in crude brain suspensions (29) and is widely distributed in the brain in synapse-rich regions and some nerve fibers. Predominant expression in the brain is a common feature of both BIT (9, 10) and SH-PTP2 (34). Thus, the interaction reported here provides an important clue to the functions of BIT and SH-PTP2 in neural signal transduction. Since BIT is a major tyrosine-phosphorylated protein associated with SH-PTP2 in brain lysates, BIT is probably an important receptor located just upstream of SH-PTP2 in the brain.

SH-PTP2 binds to tyrosine-phosphorylated BIT-TAM directly via its SH2 domains undoubtedly because two SH-PTP2 cDNA fragments cloned from a λgt11 expression library encoded only tandem SH2 domains and lacked a phosphatase domain. For SH-PTP2, the SH2 domains exert a negative regulatory influence, and binding of phosphopeptide ligands to these domains have been shown to stimulate catalytic activity (35, 36). Recently, phosphopeptides corresponding to the sequence surrounding Tyr-1172 and Tyr-1222 of IRS-1 were shown to bind with high affinity to the N- and C-terminal SH2 domains of SH-PTP2, respectively, and stimulate its catalytic activity 9–16-fold (35). The binding to the two SH2 domains of a bisphosphorylated peptide, produced by introducing a chemical spacer connecting these two monophosphorylated peptides, potently stimulates catalytic activity of SH-PTP2 (37-fold) (37). Thus the simultaneous occupancy of both SH2 domains with two phosphotyrosine residues was important for activation. Similarly, synthetic tyrosine-phosphorylated BIT-TAMs strongly stimulated the phosphatase activity of SH-PTP2 (33–35-fold). Each BIT-TAM has two tyrosine residues (N-TAM, Tyr-436 and Tyr-460; C-TAM, Tyr-477 and Tyr-501).

The amino acid sequences following Tyr-436 (YADL) and Tyr-460 (YASI), or Tyr-477 (YADL) and Tyr-501 (YAVS) are quite similar to the sequences following Tyr-1172 (YIDL) and Tyr-1222 (YASI) of IRS-1. These similarities suggest that Tyr-436 (or Tyr-477) and Tyr-460 (or Tyr-501) of BIT probably bind to N- and C-terminal SH2 domains of SH-PTP2, respectively, and that simultaneous interaction produces activation. The bisphosphorylated BIT-TAMs reported here are the first native motifs which potently stimulate SH-PTP2 phosphatase activity. More recently, the structure of the tandem SH2 domains of SH-PTP2 was reported (38). In this report, it was suggested that orientation and spacing between two phosphotyrosine residues, each of which bind to the SH2 domain, are critical for enzymatic activation. Typical TAMs have two tyrosine phosphorylation sites separated by 9–11 amino acid residues (2), and the TAMs of BIT are longer variants in which the tyrosine residues are separated by 23 amino acids. We consider that the ternary structure of these long spacer sequences are suitable for binding to the tandem SH2 domains. Strong stimulation of the phosphatase activity suggests that the interaction between BIT and SH-PTP2 is functionally significant and the association actually occurs in vivo. This activation is probably due to the conformational change in SH-PTP2 triggered by binding of the two SH2 domains to two phosphotyrosine residues (37). Other investigators have also demonstrated stimulation of the enzymatic activity by the simultaneous occupancy of both of the tandem SH2 domains of phosphatidylinositol 3-kinase with two phosphotyrosine residues (39). Some enzymes that have tandem SH2 domains may also have common mechanisms of regulation.

Another SH-PTP2-activating mechanism, triggered by recruitment of SH-PTP2 by the membrane protein BIT, may be considered. SH-PTP2 is activated by phospholipids in vitro (40). The association of SH-PTP2 with BIT may facilitate interaction with phospholipids in the plasma membrane which could continuously stimulate the enzymatic activity. A structural model in which BIT binds two SH-PTP2 molecules at its cytoplasmic domain is reminiscent of receptor-type tyrosine phosphatases, which have two phosphatase domains in a cytoplasmic region (Fig. 6). In the case of T cell receptor signaling, CD45, the best characterized receptor-type tyrosine phosphatase may dephosphorylate the inhibitory C-terminal tyrosine phosphorylation site on the Src family protein-tyrosine kinases, Fyn and/or Lck, allowing activation of their kinase activity (41, 42). The phosphopeptide containing the inhibitory C-terminal tyrosine phosphorylation site of Src (Tyr-527) is a good substrate for SH-PTP2 in vitro (12). In the present study, we demonstrated that BIT-TAMs strongly stimulate the phosphatase activity of SH-PTP2 for a peptide sub-
strate, Src-C, containing the inhibitory tyrosine phosphorylation site. Recent studies indicated that Src kinase associated with SH-PTP2, and SH-PTP2 dephosphorylated Src at Tyr-527 in vitro (43). Therefore, direct activation of SH-PTP2 by tyrosine-phosphorylated BIT-TAMs could induce activation of Src or other Src family kinases. The mammalian brain contains high levels of Src family protein-tyrosine kinases and recent observations have suggested that a Src family kinase, Fyn, functions in development of neural cells, modulation of long-term potentiation and regulation of behavior (44, 45). The idea that Src family kinases may be involved in BIT regulation, is supported by our observation that recombinant GST-BIT fusion protein was tyrosine-phosphorylated by immunoprecipitated rat brain Src and Fyn in vitro (data not shown). The potential of BIT as a substrate of Src family kinases and of SH-PTP2 as a positive regulator of Src family kinases in mammalian brain is very attractive in view of the functional relationship among these three molecules.

Grb2 was also immunoprecipitated with tyrosine-phosphorylated BIT. Grb2 is an adapter molecule and one of the functions of this molecule is to link growth factor receptors to downstream effector proteins such as Ras (46). Grb2 is known to bind tyrosine-phosphorylated SH-PTP2 in response to platelet-derived growth factor (17, 18). Thus, we supposed that the observed immunoprecipitation of Grb2 with tyrosine-phosphorylated BIT may result from an indirect interaction. But the real mechanism and the significance of the observed interaction among BIT, SH-PTP2 and Grb2 was not yet understood.

Our results suggest a model for a signal transduction mechanism of BIT (Fig. 6). Activated (tyrosine-phosphorylated) BIT recruits SH-PTP2, and binding of BIT-TAM with SH2 domains of SH-PTP2 stimulates its catalytic activity. The activated SH-PTP2 functions as a positive mediator of signaling via BIT. The extracellular ligand that stimulates tyrosine phosphorylation of BIT-TAM is as yet unknown. From the structural similarity of BIT to the T cell and B cell receptors of the immune system and from our in vitro observations, we speculate that a Src family kinase is involved in the tyrosine phosphorylation of BIT-TAMs. BIT may participate in a major signal transduction pathway involving SH-PTP2 in the mammalian brain and might be associated with the regulation of synaptic function.

Acknowledgments—We thank Dr. Akira Omori for sequence analysis and Miss Sachio Yoshida for fast atom bombardment-mass spectrometry measurements. We thank Dr. Michael J. Seagur for critical reading of this manuscript.

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