BIT, an immune antigen receptor-like molecule in the brain

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Abstract **We previously found a brain-specific glycoprotein in the rat brain. It postnatally increases and is rich in the mature brain. We cloned cDNA of this protein. It is composed of a signal peptide, a V-type immunoglobulin domain, two C1-type immunoglobulin domains, a transmembrane segment and a cytoplasmic region containing two tyrosine-based activation motifs (TAM) that are variants of the antigen receptor signaling motifs. The overall structure is similar to those of immune antigen receptors. This molecule, BIT (*b*rain *i*mmunoglobulin-like molecule with *T*AMs), is a major endogenous substrates of brain tyrosine kinases in vitro. Cerebral cortical neurons could extend their neurites on BIT-coated substrate and anti-BIT monoclonal antibody specifically inhibited the effect. These findings and our recent study concerning BIT signal transduction mechanism suggest that BIT, an immune antigen receptor-like molecule of the brain, functions as a membrane signaling molecule that may participate in cell–cell interaction.**

Key words: Immunoglobulin superfamily; Immune antigen receptor-like molecule; Tyrosine phosphorylation; Neurite extension; Rat brain

1. Introduction

Many immunoglobulin superfamily molecules have been found in the nervous system as in the immune system. They are generally divided into two groups. The first group consists of adhesion molecules such as neural cell adhesion molecule (NCAM) and L1, and the second group consists of growth factor receptors such as platelet-derived growth factor receptor and fibroblast growth factor receptor. The immune system has another group, antigen recognition molecules, which consists of immunoglobulins, T-cell receptors (TCR) and major histocompatibility complex (MHC) molecules, and this group possesses C1-type immunoglobulin domains. Neural molecules that show a high degree of structural homology to these immune molecules have not been reported. The antigen recognition molecules appear to have evolved quite recently through vertebrate evolution in comparison with other immunoglobulin superfamily molecules [1]. Then, they have become to bear the most specialized functions in the mammalian immune system. Thus, if a molecule that has structural homology to the antigen recognition molecules is present in the brain, it will be very interesting to determine what role it plays in the mammalian brain.

We previously found a brain-specific glycoprotein in the rat brain using monoclonal antibody 1D4 [2,3]. This glycoprotein postnatally increased and was highly expressed in the mature brain [2]. The time course of the protein increase appears to be related to that of synaptogenesis in the brain. In the present study, we cloned the cDNA encoding this protein. The deduced amino acid sequence shows that it is composed of a signal peptide, a V-type immunoglobulin domain, two C1-type immunoglobulin domains, a transmembrane segment and a cytoplasmic region containing two tyrosine-based activation motifs (TAM) that are variants of antigen receptor signaling motifs. The overall structure is similar to those of the immune antigen receptors. This molecule is named BIT (*b*rain *i*mmunoglobulin-like molecule with *t*yrosine-based activation motifs). Each immunoglobulin domain of BIT shows high degrees of homology to immunoglobulin domains of immunoglobulins, TCRs and MHC molecules. Moreover, BIT contains two longer variants of typical TAM, which has been characterized as a signaling motif of T- and B-cell receptors [4–7]. Furthermore, each cytoplasmic TAM of BIT contains two sequences homologous to those of insulin receptor substrate-1 (IRS-1), which interact with SHP-2 protein tyrosine phosphatase. Our recent study concerning signal transduction of BIT showed that BIT actually interacts with SHP-2 [8].

In the present study, we demonstrated that BIT is one of major endogenous substrates of brain tyrosine kinases in vitro, indicating that BIT is a quite important molecule in signal transduction in the brain. Furthermore, we showed that cultured cerebral cortical neurons could extend their neurites on BIT-coated plate. Anti-BIT monoclonal antibody 1D4, which recognizes the extracellular part of BIT, specifically inhibited the neurite extension on BIT, but it did not on laminin. These findings demonstrate that an immune antigen receptor-like molecule exists in the brain, and suggest that the molecule, BIT, functions as a membrane signaling molecule that may participates in cell–cell interaction.

2. Materials and methods

2.1. Cloning of BIT cDNA

Purified BIT [3] was digested with lysyl-endopeptidase and the peptide isolated by HPLC was microsequenced. The N-terminal sequence of the purified BIT was also analyzed. Specific antibodies for BIT were isolated from rabbit anti-BIT serum [2] using a column prepared with CNBr-activated Sepharose 4B and purified BIT. The antibodies were used to screen a rat brain cDNA library in λgt11 (RL1043a, Clontech). A clone was obtained and its insert was used for further screening of a rat brain cDNA library in λgt10 (RL1025b, Clontech).

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Abbreviations: TCR, T-cell receptor; MHC, major histocompatibility complex; TAM, tyrosine-based activation motif; SH2, src homology 2; IRS-1, insulin receptor substrate-1; PMSF, phenylmethylsulfonyl fluoride; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank databases with the accession number D38468.

PII S0014-5793(97)00724-2

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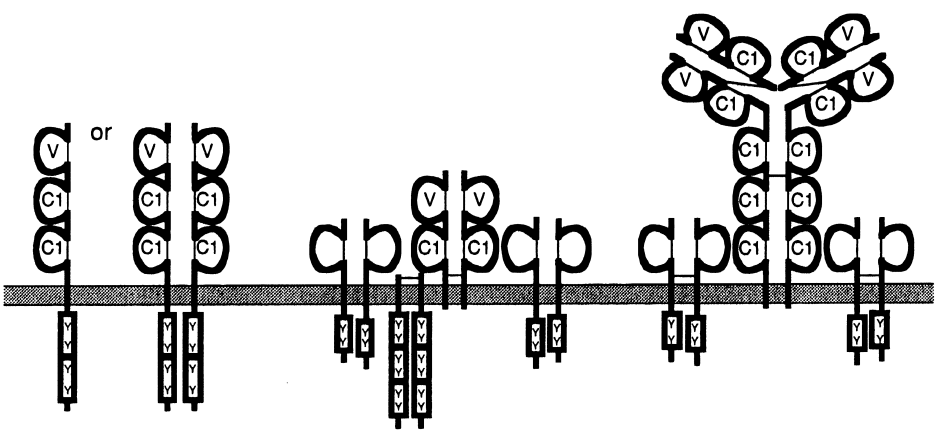
B



Some positive clones were obtained. After subcloning the inserts into the Bluescript II KS$^-$ plasmid (Stratagene), the nucleotide sequences of the cDNA inserts were determined by dideoxy chain termination in both directions. Overlapping sequences of the cDNA clones gave a single open reading frame and included two amino acid sequences obtained by peptide microsequencing.

2.2. Northern blot analysis

Total RNAs (5.4 μg) from rat several tissues were resolved by electrophoresis through 1% agarose formaldehyde gel, transferred to Hybond N (Amersham) and hybridized with the 1.7-kb BIT cDNA insert of pRB10.7. Hybridization proceeded at 65°C using rapid hybridization buffer (Amersham). The blots were washed at room temperature with 2×SSC and 0.1% SDS, then at 65°C with 0.1×SSC and 0.1% SDS.

2.3. Immunoblot analysis of BIT expression

Rat tissues were homogenized in 20 mM Tris-HCl (pH 6.8) containing 2% SDS and 2% 2-mercaptoethanol, and the homogenates (10 μg protein) were resolved on a 10% SDS-polyacrylamide gel, transferred onto a PVDF membrane (Millipore), and immunoblotted with anti-BIT serum [2]. Detection proceeded using peroxidase–antiperoxidase complex as described previously [3] except that peroxidase activity was detected in the presence of 5 mg/ml of $(NH_4)_2Ni(SO_4)_2{\cdot}6H_2O$ for high sensitivity.

2.4. Tyrosine phosphorylation of BIT in vitro

Rat brain homogenate (8%) in solution A containing 15 mM HEPES-Tris (pH 7.5), 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 μg/ml antipain, 0.7 μg/ml pepstatin, 0.1 mM PMSF and 200 μg/ml 1,10-phenanthroline monohydrate was centrifuged at 1000×*g* for 10 min. The supernatant diluted with the same volume of solution A was incubated with or without 10 mM ATP in the presence of 5 mM $MgCl_2$ and 5 mM $MnCl_2$ at 30°C for 10 min. The reaction was stopped with the same volume of NEHPF solution containing 150 mM NaCl, 5 mM EDTA, 15 mM HEPES-Tris, 100 mM sodium phosphate (pH 7.4) and 50 mM NaF. Proteins were solubilized with 1% CHAPS at 4°C. The solubilized CHAPS lysates (1 ml) were mixed with 30 μl of monoclonal antibody 1D4–Sepharose suspension at 4°C overnight. The CHAPS lysates, the unadsorbed supernatants and the washed immunoprecipitates were immunoblotted as described above with monoclonal anti-phosphotyrosine (4G10) antibody (UBI) and monoclonal anti-BIT (1D4) antibody [2].

2.5. Cerebral cortical cell culture

96-well plates were coated with nitrocellulose as described [9]. Bovine serum albumin (BSA) (10 mg/ml), poly-L-lysine, (0.1 mg/ml), laminin (Collaborative Research) (0.4 mg/ml), or purified BIT (0.15 mg/ml) were applied to the wells. After 10 min, the solutions were removed and free nitrocellulose was blocked by washing with culture medium (Eagle's minimum essential medium (MEM) with 19.4 mM glucose, 6 mM $NaHCO_3$, 60 nM Na_2SeO_3, 84 μg/ml gentamicin sulfate and 10% fetal bovine serum). Cerebral cortex cells were prepared from embryonic day 17 rat embryos as described [10] and added to each well at a concentration of 1×10^5 cells/well. The cells were maintained in the culture medium at 37°C in 5% CO_2. After 45 h incubation, cultures were fixed with 4% paraformaldehyde in PBS. For antibody inhibition test, prior to addition of cells, 0.1 mg/ml monoclonal anti-BIT antibody (1D4) or control monoclonal antibody (3B12a) in PBS were added into the wells and allowed to incubate for 1 h. The wells were then washed with the culture medium and cells were added as described. After 24 h incubation, cultures were fixed as described.

Table 1
Immunoglobulin-like domains homologous to those of BIT

BIT domain	Homologous domain	Residue identity
V-like domain (32–147)	TCR Vδ sheep	32%
	TCR Vα human	31%
	Ig Vκ human	31%
	Ig Vλ human	31%
C1-like domain (152–249)	Ig Cκ pig	35%
	Ig CH4 horned shark	31%
	IgG3 CH1 mouse	31%
	MHC II β wild mouse	31%
C1-like domain (254–352)	IgG4 CH3 human	30%
	Ig CH4 ladyfish	29%
	IgG1 CH3 human	29%
	IgG3 CH3 mouse	28%

The search was performed using the IG-Suite FastDB. Analyzed sequences of BIT are indicated by amino acid positions in parentheses.

3. Results

3.1. Cloning of BIT cDNA

To clone cDNA of this molecule, initially we screened a rat brain cDNA library in λgt11 using the specific polyclonal antibody, and then an insert from a positive clone was used for further screening of a rat brain cDNA library in λgt10. Some positive clones were obtained and overlapping cDNA clones gave a single open reading frame. It included two amino acid sequences obtained by peptide microsequencing (Fig. 1A). This molecule, BIT, is composed of a signal sequence, a V-type immunoglobulin domain, two C1-type immunoglobulin domains, a transmembrane segment and a cytoplasmic region containing two TAMs. The overall structure is similar to those of immune antigen receptors (Fig. 1B). We illustrate BIT as a monomer and as a dimer. The possibility of BIT dimer formation is supported by the facts that a part of purified BIT sometimes migrates at the dimer position in SDS-PAGE even after boiling in the presence of 1% 2-mercaptoethanol, and that the putative BIT dimer is detected by immunoblotting after incubation of the brain membrane fraction with chemical cross-linker. Mature BIT without a signal peptide is composed of 478 amino acids and has a calculated molecular weight of 52 714. Rat brain BIT is highly glycosylated and its molecular weight is about 90 kDa [3]. This is consistent with the finding that the extracellular structure of BIT has fifteen putative N-glycosylation sites.

3.2. Properties of immunoglobulin-like domains of BIT

The first immunoglobulin-like domain has characteristics of the V-type [11], which is larger than C1 and C2-types and has the unique consensus amino acids, as shown in Fig. 2A. The V-type sequence shows a high degree of homology to those of the TCRα and -δ chains and immunoglobulin light chains (Table 1). Judging from the size and the consensus amino acids, the two immunoglobulin-like domains are of the C1-

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Fig. 1. Structure of BIT. A: Nucleotide sequence and amino acid sequence of BIT. N-terminal amino acid sequences of purified BIT and of a peptide obtained by lysyl-endopeptidase digestion are indicated by dotted lines. A putative signal sequence and a hydrophobic transmembrane region are underlined. Possible N-glycosylation sites (Asn–X–Ser/Thr) are indicated by open triangles. Cysteines likely to form disulfide bonds in the three immunoglobulin-like domains are circled. Putative phosphorylation sites for protein kinase C and casein kinase II are indicated by open circles and open squares, respectively. The tyrosine residues of two TAMs are indicated by closed circles. B: Structural similarity between BIT, T-cell receptor (TCR) and B-cell receptor (BCR). BIT is illustrated as a monomer and as a dimer.

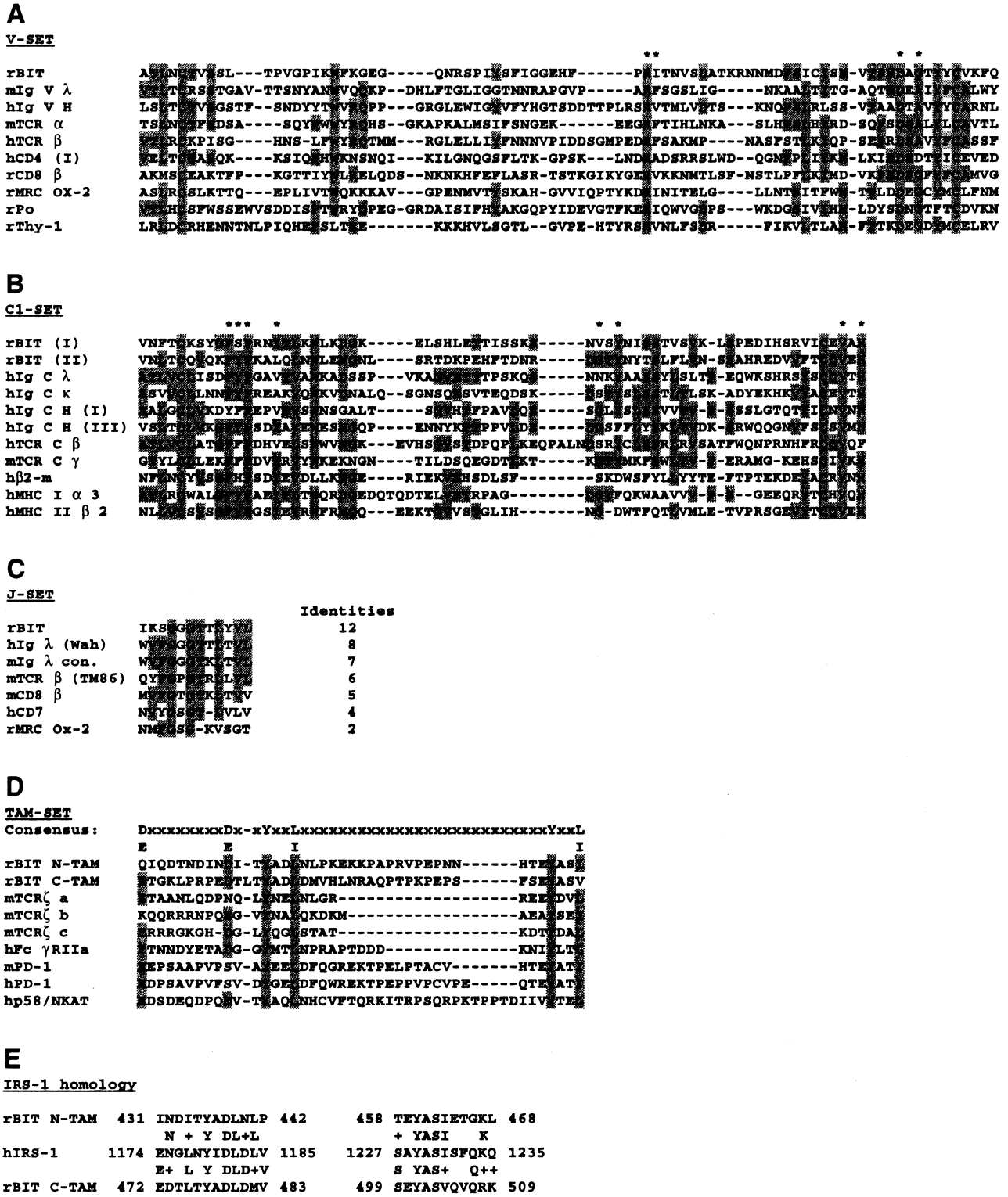

Fig. 2. Alignment of BIT with homologous sequences. Gaps are indicated by −. r, rat; m, mouse; h, human. Alignment of the first immunoglobulin-like domain (51–126) of BIT with V domains (A) and the second (168–233) and third (270–336) immunoglobulin-like domains with C1 domains (B). All the sequences shown here were taken from Williams and Barclay [11]. The characteristic residues for each domain are indicated by asterisks. Four or more identical residues are shaded. C: Alignment of the J-like sequence (136–147) of BIT with those of other molecules. The numbers of residues identical with BIT are indicated. The sequences of hIg λ (Wah), hCD7, rMRC Ox-2 and all others are taken from [26], [13], [14] and [12], respectively. The J-region sequence of mouse λ chain corresponds to the consensus sequence given in ref.[12]. D: Alignment of the N-TAM (424–463) and C-TAM (464–504) of BIT with those of other molecules. Consensus residues are indicated at the top and are shaded in the sequences. The sequences shown here are taken from [5], [16] and [17]. E: Alignment of the cytoplasmic sequences of BIT with the sequences of human IRS-1. The alignments were obtained by BLAST search. Conservative amino acids are indicated by +.

type [11] (Fig. 2B), which have been found only in molecules involved in antigen recognition. The first C1-type domain shows a high degree of homology to the C1 domains of the immunoglobulins and MHC molecules (Table 1). The second C1-type domain shows a high degree of homology to the C-terminal C1 domains of immunoglobulins and MHC molecules (Table 1), and has a consensus sequence pattern (Phe/Tyr–X–Cys–Val/Ala–X–His) found in the C-terminal C1 domains of immunoglobulins and MHC molecules. Moreover, BIT has a sequence homologous to the J sequences of immunoglobulins and TCRs (Fig. 2C). J-like sequences were also found in CD8 β [14], CD7 [15] and MRC Ox-2 [16], but that of BIT has more amino acids identical to those of immunoglobulins and TCRs. Thus, the extracellular part of BIT is highly homologous to those of the immune antigen receptors.

3.3. Properties of BIT-TAM

The cytoplasmic region includes two variants of TAM (Fig. 2D). This motif exists in the cytoplasmic portions of several molecules constituting an antigen receptor complex [4–7] and is involved in the generation of antigen receptor signaling. TCRζ contains three motifs, η contains two, and CD3γ, -δ and -ε, Igα, Igβ and some Fc receptors contain one. The motifs have recently been referred to as immunoreceptor TAM (ITAM), and in this paper ITAM and its variants are called TAM.

The two motifs of BIT differ from the typical motifs. The former have 20 amino acids between two YxxL/I, while the latter have 6–8. Longer variant motifs have also been identified in non-antigen receptor molecules, such as mouse and human homologues of PD-1, a molecule related to the programmed cell death of immune cells, (19 and 21 amino acids, respectively) [15,16] and human natural killer cell receptor, p58/NKAT, associated with MHC class I recognition (26 amino acids) [17,18]. TAMs have only been identified in molecules in the immune system and in viruses infecting immune cells [19]. The motifs in BIT were the first found in the nervous system.

BLAST search reveals that sequences around two tyrosine residues in each TAM are quite similar to the IRS-1 sequences around two tyrosine residues that are reported to be binding sites of two SH2 domains of SHP-2 tyrosine phosphatase [20,21] (Fig. 2E). We recently demonstrated that TAM of BIT actually interacts with SHP-2 [8]. Bisphosphotyrosyl peptide corresponding to N-TAM or C-TAM potently stimulates SHP-2 phosphatase activity (33–35-fold) [8], as a tethered peptide containing the two IRS-1 phosphorylation sites does [20,21].

3.4. Tissue distribution of BIT

We examined BIT mRNA expression in several rat tissues by Northern blotting (Fig. 3A), and a transcript of 4.0 kb was detected in the brain, a 3.7 kb transcript was found in the spleen, and faint 3.7 kb transcripts were also detected in the lung and heart. We previously detected BIT protein only in the brain among 17 different tissues by immunoblotting [2,3]. Thus, we re-examined BIT protein expression in various tissues by highly sensitive immunoblotting with polyclonal antibody against the purified BIT (Fig. 3B). In addition to an intense 90 kDa band in the brain, a faint 130 kDa band was found in the spleen and a fainter 120 kDa band was detected in the lung. Immunoblotting with polyclonal antibody against a BIT cytoplasmic peptide showed apparently the same results (data not shown), indicating that the result shown in Fig. 3B did not strongly reflect the antibody reactivity to brain-specific modification, such as glycosylation. Thus, the 3.7 kb transcripts seem to be translated at very low levels.

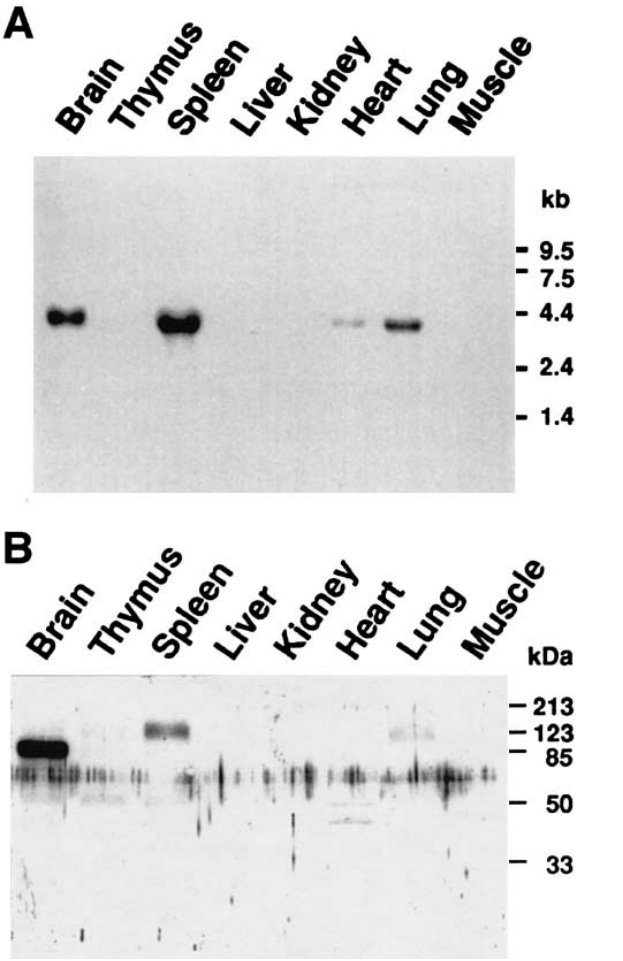

Fig. 3. Tissue distribution of BIT. A: Northern blot analysis of BIT mRNA expression. Total RNAs (5.4 μg) from the indicated tissues of the adult rat were resolved by electrophoresis through 1% agarose formaldehyde gel, transferred to a nylon membrane and hybridized with the BIT cDNA probe. RNA samples were electrophoresed on a separate gel and stained with ethidium bromide to ensure that quality and quantity of the tissue RNA were equivalent. B: Immunoblot analysis of BIT expression. Homogenates (10 μg protein) of the indicated tissues of the adult rat were resolved on a 10% SDS-polyacrylamide gel, transferred to a PVDF membrane and immunoblotted with anti-BIT serum.

3.5. Tyrosine phosphorylation of BIT in brain suspension

The in vitro tyrosine phosphorylation of BIT was examined using crude brain suspension. One of the major tyrosine-phosphorylated proteins, a molecule of about 90 kDa in the CHAPS lysate, was immunoprecipitated with anti-BIT antibody (Fig. 4), showing that BIT is a major endogenous substrate for protein tyrosine kinase(s) in the brain. This suggests that BIT plays an important role in signal transduction in the brain. Phosphorylation appears to cause a structural change, since phosphorylated BIT migrated more slowly than non-phosphorylated BIT in SDS gels.

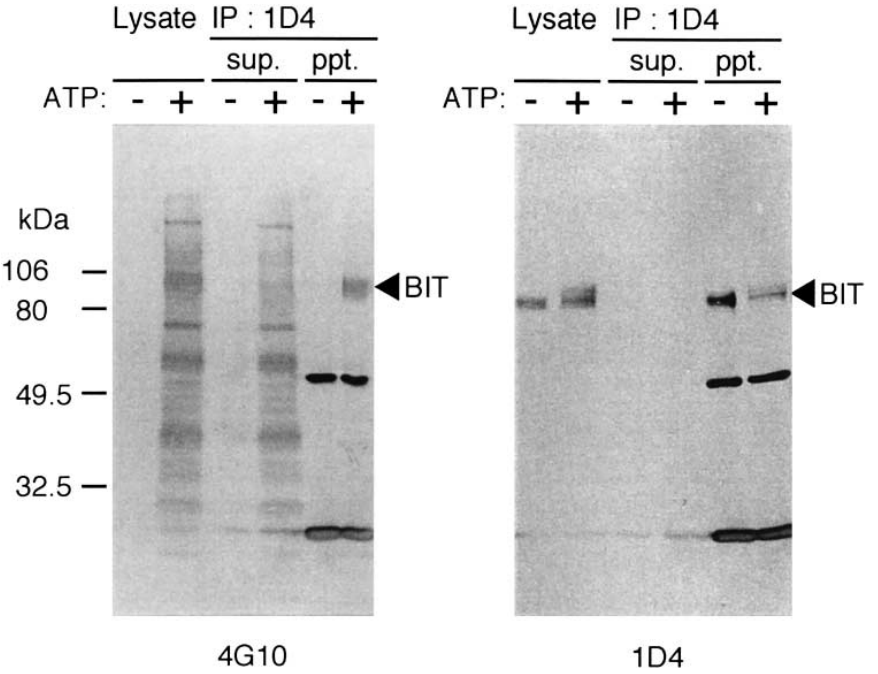

Fig. 4. BIT is one of major endogenous substrates of brain tyrosine kinases in vitro. After incubation of rat brain samples with (+) or without (−) ATP, proteins were solubilized with 1% CHAPS, and BIT was immunoprecipitated with monoclonal antibody 1D4 attached to Sepharose 4B. The solubilized CHAPS lysates, the unadsorbed supernatants and the immunoprecipitates were immunoblotted with monoclonal anti-phosphotyrosine (4G10) (left) and anti-BIT (1D4) (right) antibodies. The bands below 80 kDa in the immunoprecipitates were those of the mouse immunoglobulin chains reacted with a second antibody. IP, immunoprecipitation.

3.6. Culture of cerebral cortical neurons on BIT

Some of immunoglobulin superfamily molecules have been reported to have ability to promote neurite extension [23]. Thus, we examine the effect of BIT as a substrate on primary cultured neurons. Primary cerebral cortical neurons from E17 rat were cultured on the plates that were coated with BSA, poly-L-lysine, laminin or BIT (Fig. 5A). BSA could not support cell attachment and neurite extension. Poly-L-lysine that is usually used for the primary culture, laminin that is known as a potent substrate for neurite extension, and BIT could support cell attachment and neurite extension. Furthermore, we examined specific involvement of BIT in this effect using monoclonal anti-BIT antibody that recognize an extracellular part of BIT [3]. Pretreatment of the BIT-coated plate with this antibody specifically blocked the neurite extension, but the pretreatment of the laminin-coated plate showed no effects (Fig. 5B). These results suggest that BIT on the plate interacts with the ligand on cerebral cortical cells and this interaction might support cell attachment and neurite extension.

4. Discussion

BIT is a new type immunoglobulin superfamily molecule found in the brain. It has three immunoglobulin-like domains (V, C1, C1) and two TAMs. The overall structure is similar to those of the immune antigen receptors. Each immunoglobulin domain of BIT shows high degrees of homology to immunoglobulin domains of immunoglobulins, TCRs and MHC molecules (Table 1). Moreover, the immunoglobulin domains of BIT show high levels of homology to those of primitive immunoglobulins (Table 1). BIT probably have evolved from the same ancestor containing C1-type domain(s) as the antigen recognition molecules. BIT structurally belongs to a family that consists of immunoglobulin, TCR and MHC molecule.

However, there are two differences between BIT and the immune antigen receptors. The first is that BIT has more glycosylation sites than the immune antigen receptors. About 30% of the BIT molecular weight calculated using SDS-PAGE is actually carbohydrate [3]. It is possible that the oligosaccharide structure of BIT is related to physiological role of BIT. The second is that the diversity of the V-type domain of rat BIT has not been found so far.

In the present study we showed that BIT could support cell attachment and neurite extension of cerebral cortical neurons. BIT showed no homophilic adhesion activity when transfected CHO cells expressing BIT at the cell surface were used for the aggregation assay in the absence and presence of 1 mM Ca^{2+} and 1 mM Mg^{2+} (data not shown). These findings suggest that BIT may be a heterophilic adhesion molecule, which interact with a ligand on cerebral cortical cells. Further studies are required for elucidating the extracellular function of BIT.

BIT has two longer TAMs. It was reported that molecules with more TAMs tend to generate more intense signals [22].

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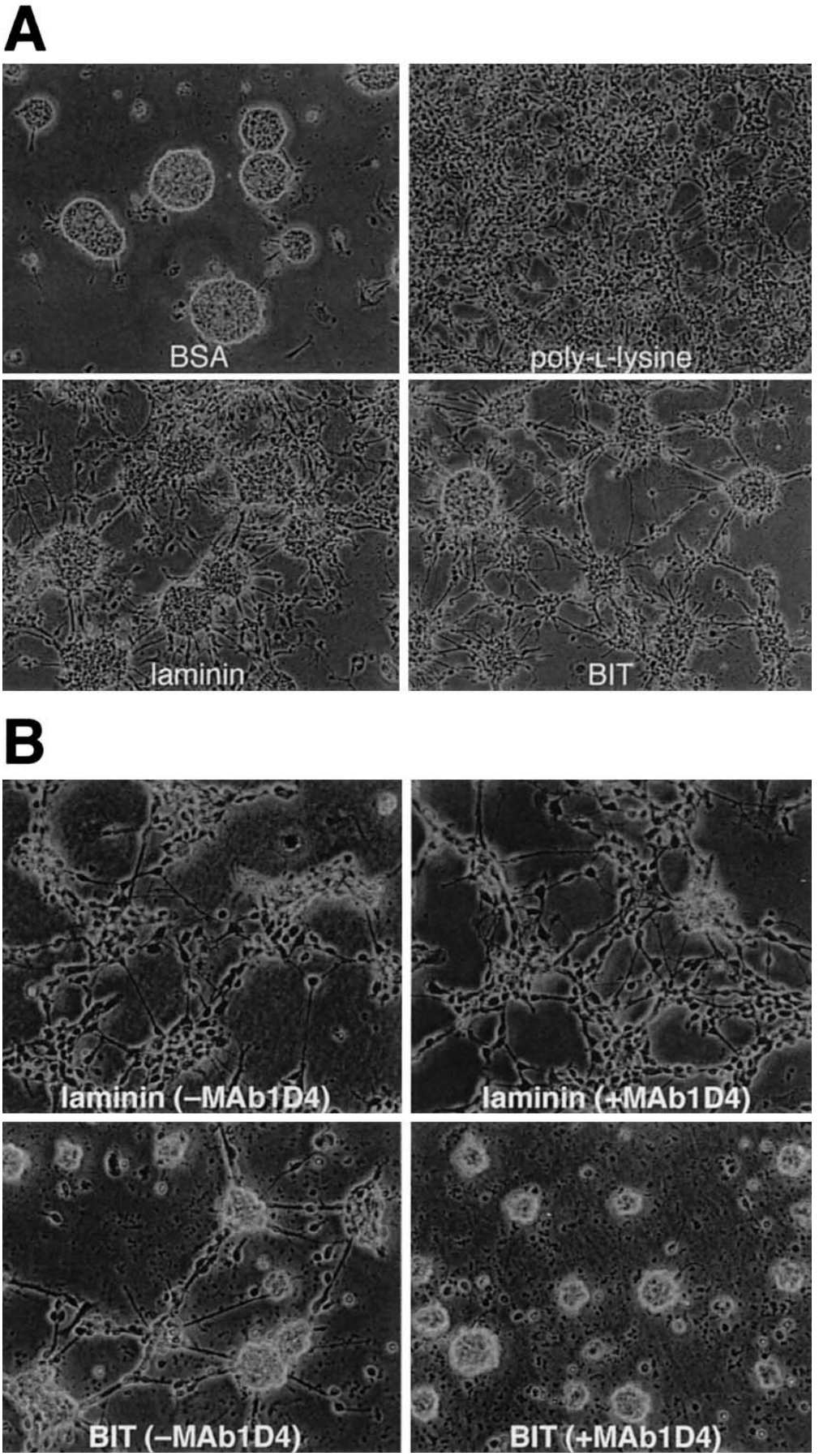
Fig. 5. Culture of cerebral cortical neurons on BIT. A: Cerebral cortical cells prepared from E17 rat were cultured on the plates coated with BSA, poly-L-lysine, laminin or BIT. Phase contrast micrographs were taken after 45 h culture. B: Pretreatment of the BIT-coated plate with anti-BIT monoclonal antibody inhibited the neurite extension, but pretreatment of the laminin-coated plate with the same antibody showed no effects. Control plates were pretreated with control monoclonal antibody (3B12a). Phase contrast micrographs were taken after 24 h culture.


A
BSA
poly-L-lysine
laminin
BIT
B
laminin (–MAb1D4)
laminin (+MAb1D4)
BIT (–MAb1D4)
BIT (+MAb1D4)

Thus, strengthening signal is probably the reason why BIT has two TAMs. The longer TAMs tend to have higher contents of proline between the two YxxL/I sequences; human PD-1 (5 prolines), p58/NKAT (4 prolines) and BIT (5 and 4 prolines) (Fig. 2D). The long motifs may require proline residues for functional conformation.

We recently searched for a molecule that interacts with the tyrosine-phosphorylated BIT in the brain and found that SHP-2 specifically bound to the phosphorylated BIT through its SH2 domain in vitro and in living cells [8]. The amino acid sequences around the tyrosine residues in each TAM are quite similar to the sequences around the SHP-2 binding sites of IRS-1 (Fig. 2E). Furthermore, BIT was a major tyrosine-phosphorylated protein associated with SHP-2 in phosphorylated rat brain lysate, suggesting that BIT is a major signaling molecule just upstream of SHP-2 in the brain [8]. In the present study, we demonstrated that BIT is a major substrate of protein tyrosine kinases in the brain. These findings suggest that BIT is a quite important molecule in signal transduction in the brain.

We previously reported BIT as a brain-specific protein. Western blotting of several different tissues with monoclonal anti-BIT (1D4) or polyclonal anti-BIT showed that BIT is specific for the brain [2,3]. However, mRNA of BIT did not appear to be specific for the brain in the present study. Regulation at post-transcriptional revel is probably important for the specific expression of the BIT protein.

The results in the present study and our recent findings suggest that BIT functions as a membrane signaling molecule that might participate in heterophilic cell adhesion. BIT increases postnatally and is highly expressed in the mature brain [2]. Furthermore, BIT distributes widely and selectively in synapse-rich regions in the brain, including cerebral cortex and hippocampus [[2], in preparation]. BIT is also present in some fiber tracts. These findings suggest that BIT may play a key role in cell–cell interaction in the neural network and may be involved in regulation of neuronal function in the mammalian brain. BIT is an immune antigen receptor-like molecule first found in the nervous system. The structural homology indicates that BIT may have evolved quite recently through vertebrate evolution as immune antigen receptors. Thus, it is quite interesting to consider the possibility that BIT participates in brain function that have evolved recently through vertebrate evolution.

Quite recently SHP-2 binding proteins, SHPS-1 and SIRPα, have been cloned [24,25], and they are the same molecules as BIT. SIRPα, a human homologue of BIT, has been found as a family of proteins, and 15 different sequences of the first immunoglobulin-like domain have been reported. This diversity of the V-type immunoglobulin domain of human BIT (SIRP family) reminds us of its homology to the immune antigen receptors, and of its possible evolutionary divergence. We think that it is very important to elucidate the physiological role of this quite unique molecule.

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