Gene structure of mouse BIT/SHPS-1

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BIT/SHPS-1/SIRP α /P84 is a unique molecule with a high degree of homology with immune antigen recognition molecules (immunoglobulin, T-cell receptor and MHC), and is highly expressed in the brain. The extracellular region contains three immunoglobulin-like domains (V-type, C1-type and C1-type), and the intracellular region contains two signalling motifs that interact with SHP-2 protein tyrosine phosphatase. BIT-coated plates support cell-substrate adhesion and neurite extension of neurons, and BIT participates in neuronal signal transduction. Diversity of the V-type domain sequences of human BIT has been reported. In the present study we analysed the structure of the mouse BIT gene (*Bit*). The protein coding region consists of eight exons corresponding to a signal peptide, a V-type domain, a C1-type domain, a C1-type domain, a transmembrane region

and three parts of one cytoplasmic region. The two signalling motifs are encoded in one exon. Four splicing forms of mouse BIT were revealed. We also found the sequence diversity in three mouse strains, namely BALB/c, 129/Sv and C57BL/6. The substitution patterns of amino acids and nucleotides indicate positive pressure to alter the amino acids in the V-type domain in evolution. Immunoblot analyses showed that mouse BIT and human BIT α are predominantly expressed in the brain. On the bases of these findings we discuss the possibility that BIT contributes to the genetic individuality and diversity of the brain.

Key words: brain, evolution, immunoglobulin superfamily, polymorphism, SHP-2.

INTRODUCTION

We previously identified BIT as a 90 kDa glycoprotein in rat brain by using the monoclonal antibody 1D4 [1,2]. BIT is a highly N-glycosylated protein recognized by several lectins and contains sialic acid [2]. Molecular cloning revealed that BIT has three immunoglobulin-like domains (V-type, C1-type and C1-type [3]) in the extracellular part and two signalling motifs in the intracellular part [4–7]. The intracellular signalling motif containing two tyrosine phosphorylation sites interacts with SHP-2 protein tyrosine phosphatase in response to its tyrosine phosphorylation [5–7], and the tyrosine-phosphorylated motif potentially stimulates SHP-2 phosphatase activity [5]. The immunoglobulin-like domains of BIT show a high degree of homology with those of immunoglobulins, T-cell receptors and MHC molecules [4]. BIT has a J-like sequence in the first immunoglobulin-like (V-type) domain and a consensus sequence (Phe/Tyr-Xaa-Cys-Xaa-Val/Ala-Xaa-His), which has been found in the C-terminal C1 domains of immunoglobulins and MHC molecules, in the third immunoglobulin-like (C1-type) domain [4]. It seems likely that BIT and these immune molecules have a recent common origin.

BIT-coated plates support the cell adhesion and neurite extension of neurons [4,8]. We recently found that brain-derived neurotrophic factor (BDNF) induces tyrosine phosphorylation of BIT and its association with SHP-2 in primary cultured neurons [9]. BIT might participate in the formation, maintenance and/or plasticity of neuronal networks. It was recently reported

that BIT is also abundantly expressed in macrophages and monocytes, where it associates with SHP-1 [10,11].

BIT has also been reported as SHPS-1 [6], SIRP α [7] and P84 [12]. SHPS-1 and SIRP α were cloned as a SHP-2-binding protein and were shown to be tyrosine-phosphorylated through the activation of receptors of insulin, epidermal growth factor and platelet-derived growth factor. Human SIRP α (BIT α) and SIRP β (BIT β), which lacks the cytoplasmic region, were reported as a family of proteins [7]. A total of 15 V-type domain sequences of this family from several human sources were reported. P84 was originally found in the mouse central nervous system with the use of a rat monoclonal antibody [8].

We previously determined the cDNA sequence of BALB/c mouse BIT and mapped mouse *Bit* to chromosome 2 [13]. In the present study, the gene structure of 129/SvJ mouse BIT was analysed. The gene encoding BIT protein is composed of eight exons. We also found BIT polymorphism, and we determined relative amounts of BIT protein in mouse and human tissues.

EXPERIMENTAL

Analysis of 129/SvJ mouse BIT gene

A 129/SvJ mouse genomic library in the λ FIXII vector (946306; Stratagene) was screened by hybridization with dioxigenin (DIG)-labelled cDNA probes of BALB/c mouse BIT [13], initially with probe b (nucleotides 304–963) and secondly with probe a (nucleotides 1–303). The DIG-labelled probes were

Abbreviations used: DIG, dioxigenin; UTR, untranslated region; ORF, open reading frame; ITAM, immunoreceptor tyrosine-based activation motif; BDNF, brain-derived neurotrophic factor.

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The nucleotide sequence data reported here have been submitted to the DDBJ, EMBL and GenBank® Nucleotide Sequence Databases under the accession numbers AB018194 (C57BL/6 BIT), AB023430 (human BIT α 2), AB024500 (exon 1), AB024501 (exon 2), AB024502 (exon 3), AB024503 (exon 4), AB024504 (exon 5), AB024505 (exon 6), AB024506 (exon 7) and AB024507 (exon 8).

synthesized using a DIG DNA Labelling Kit (Boehringer), and the conditions for the detection were essentially the same as the standard protocol in the DIG System User's Guide (Boehringer). We isolated clones #1, #2 and #3 with probe b, and clones #4, #5 and #6 with probe a. Clones #1, #2 and #4 were analysed in detail. The genomic DNA encoding the C-terminal region was obtained by PCR using the genomic library as a template with LA Taq (TAKARA), the 5' primer (TTGGTGGGTCAATAG-GCTGGGGCTCCAGAATGA) corresponding to a sequence near the 3'-terminal of clone #1, and the 3' primer (GTAGG-ACAAGAAGACTTGTGGGGATGGGGCCTA) corresponding to the 3' untranslated region (UTR) of BIT cDNA. A PCR product of the same length was also seen using 129/Sv genomic DNA as a template. The sequence (298 bp) between the primers was determined by obtaining the same sequence products from independent PCR. Since there was a gap between clones encoding the signal peptide (clone #4) and the V-type domain (clone #2), the length between clone #4 and clone #2 was estimated by PCR using 129/Sv mouse genomic DNA as a template with LA Taq, the 5' primer (ATCTGAAATTGTGCTTCGCAGGCCTGGA-AAGCT) corresponding to a sequence near the 3'-terminal of clone #4, and the 3' primer (TGGTGAAGTAAAAAGCAGC-ATCTGGGCTGGAGA) corresponding to a sequence near the 5'-terminal of clone #2. The confirmation of the estimated length was made by PCR with different primer sets and Southern blotting of the PCR products. Clones #1, #2 and #4 were digested with restriction enzymes, and the fragments hybridized with cDNA probes were subcloned into Bluescript KS⁻ (Stratagene) and sequenced.

cDNA sequence analysis of C57BL/6N mouse BIT

Total RNA was purified from adult mouse brain of C57BL/6N (CLEA JAPAN, Tokyo, Japan) using an RNeasy Kit (Qiagen, Hilden, Germany), and first-strand cDNA was synthesized using SuperScript II RNase H⁻ Reverse Transcriptase (Gibco BRL) and a random primer. The total RNA was then used for the synthesis of BIT cDNA with Vent DNA polymerase (New England BioLabs, Beverly, MA, U.S.A.) using the 5' UTR primer (TCACCGCCGCTCTCCCTCCTTGCTCTGCA) and 3' UTR primer (GTAGGACAAGAAGACTTGTGGGGATGGGGCCTA). The product of 1.6 kb was sequenced after subcloning into pCR-Script vector (Stratagene). The BIT cDNA sequence was determined by obtaining the same sequence from the independent PCR.

Analysis of splicing variants of mouse BIT

Total RNA was purified from adult BALB/c mouse brain, and BIT cDNA was synthesized with SuperScript II RNase H⁻ Reverse Transcriptase (Gibco BRL) and Vent DNA polymerase (New England BioLabs) using the 5' UTR primer and 3' UTR primer as described above. The products of 1.6, 1.0 and 0.6 kb were sequenced after subcloning.

Southern blotting

Genomic DNA (7.5 µg) from 129/Sv mouse kidney or brain was digested with *Eco*RI, *Bam*HI or *Hind*III and electrophoresed in 0.8% agarose gel. The DNA was then blotted on to a Hybond N (Amersham) membrane, and detected using the DIG system (Boehringer) with the BIT cDNA probes shown in Figure 1(A), namely probe a (nucleotides 1–303), probe b (304–963), probe c (964–1519) or probe d (1520–2128). Hybridization was performed in high-SDS-concentration buffer (the DIG System User's Guide, Boehringer) [5 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium

citrate)/50 % (v/v) formamide/50 mM sodium phosphate (pH 7.0)/7 % (w/v) SDS/2 % (w/v) blocking reagent/0.1 % *N*-lauroylsarcosine/50 µg/ml salmon sperm DNA] at 42 °C overnight. The filters were washed twice in 2 × SSC/0.1 % SDS at room temperature and then in 0.1 × SSC/0.1 % SDS at 68 °C twice. Anti-DIG-alkaline phosphatase Fab fragments and CDP-Star [disodium 4-chloro-3-(methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan-4-yl)phenyl phosphate; Boehringer] were used for detection.

Cloning of human BIT cDNA

We first analysed human BIT cDNA by PCR. A human brain cDNA library in λgt11 (HL3002b; ClonTech, Palo Alto, CA, U.S.A.) was used as a template for PCR. Primers that amplify human BIT cDNA were synthesized on the basis of putative partial human BIT sequences in the database, CCA53 (accession number U06701) for the 3' UTR primer near the stop codon (TGTGGGACAAGAAAGCGCGTAGAGATGGGTGCT) and CpG73d2 (accession number Z62810) for the 5' UTR primer near the initiation codon (AAAGTGGAGCGGGGACCCGG-CCTCTGGGCA). Another 5' UTR primer (GCACTCACGG-CCGCTCTCCCTCCTCGCT) was also synthesized on the basis of the cDNA sequence obtained from PCR between the λ phage primer and the BIT open reading frame (ORF) primer. The PCR products were sequenced after subcloning. We obtained cDNA sequences encoding α1- and α2-types of BIT using either 5' UTR primer.

To obtain full-length human BIT cDNA, we cloned BIT cDNA from a SuperScript human brain cDNA library (Gibco BRL) using a GeneTrapper cDNA Positive Selection System (Gibco BRL) with a primer (TGAGGAGGAGCTGCAGGT-GATTGAGCCTGA) corresponding to the sequence near the 5' end of the BIT V-type domain where the α1-type and α2-type cDNA sequences are identical. The cloning procedure used is essentially the same as that given in the instruction manual. For the identification of cDNA clones by colony hybridization, we used a DIG-labelled cDNA probe mixture of human BIT V-type domain; 14 clones obtained were characterized.

Antibody production and Western blotting

For antibody production, rabbits were immunized with 550 µg of rat BIT C-terminal 40-mer peptide ('C-TAM') [5] in Freund's complete adjuvant. A booster of the same dose in complete adjuvant was injected 1 month later, and the antiserum was prepared 7 days after the booster. The antibody was purified from the antiserum using a column of a fusion protein of glutathione S-transferase and the BIT cytoplasmic region [5]. This purified antibody was used as a primary antibody for Western blotting. Western blotting of several tissues from C57BL/6N mouse and from human (Protein Medley; ClonTech) was performed by the enhanced chemiluminescence (ECL[®]) detection system (Amersham) as described previously [5]. The mouse samples for SDS/PAGE were prepared by homogenizing the tissues in 20 mM Tris/HCl, pH 6.8, containing 2 % SDS and 2 % mercaptoethanol, boiling them for 3 min and adding glycerol to a final concentration of 20 %. Relative amounts of BIT protein were determined by densitometry using Densitograph AE-6900 (ATTA, Tokyo, Japan) with different amounts of the brain homogenate for the standard curve. Deglycosylation by N-glycosidase F (Boehringer) was carried out as described previously [2].

Figure 1 Gene structure of mouse *Bit*

(A) The genomic clones analysed are schematically aligned. The cDNA structure of BIT and cDNA regions used as probes are also shown. SP, V, C1, TM and Cyt. indicate signal peptide, V-type domain, C1-type domain, transmembrane region and cytoplasmic region respectively. (B) Southern-blot analysis of 129/Sv mouse *Bit*. The source of DNAs and the enzymes used are indicated above the lanes.

RESULTS

Gene structure of 129/SvJ mouse BIT

First, a 129/SvJ mouse genomic library in the λ FIXII vector was screened by hybridization with a cDNA probe of BALB/c mouse BIT (probe b in Figure 1A; nucleotides 304–963) [13]. Three clones (clones #1, #2 and #3) were isolated from 5×10^5 plaques. Since clone #3 appeared to be identical with clone #2, clones #1 and #2 were analysed further. These clones contained BIT exons (exon 2–exon 7), except for those corresponding to the signal peptide with the 5' UTR (exon 1) and C-terminal cytoplasmic region (exon 8). To obtain exon 1 we used a second probe, probe a (a 5' UTR region probe, nucleotides 1–303). Three clones (clone #4, #5 and #6) encoding this region were isolated from 5×10^5 plaques, and among them clone #4 was analysed further. The C-terminal region (exon 8) was obtained by PCR, as

described in the Experimental section. The gene structure, and exon and intronic sequences of 129/Sv mouse *Bit* are shown in Figure 1(A) and Figure 2. A signal peptide with the 5' UTR, a V-type domain, a C1-type domain, a C1-type domain, and a transmembrane region were encoded by distinct exons (exon 1–exon 5). The cytoplasmic region was divided into three exons (exon 6–exon 8). Two cytoplasmic signalling motifs were encoded by one exon (exon 8). The distance between exon 1 and exon 2 was estimated to be about 15 kb by PCR as described in the Experimental section. Exon 1–exon 9 of the *Bit* gene cover over 30 kb. The Southern-blot analysis of kidney and brain DNAs using the probe a, b, c or d was performed (Figure 1B), and the blot patterns of kidney and brain DNAs were identical. Exon 7 has two splice donor sites, causing a splice variant that has a sequence (GTACAGTCTTTG) corresponding to the VQSL amino acid sequence in the cytoplasmic region. This cytoplasmic

Figure 3 Splicing variants of mouse BIT

(A) Analysis of RT-PCR products of mouse BIT. RT-PCR products from BALB/c mouse brain with BIT 5' and 3' UTR primers were electrophoresed in agarose gel and stained with ethidium bromide. Band a (1.6 kb) and other short bands, b (1.0 kb) and c (0.6 kb), were sequenced after subcloning. (B) Schematic structure of mouse BIT splicing variants. a and a' represent the structures from sequences of band a. a' has a VQSL amino acid insertion in the cytoplasmic region. b represents a short form without the regions encoded by exons 3 and 4, and c represents a shorter form without the regions encoded by exons 2, 3 and 4.

insertion sequence is frequently found in BIT cDNAs, as shown in Figure 3 and as reported previously [14].

Splicing variants of mouse BIT

Figure 3 shows mouse BIT splicing variants expected from reverse-transcription (RT-) PCR products with the primers corresponding to the 5' UTR and 3' UTR sequences. One variant has the cytoplasmic VQSL insertion sequence from exon 7, as described above. Another variant has only a signal peptide and a V-type domain in the extracellular part (absence of exon 2 and 3). The same form was also reported by another group [12]. A third variant, which was a rare RT-PCR product, has no immunoglobulin-like domain in the extracellular part (absence of exons 2, 3 and 4).

Substitutions of amino acids and nucleotides among BALB/c, 129/Sv and C57BL/6 BIT are highly concentrated in the V-type domain

The protein coding sequence constructed using the exons of 129/Sv *Bit* is different from the BALB/c BIT sequence we

previously determined (accession number D85785) [13] and is identical with the mouse BIT cDNA sequence reported as mouse SHPS-1 [14] and P84 [12]. We further determined the cDNA sequence of C57BL/6 BIT by sequencing the RT-PCR products for comparison (accession number AB018194). The comparison of the sequences of BALB/c (type I), 129/Sv (type II) and C57BL/6 (type III) revealed the concentration of amino acid and nucleotide substitutions in the V-type domain (Table 1). Similar sequence variation in the mouse was recently reported, and it was suggested that the variation may reflect some degree of BIT polymorphism [10]. We obtained different exon 2 sequences from different strain genomic DNAs by PCR using the same primer sets corresponding to the intronic sequences near exon 2 (results not shown). This result confirmed that these different sequences are from the same gene of different strains.

Sequence comparison of human BIT

We first obtained the $\alpha 1$ and $\alpha 2$ types of human BIT cDNA by PCR using a brain cDNA library as a template as described in the Experimental section. Nucleotide substitutions between $\alpha 1$ and $\alpha 2$ ORF sequences were found only in the region encoding the V-type domain. Figure 4(A) shows the alignment around the V-type domains of the $\alpha 1$ and $\alpha 2$ types of BIT. There are 20 nucleotide substitutions and three nucleotide insertions in the V-type domain. Among these substitutions, only three nucleotide substitutions are related to unsubstituted amino acids. To obtain full-length human BIT cDNA, we cloned BIT cDNA from another human brain cDNA library. A total of 14 clones obtained were characterized. All of these clones encoded the $\alpha 2$ -type BIT, whose nucleotide sequence has not been reported. Two long clones (GH9 and GH13) of 3.9 kb and one clone (GH4) of 2.5 kb containing complete ORF sequences and poly(A) tails were further analysed. The sequence of the clone GH13 is shown in Figure 4(B) and the poly(A) tail of the 2.5 kb clone GH4 starts at the residue corresponding to residue 2436 of the clone GH13. These results suggest that these clones with different lengths of 3'UTR correspond to bands of 3.9 kb and 2.5 kb in Northern blots [7].

Mouse BIT and human BIT α are expressed predominantly in the brain

The high expression of BIT protein in the brain and spleen have been reported, but the relative amounts of BIT protein in tissues and the tissue specificity of the protein in human have not been reported. Thus, the tissue distribution in mouse and in human was quantitatively analysed by Western blotting (Figure 5). The polyclonal antibody against a well-conserved cytoplasmic sequence of rat BIT recognized mouse BIT (about 95 kDa) (Figure 5A) and human BIT α (about 80 kDa) (Figure 5D). This antibody does not recognize human BIT β , which has a truncated cytoplasmic region [7]. The antibody revealed that BIT protein was expressed predominantly in the brain in both species, as in the rat [1,4]. BIT was also seen in mouse spleen (about 130 kDa). The bands of about 130 kDa were seen in all the tissues except skeletal muscle. These were clearly observed by long exposure, and N-glycosidase F treatment converted the molecular mass to 65 kDa (Figure 5B). A band of about 75 kDa in the mouse brain might be a degradation product of the 95 kDa form. We also observed BIT minor bands of 130 kDa and 45 kDa in the mouse brain (Figure 5A). N-glycosidase F digestion appeared to convert the molecular masses of the bands from both 130 and 95 kDa to 65 kDa, from 75 to 47 kDa and from 45 to 35 kDa. A similar deglycosylation result was observed using immunoprecipitated BIT [10]. A sharp band of 48 kDa did not change the molecular

Table 1 Number of amino acid (aa) and nucleotide substitutions in BIT

SP, V, C1, TM, Cyt. and E indicate signal peptide, V-type domain, C1-type domain, transmembrane region, cytoplasmic region and exon respectively. The numbers of amino acids in the mouse BIT regions are indicated at the top. Rat and human BIT regions corresponding to mouse BIT regions are compared.

	SP (E1) 28 aa	V (E2) 119 aa	C1 (E5) 106 aa	C1 (E4) 111 aa	TM (E5) 39 aa	Cyt. (E6 + 7 + 8) 106 aa
BALB/c and 129/Sv						
Amino acid	0	10	2	1	0	1
Nucleotide	0	11	2	2	0	2
BALB/c and C57BL/6						
Amino acid	0	12	0	2	1	1
Nucleotide	0	16	0	3	1	2
129/Sv and C57BL/6						
Amino acid	0	12	2	1	1	0
Nucleotide	0	15	2	1	1	0
BALB/c and rat						
Amino acid	2	27	32	31	3	3
Nucleotide	2	45	54	48	9	11
BALB/c and human (clone GH13)						
Amino acid	4	43	38	48	15	12
Nucleotide	8	98	79	97	34	47

mass, indicating that this protein is not directly related to BIT. The 45 kDa band may be the short form that has one immunoglobulin-like domain, because COS-7 cells transfected with this form of cDNA expressed a 45 kDa molecule, whose molecular mass was converted into 35 kDa by N-glycosidase F digestion, as reported in [10]. The amounts of about 130 kDa (and 120 kDa) bands in the mouse spleen, lung and thymus were 9.5, 1.4 and 1.1 % of the mouse brain 95 kDa band respectively, and those in the other mouse tissues were less than 1 % (Figure 5C). In mouse brain the amounts of 130, 75 and 45 kDa bands were 0.41, 11.3 and 1.1 % respectively. The human brain has a major 80 kDa band and a 52 kDa band (Figure 5D). The bands of about 86 kDa were also seen in all the human tissues, except the skeletal muscle, by long exposure (results not shown), and the relative amounts of the bands were less than 1 % of the human brain 80 kDa band. It might be possible that some human BIT α was degraded prior to the electrophoresis.

DISCUSSION

BIT is a unique immunoglobulin superfamily molecule that has one V-type and two C1-type immunoglobulin domains. The amino acid sequence of the first immunoglobulin-like domain (V-type) has a high degree of homology with those of the V-type domains of immunoglobulin light chains and T-cell receptors [4]. The two C1-type domains show a high degree of homology with the C1 domains of the immunoglobulins and MHC molecules. The second C1-type domain has a consensus sequence pattern (Phe/Tyr-Xaa-Cys-Xaa-Val/Ala-Xaa-His) found in the C-terminal C1 domains of immunoglobulins and MHC molecules. This consensus pattern is also conserved in the BIT of three mouse strains, while a change of phenylalanine/tyrosine to leucine in the sequence occurs in the human BIT (Figure 4B). The cytoplasmic signalling motifs of BIT are also similar to those of T-cell receptor and B-cell receptor [4,5]. The former interacts with tandem SH2-containing tyrosine phosphatase, SHP-2, and the latter interacts with tandem SH2-containing tyrosine kinase, ZAP70 or Syk. These structural features prompted us to investigate the gene structure and the diversity of mouse BIT.

We analysed the mouse *Bit* gene and found that the protein-coding region consists of eight exons corresponding to a signal peptide, one V-type domain, two C1-type domains, a transmembrane segment and three parts of one cytoplasmic domain. The coding-region of the J-like sequence is present as a part of the V-type domain exon (exon 2). The two cytoplasmic signalling motifs are encoded in one exon (exon 8). Since the two motifs resemble each other [4], the exon 8 might be generated by the duplication of an ancestral motif gene and the removal of an intron between them. The gene structure of the signalling motifs of BIT is different from that of immunoreceptor tyrosine-based activation motifs (ITAMs) of the T-cell-receptor ζ -chain. Each ITAM straddles the boundary of two exons [15,16].

Our Southern-blot results suggest that mouse *Bit* is a single-copy gene. The Southern-blot patterns of brain and kidney *Bit* appeared to be identical. These findings indicate that mouse *Bit* may not diversify by gene rearrangement. When we analysed the mouse BIT cDNA sequence obtained by RT-PCR, we found four splicing variants (Figure 2). Considering the structural similarity to T cell receptor ζ -chain, a variant with no immunoglobulin-like domain is interesting, but the presence of the protein has not been demonstrated.

Different BIT proteins are expressed in the mouse strains BALB/c (type I), 129/Sv (type II) and C57BL/6 (type III), and the substitutions of amino acids and nucleotides are concentrated in the V-type domain. There are 10–12 amino acid substitutions and 11–16 nucleotide substitutions in the V-type domain between any two strains, and the V-type domain that is only 23 % of the ORF has 72–75 % of the amino acid substitutions and 65–79 % of the nucleotide substitutions (Table 1). The substitutions in codons show a pattern different from the general pattern, in which the third position in codons has the most substitutions, which generate mostly silent (synonymous) substitutions. In the V-type domain, the substitutions of the third position are the fewest. Thus about 81 % of the substitutions in the V-type domain are amino-acid-altering (non-synonymous) substitutions. Therefore the present findings suggest that BIT alters the amino acid sequence of the V-type domain positively among mouse strains. This feature has some similarity to that of MHC, whose

Figure 5 Mouse and human BIT proteins are predominantly expressed in the brain

(A) Western-blot analysis of BIT tissue distribution in C57BL/6 mouse. Homogenates (20 μ g of protein) of the indicated tissues were resolved on a SDS/4–20%-polyacrylamide gel and blotted as described in the Experimental section. (B) Deglycosylation of mouse BIT by N-glycosidase F. Homogenates of the indicated tissues were incubated with (+) or without (–) N-glycosidase F. Samples (20 μ g of protein) of digests were subjected to Western blotting. (C) Relative amounts of BIT protein were determined by densitometry. The amounts of about 130 kDa (and 120 kDa) bands in the mouse thymus, spleen, lung, liver, kidney, heart, skeletal muscle, stomach and intestine were 1.1, 9.5, 1.4, 0.28, 0.24, 0.12, 0, 0.61 and 0.75% of the mouse brain 95 kDa band respectively. In the mouse brain, the amounts of 130 kDa and 75 kDa were 0.41 and 11.3% respectively. (D) Western-blot analysis of BIT tissue distribution in human. Homogenates (20 μ g of protein) of the indicated tissues were resolved on a SDS/4–20%-polyacrylamide gel and blotted as described in the Experimental section. Rat brain homogenate (20 μ g of protein) was also loaded.

substitutions of amino acids and nucleotides are present between mouse (BALB/c) and human (α 2-type) BIT. The amino acid sequences show only 66.6% identity (60.8% in the extracellular part and 89.6% in the cytoplasmic region). Considering that the amino acid sequences of human and mouse L1 are 87.7% identical (86.3% in the extracellular part and 100% in the

cytoplasmic region) [20], the substitution rate of BIT, especially in the extracellular part, is quite high.

Immunoblot analyses of rat tissue distribution using the same amounts of protein have revealed that BIT protein is predominantly expressed in the brain [1,4]. A small amount of the protein is also expressed in the spleen and a smaller amount is

expressed in the lung [4]. However, BIT mRNA is expressed more widely, and the highest expression levels are observed in the spleen and the brain [4,7,14]. We detected BIT RT-PCR products of normal size and short size (one- immunoglobulin-domain-form size) from all rat tissues analysed. We speculate that the regulation of the post-transcriptional level is important for the specific expression of BIT protein. In the present study we analysed BIT protein expression in mouse and human and determined the relative amounts. Mouse BIT and human BIT α is expressed dominantly in the brain, as is rat BIT. In addition to transcriptional level regulation, the expression of BIT protein is probably regulated at the post-transcriptional level in these species.

Considering mouse BIT polymorphism, some of the different human V-type domain sequences reported [7] may also be generated from human BIT polymorphism. We obtained both BIT α 1 and BIT α 2 cDNAs by PCR from one individual brain cDNA library, while we obtained only BIT α 2 cDNA clones from another individual brain cDNA library by screening. We could not find the α 1-type sequence in PCR products from the latter library. Thus, it seems likely that the α 1 and α 2 types are from the same allele.

BIT is expressed at a high level in the adult brain, and the time course of the increase after birth appears to correspond to that of synaptogenesis in the brain [1]. BIT is distributed in synaptic regions and fibres. BIT is also a major substrate for protein tyrosine kinases in the brain [4] and participates in tyrosine phosphorylation signalling [5]. BIT supports the cell adhesion and neurite extension of neurons *in vitro* [4,8]. BIT also participates in BDNF signalling in neurons [9]. These findings indicate that BIT may be involved in the formation, maintenance and/or plasticity of neuronal networks. We hypothesize that the differences in the BIT V-type domain contribute to differences in cell-cell interactions and cause differences in network formation or in plastic change. This might generate the genetic individuality and diversity of the brain.

It was recently reported that MyD-1, which is the same molecule as BIT, is expressed on monocytes and a subpopulation of dendritic cells and mediates binding to CD4 T-cells [21]. Another group reported that one major binding protein for SH2-containing protein phosphatase SHP-1 in macrophages was identified as BIT [11]. SHP-1 is a critical regulator of macrophage biology. These findings suggest that BIT plays some important roles in the immune system. Thus it is also likely that the diversity of the V-type domain of BIT is implicated in the

immune system, and positive pressure to alter the amino acids in evolution might occur in the immune system.

Among the amino acid sequences of mouse, rat and human BIT, the substitution rate of BIT, especially in the extracellular part, is quite high, indicating that BIT might have evolved recently through vertebrate evolution as immune antigen recognition molecules [22,23]. The possibility that BIT participated in the recent brain evolution is quite interesting. Further studies to elucidate the functions of BIT in the central nervous system and the immune system are required, and these studies will help to resolve the physiological significance of the diversity of BIT.

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