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Cloning and Characterization of BAP3 (BAI-Associated Protein 3), a C2 Domain-Containing Protein That Interacts with BAI1

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BAI1 (brain-specific angiogenesis inhibitor 1), a p53-target gene specifically expressed in brain, encodes a seven-span transmembrane protein considered to be a member of the secretin receptor family. Using a two-hybrid system, we isolated a cDNA encoding a product that interacts with the cytoplasmic region of BAI1 and designated it BAP3 (BAI1-associated protein 3). The BAP3 product is a novel C2 domain-containing molecule with homology to Munc13 and synaptotagmin. As with Munc13, BAP3 is expressed predominantly in brain. Deletion-mutant analysis revealed that the interaction between BAI1 and BAP3 was not mediated by the C2 domains. Its predominant expression in brain and homology to Munc13 indicate that BAP3, by interacting with BAI1, might be involved in some neuronal function such as regulating release of neurotransmitters.

We recently isolated a novel brain-specific gene, BAI1 (brain specific angiogenesis inhibitor 1), using an approach involving identification of genomic DNA fragments containing "functional p53-binding sites" (Nishimori 1997, Tokino 1994). The BAI1 gene contains at least one functional, intronic p53-binding sequence, and its expression is directly inducible by wild-type p53 (Nishimori 1997). The predicted BAI1 protein includes a seven-span transmembrane region as well as extended extracellular and cytoplasmic domains, and appears to be a member of the secretin-receptor family. In its extracellular domain BAI1 possesses thrombospondin (TSP) type 1 repeats which can inhibit bFGF-induced neovascularization in rat cornea (Nishimori 1997). TSP-type 1 repeats are features of several proteins thought to be adhesion molecules; among these are UNC-5 and F-spondin (Klar 1992, Leung-Hagesteijn 1992), both of which are considered to play important roles in growth and guidance of axons. Analysis of subcellular fractions of fetal rat brain indicated that BAI1 was enriched in the growth-cone fraction (Oda 1998). These features suggest that BAI1 might be a multi-functional protein involved in cell-cell or cell-matrix interactions, transmitting extracellular signals to intercellular signal-transduction cascades in the nervous system.

To elucidate the functions of BAI1, by means of a yeast two-hybrid screening system we have been isolating proteins that associate with BAI1 (Shiratsuchi 1998). The first of these isolates, BAP1 (BAI1 associated protein 1) proved to be a member of the MAGUK (membrane-associated guanylate kinase homologue) family of molecules. MAGUKs are considered to serve important functions in organization of receptors, ion-channels and signaling molecules at cell-cell junctions, including synapses (Kim 1995, Kornau 1995, Tsunoda 1997). The PDZ domains of BAP1 recognize and bind to the QTEV motif in the carboxy-terminal region of BAI1 in the same way as similar domains of PSD-95 and DLG do (Shiratsuchi 1998). Another novel gene in our series, BAP2, was found to encode the human homologue of a 58/53-kd substrate of hamster insulin-receptor tyrosine kinase (IRSp53) (Yeh 1996). The BAP2 product contains an SH3 (Src homology 3) domain that interacts directly with the proline-rich cytoplasmic region of BAI1 (Oda 1998). The biological function of BAP2 remains to be elucidated, but it is likely that BAI1/BAP2 interaction mediates membrane-cytoskeletal interactions or signal transduction during neuronal growth.

In the work reported here, we isolated a third gene in the series, which encodes a novel protein (BAP3) that

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interacts with the cytoplasmic region of BAI1. BAP3 possesses two C2 domains and bears sequence homology to Munc13 and synaptotagmin, molecules that are considered to have essential signaling roles during release of neurotransmitters.

MATERIALS AND METHODS

Yeast two-hybrid screening. We have been using a yeast two-hybrid screening system (Durfee 1993) to isolate cDNAs encoding proteins that interact with the cytoplasmic region of BAI1 (Shiratsuchi 1998). In the present experiments the bait vector, constructed by subcloning the entire cytoplasmic region of BAI1 into a SmaI site of pAS2-1, was used to screen an oligo dT-primed human fetal-brain cDNA library in pACT2 vector (Clontech), according to the manufacturer's instructions. Positive clones were co-transformed with either the bait vector or the original pAS2-1 vector into yeast; beta-galactosidase activity served as a marker to confirm the interaction.

Isolation of full-length BAP3 (BAI-associated protein 3) cDNA. A cDNA fragment obtained in the two-hybrid screening was radiolabeled and used as a probe to screen a human fetal brain cDNA library (Stratagene). By re-screening the same library with a partial cDNA, we assembled nucleotide sequences of representative positive clones into a nearly full-length cDNA. The remaining 5′ portion of BAP3 cDNA was cloned by a modified rapid amplification of cDNA ends (RACE) protocol (Marathon cDNA amplification kit, Clontech), based on two successive rounds of PCR amplification using nested primers, according to the manufacturer's instructions. DNA sequencing experiments were performed by the dideoxy nucleotide termination method using an ABI377 DNA sequencer.

Sequence analysis. Multiple sequence alignments were calculated using the clustalW program. Analysis of the degree of relatedness among C2-domain sequences was performed using the DNAsis program and the results were depicted as a dendrogram.

Northern-blot analysis. Northern blots containing poly(A)+ RNA from 16 normal human tissues and 16 different human brain sections were purchased from Clontech. The blots were hybridized with a random-primed [32P]-labeled DNA probe (nucleotides 172–1156 of BAP3 cDNA) according to the manufacturer's instructions. The blots were washed with 0.1× SSC/0.1% SDS at 60°C and exposed for autoradiography at −80°C for 3 days.

Construction of expression vectors of BAI1 and myc-tagged BAP3. The entire coding sequence of BAI1 cDNA was subcloned into the pcDNA3.1 (Invitrogen) expression vector (Shiratsuchi 1998). To construct myc-tagged BAP3, an oligonucleotide duplex which encoded Kozak and myc-epitope sequences was inserted into the HindIII-EcoRI site of pcDNA3.1 (myc-pcDNA3.1), and BAP3 cDNA was ligated in-frame into an EcoRI-XhoI site of myc-pcDNA3.1. The deletion mutants of BAP3 were created by PCR using specific primers and ligated in-frame into the EcoRI-XhoI site of myc-pcDNA3.1. All of the constructs were confirmed by sequencing.

Cell culture and transfection. COS-7 cells were cultured in DMEM containing 10% fetal bovine serum and antibiotic/antimycotic solution (Sigma). 1×10^6 cells were plated in 10-cm culture dishes 24 h before transfection. Expression vectors (15 μg per 10-cm culture dish) were transfected using Trans IT-LT1 (Mirus) according to the manufacturer's instructions. The cells were harvested 16-24 hours after transfection.

Immunoprecipitation. Transfected COS-7 cells were washed with PBS(−) and lysed in a lysis buffer containing 50 mM Tris–HCl at pH 7.5, 140 mM NaCl, 1% Triton X-100, and proteinase inhibitors (complete tablet, Boehringer). The lysates were precleared with protein A Sepharose, then incubated with anti-BAI1 antibody (Shiratsuchi 1998) at 4°C for 2 h. Immune complexes were precipitated with 20 μl of protein A Sepharose and washed extensively with the lysis buffer. The precipitates were eluted with SDS–PAGE sample buffer, separated by means of SDS–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to PVDF membrane, and immunoblotted with an anti-myc tag antibody (9E10, Pharminogen). Labeled bands were visualized with enhanced chemiluminescence (Amersham).

Fluorescence in situ hybridization. Two genomic clones encoding the BAP3 gene were isolated from the human BAC library (Research Genetics, Inc.) by PCR screening. The BAC clones were used as probes for direct R-banding fluorescence *in situ* hybridization (FISH), which is based on FISH combined with replicated prometaphase R-bands (Takahashi 1990, 1991). For suppression of the repetitive sequences contained in the BAC clones, we used Cot-1 DNA (BRL) as described by Lichter *et al.* (1990) with slight modifications (i.e., Cot-1 DNA was used in about 2-fold excess). Labeling, hybridization, rinsing and detection were carried out in a routine manner. Images were captured using CytoVision (Applied Imaging).

RESULTS

Isolation of BAP3. In searching for proteins capable of associating with the cytoplasmic region of BAI1, we used a polypeptide corresponding to the entire cytoplasmic region of BAI1 as a bait for a yeast two-hybrid screening (Shiratsuchi 1998). By screening 4.2×10^6 colonies from a human fetal brain cDNA library, we isolated and sequenced several positive clones that revealed specific interaction with the cytoplasmic domain of BAI1. One of them contained a cDNA sequence encoding a C2 domain similar to that of Munc13 and synaptotagmins. To isolate the full-length cDNA, which we named BAP3, we screened a human fetal brain cDNA library using as the probe a cDNA fragment obtained from the two-hybrid screening. We determined the nucleotide sequences of representative positive clones and assembled them by computer analysis. Alignment of overlapping clones allowed us to define the cDNA of a novel gene that consisted of 4668 nucleotides with an open reading frame of 3561 bp. We assumed that this was almost the full-length cDNA, since Northern-blot analysis revealed a main transcript of about 4.8 kb in brain. Detailed structural analysis of the predicted product (1187 amino acids; Fig. 1a) revealed that BAP3 possessed two C2 domains with homology to the Ca^{2+}/phospholipid-binding domain in protein kinase C. A search for homologies in the non-redundant nucleotide database revealed similarities between BAP3 and Munc13 and synaptotagmins.

Analysis of the C2 domain of BAP3. No domain structures other than two C2 domains were defined in BAP3. C2 domains are found in a large number of proteins; more than 50 are already known (Fig. 1b). Many C2-containing proteins are involved in signal-transduction or membrane-trafficking pathways, but others have only been defined at the sequence level. We systematically analyzed sequence homologies between the C2 domains of BAP3 and those of representative members of various protein families; alignment of these domains revealed a complex mosaic of conserved and variable residues (Fig. 1c). A computer-

a

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1     MRPRGAAFAAGPPGDLHLGTAIGFAGAIWRSRSPAMSTLLDIKSSVLRQV   50
51    QVCPSFRRRTEQDPGSASADPQEPATGAWKPGDGVEFFAHMRLMLKKGEG  100
101   RQGLPCLEVPLRSGSPAPPEPVDPSLGLRALAPEEVEMLYEEALYTVLYR  150
151   AGTMGPDQVDDEEALLSYLQQVFGTSLEEHTEAIERVRKAKAPTYALKVS  200
201   VMRAKNLLAKDPNGFSDPYCMLGILPASDATREPRAQKEQRFGFRKGSKR  250
251   GGPLPAKCIQVTEVKSSTLNPVWKEHFLFEIEDVSTDQLHLDIWDHDDDV  300
301   SLVEACRKLNEVIGLKGMGRYFKQIVKSARANGTAGPTEDHTDDFLGCLN  350
351   IPVREVPVAGVDRWFKLEPRSSASRVQGHCHLVLKLITTQRDTAMSQRGR  400
401   SGFLSHLLLLSHLLRLEHSAEEPNSSSWRGELSTPAATILCLHGAQSNLS  450
451   PLQLAVLHWQVSSRHHQTCTLDYSYLLGLLEDMQAHWEEAPSLPQEQEES  500
501   LADSLSAFSEFGLQLLRQLRDYFPATNSTAVHRLELLLKCLGKLQLFQPS  550
551   FEICPFESELNMDIAAALKRGNREWYDRILNAKSPREQPGPQRLPGLVVL  600
601   ADAVYDDLQFCYSVYASLFHSILNVDVFTLTFRQLERLVAEEAWVLTEEL  650
651   SPKMTLEVASGLFELYLTLADLQRFWDSIPGRDSRSLALAGIHAPFLPAV  700
701   KLWFQVLRDQAKWRLQGAVDMDTLEPVDASSRHSSSAATAGLCLSHIQEL  750
751   WVRLAWPDPAQAQGLGTQLGQDVCEATLFYTELLRKKVDTQPGAAGEAVS  800
801   EALCVVLNNVELVRKAAGQALKGLAWPEGATGPEGVLPRPLLSCTQALDD  850
851   DLQREAHTVTAHLTSKMVGDIRKYVQHISLSPDSIQNDEAVAPLMKYLDE  900
901   KLALLNASLVKGNLSRVLEALWELLLQAILQALGANRDVSADFYSRFHFT  950
951   LEALVSFFHAEGQGLPLESLRDGSYKRLKEELRLHKCSTRECIEQFYLDK 1000
1001  LKQRTLEQNRFGRLSVRCHYEAAEQRLAVEVLHAADLLPLDANGLSDPFV 1050
1051  IVELGPPHLFPLVRSQRTQVKTRTLHPVYDELFYFSVPAEACRRRAACVL 1100
1101  FTVMDHDWLSTNDFAGEAALGLGGVTGVARPQVGGGARAGQPVTLHLCRP 1150
1151  RAQVRSALRRLEGRTSKEAQEFVKKLKELEKCMEADP               1200
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b

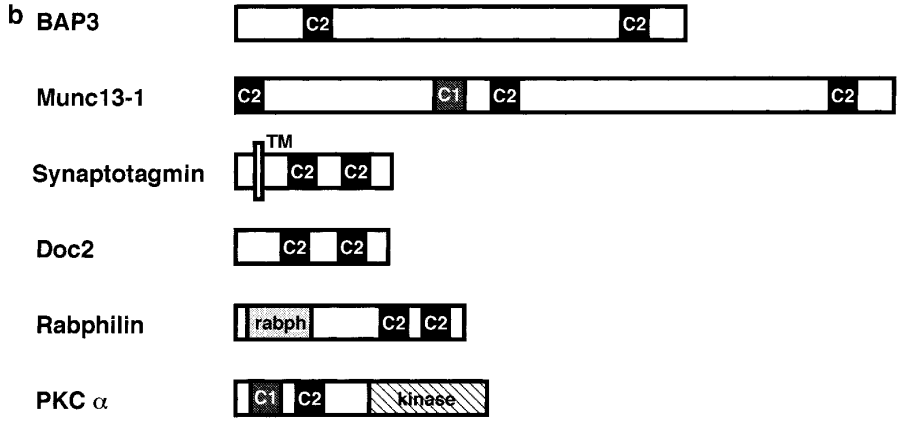

FIG. 1. (a) Amino-acid sequence of human BAP3, with two C2 domains underlined. The nucleotide sequence data that provided the deduced amino acid sequences will appear in DDBJ, EMBL and GenBank nucleotide sequence databases. (b) Domain organization of representative C2 domain-containing molecules, shown schematically. TM, transmembrane region; Rabph, rabphilin homology domain; kinase, protein kinase domain. (c) Alignment of amino acid sequences of C2 domains from representative members of the protein families depicted in (b), as determined by the clustalW program. Black background indicates amino-acid identity. Sequences are identified on the left; proteins with multiple C2 domains are labeled a, b, and c. (d) Dendrogram of the C2 domains aligned in (c), calculated by the DNAsis program. Note that the different C2 domains can be divided in subclasses on the basis of sequence alignment and the dendrogram.

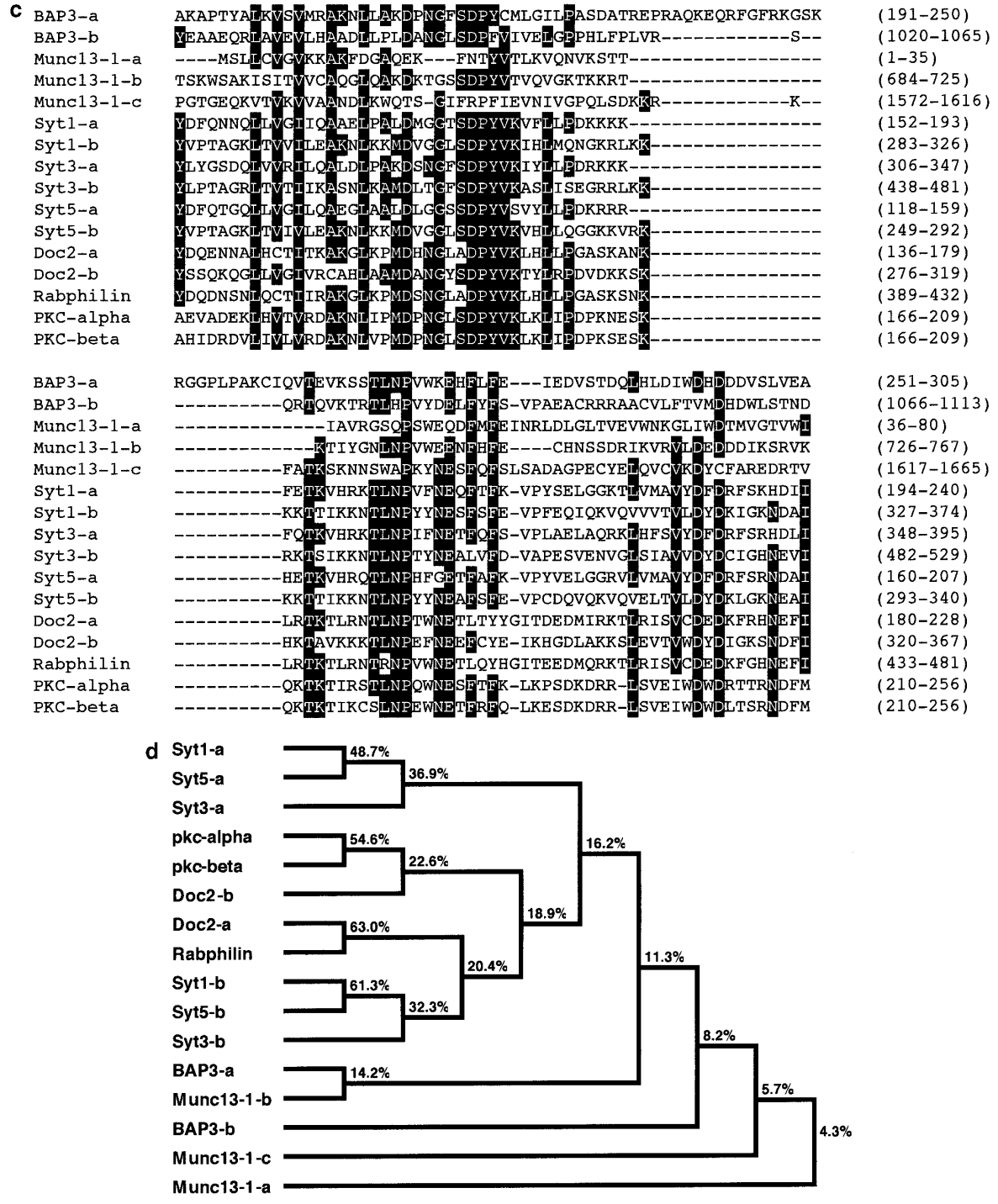

FIG. 1—*Continued*

constructed dendrogram (Fig. 1d) indicated that the C2 domains of BAP3 were evolutionally related to Munc13. Moreover, as the sequence between the two C2 domains of BAP3 also showed homology to Munc13 (20.2% identity in 726 a.a.) (data not shown), BAP3 and Munc13 are likely to be functionally related proteins, probably evolutionary descendants of a common ancestral molecule.

Tissue distribution of BAP3 mRNA. Northern-blot analysis was carried out using nucleotides 172-1156 of BAP3 cDNA as a probe, to investigate BAP3 expression in various human tissues (Fig. 2a). Like BAI1, BAP3 transcript was expressed predominantly in brain. In some tissues, notably testis, a larger band was observed in addition to the 4.8-kb transcript seen in brain. This observation might reflect alternative splic-

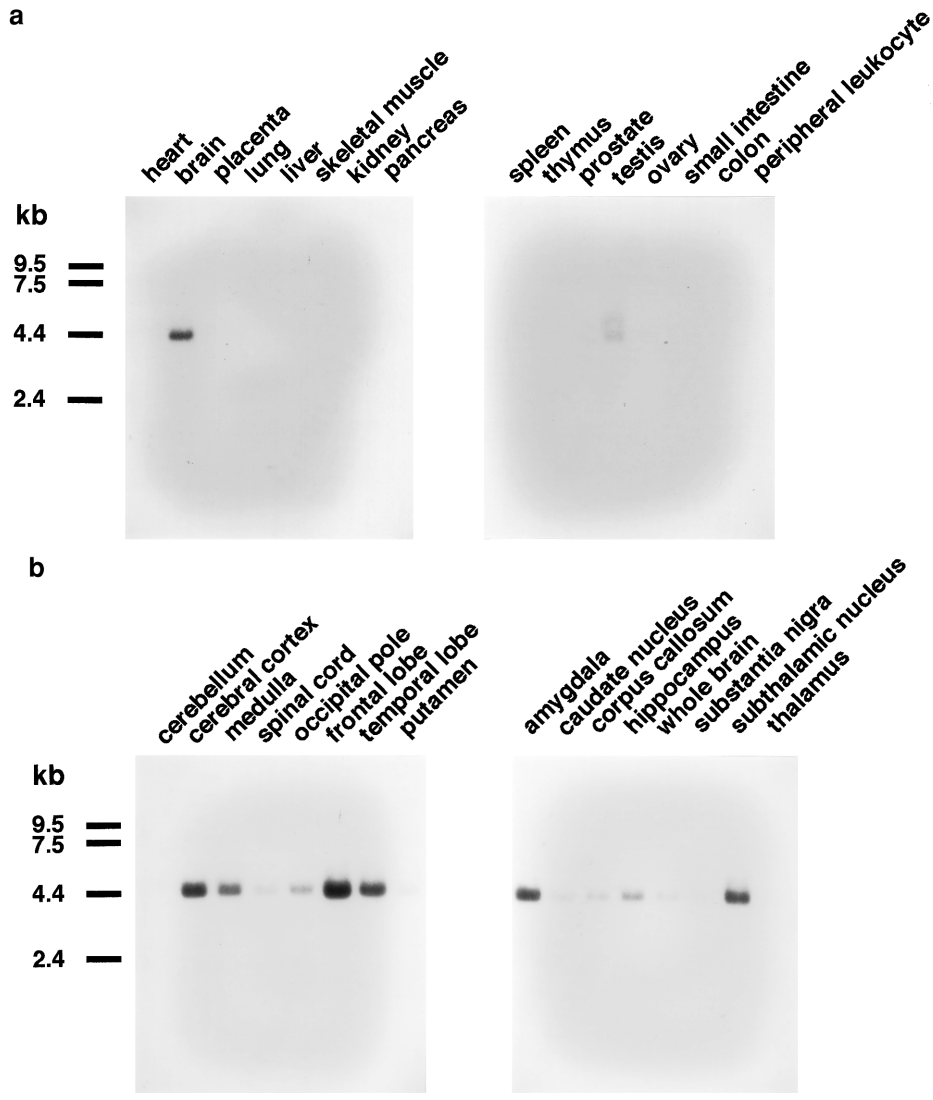

FIG. 2. (a) Distribution of BAP3 mRNA in adult human tissues. Multiple-tissue Northern blots containing 2 μg poly(A)+ RNA per lane were hybridized with a BAP3 cDNA probe. (b) Northern blots containing 2 μg poly(A)+ RNA per lane from 16 different adult human brain section were hybridized with the same BAP3 probe used in (a).

ing, because the anomalous band was also detected when another part of the cDNA was used as a probe (data not shown). In sub-regions of human brain (Fig. 2b), BAP3 expression was strongest in cerebral cortex, frontal lobe, temporal lobe, amygdala and subthalamic nucleus. However, it was undetectable in cerebellum.

Interaction of BAP3 with BAI1 in vivo. We constructed an expression vector containing myc-tagged full-length BAP3 (Fig. 3a). Extracts from COS-7 cells cotransfected with BAI1 and BAP3 were immunoprecipitated with anti-BAI1 antibody, resolved by SDS–PAGE, and immunoblotted with anti-myc antibody. As shown in Fig. 3b, myc-tagged full length BAP3 protein coprecipitated with BAI1 under these conditions.

To determine which regions of BAP3 were responsible for the interaction with BAI1, we constructed myc-tagged expression vectors of a series of deletion mutants (Fig. 3a). Myc-BAP3 TH contained the region that had been obtained from the two-hybrid screening. The fact that all deletion mutants except myc-BAP3 del 3 were coimmunoprecipitated with BAI1 (Fig. 3c) indicated that amino acids 569-882 were indispensable for interaction of this protein with BAI1.

Chromosomal localization of BAP3. We performed fluorescence *in situ* hybridization using two BAC clones corresponding to the BAP3 gene, and examined 50 typical R-banded (pro) metaphase plates (Fig. 4). All twin-spot signals were localized to the p13.3 band of chromosome 16.

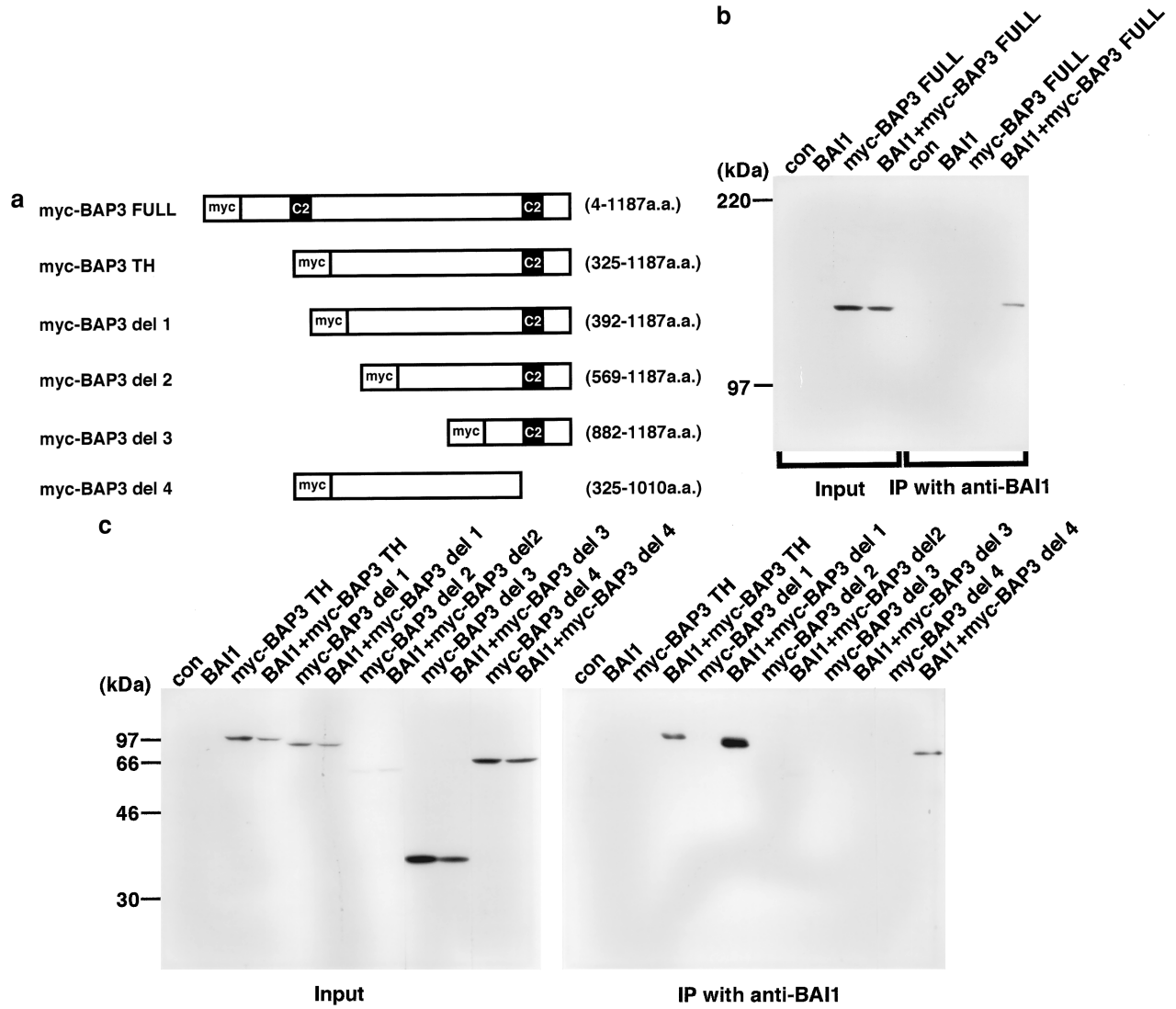

FIG. 3. (a) Design of the myc-tagged BAP3 polypeptides used in this study. Numbers refer to the amino acid residues that define the boundaries of each construct. (b) Association of BAP1 with the BAI1 C-terminus. COS-7 cells were transiently transfected with expression vectors encoding the indicated constructs. The lysates were subjected to immunoprecipitation with anti-BAI1 antibody and immunoblotted with anti-myc antibody. (c) Determination of the region in BAP3 required for interaction with BAI1. The myc-tagged deletion mutants of BAP3 shown in (a) were transfected and their interactions with BAI1 were examined as in (b).

DISCUSSION

In the work reported here, we isolated a novel C2 domain-containing protein, BAP3, which we found to be capable of interacting directly with the cytoplasmic region of BAI1. BAP3 possessed two C2 domains and showed sequence homology to Munc13 and synaptotagmins. According to a study of mutations in the unc-13 gene in Caenorhabditis elegans (Hosono 1991), Munc13 molecules seem to play an essential role in normal presynaptic function by regulating neurotransmitter release (Brose 1995). Recent studies have indicated neuronal function of two other C2 domain-containing proteins, synaptotagmin and Doc2, as Ca^{2+} sensors (Geppert 1994, Orita 1997). Our northern-blot analysis revealed that like Munc13, BAP3 transcript is expressed specifically in brain. Taken together, these data suggest that BAP3 might play an important role in synaptic functions by interacting with BAI1.

Analysis of deletion mutants of BAP3 indicated that BAI1/BAP3 interaction is mediated not by the C2 domains but by the region between them that bears sequence homology to the segment between the second and third C2 domains of Munc13-1. That portion of Munc13 is indispensable for interaction with Doc2 (Orita 1997). Hence, BAP3 and Munc13 might be structurally and functionally related.

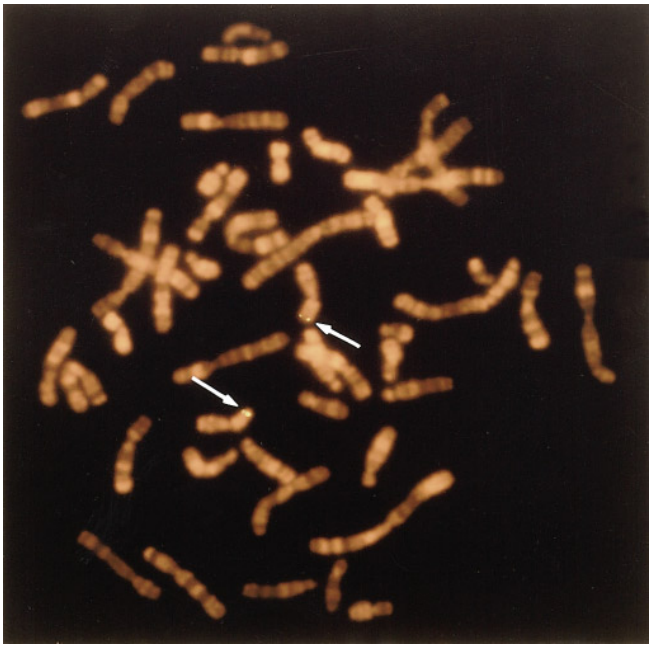

FIG. 4. Representative R-banded metaphase plate after FISH with the BAP3 gene. Arrows indicate fluorescent signals on 16p13.3.

A large number of molecules already have been implicated in synaptic function. Among them synaptotagmin, an integral transmembrane component of synaptic vesicles (Matthew 1981, Perin 1991), also possesses two C2 domains; this protein is thought to be the Ca^{2+} sensor involved in triggering Ca^{2+}-dependent fusion of vesicles to presynaptic membranes (Bennett 1994, Sollner 1993, Jahn 1994). Synaptotagmin promotes formation of filopodia in fibroblasts (Feany 1993). When transfected with BAI1 expression vector, COS-7 cells show many long filopodia-like cytoplasmic extensions reminiscent of synaptotagmin-transfected cells (Shiratsuchi 1998). Synaptotagmin is also known to bind to neurexins and syntaxins (Hata 1993, Bennett 1992); neurexin is specifically expressed in brain and possesses, in its extracellular region, repeats homologous to portions of extracellular matrix proteins such as laminin A and argin (Ushkaryov 1992). Moreover, the carboxy-terminus of neurexin binds to the PDZ domain of CASK, which is a member of the MAGUK (membrane associated guanylate kinase homolog) family (Irie 1997). BAI1, through the QTEV motif in its carboxy-terminus, also interacts with the PDZ domains of BAP1, another member of the MAGUK family (Shiratsuchi 1998). That observation suggests that BAI1 might be functionally related to neurexins.

A recently isolated membrane protein, CIRL (calcium-independent receptor of α-latrotoxin) (Krasnoperov 1997), is similar to BAI1 in some respects. CIRL and BAI1 are members of the secretin-receptor family, and show sequence homology in their seven-span transmembrane regions (24.7% identity in 676 a.a.). Both proteins possess unusually large extracellular domains containing extracellular matrix-like repeats, and large cytoplasmic regions with proline-rich sequences. CIRL is expressed in brain and is thought to be an important regulator of neurosecretion (Krasnoperov 1997). Syntaxin, which associates with synaptotagmin, forms stable complexes with CIRL *in vitro* (Krasnoperov 1997). Clearly, the mechanisms involved in regulation of neuronal functions are complex and remain to be fully understood, but BAI1/BAP3 interaction might well play a physiologically important role in these processes.

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