Novel splice variants of amphiphysin I are expressed in retina[1]

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Abstract Using RT-PCR-based cDNA cloning, we identified novel splice variants of amphiphysin I, termed amph Ir, that are expressed specifically in retina. In comparison with the prototype amphiphysin I, amph Ir contained two novel insertions (inserts A and B) and one deletion. Insert A is only 9 bp in length but appears to be a determinant for the retina-specific expression. In contrast, insert B is a large domain of 1740 bp and two shorter transcripts with 3′-truncated insert B were also expressed. All the insert sequences were present as unidentified exons in the amphiphysin I gene on human chromosome 7. Western blot analysis of various rat tissues with anti-insert B antibody confirmed the presence and tissue specificity of the variant proteins. © **2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Amphiphysin; Splice variant; Retina

1. Introduction

Amphiphysin I (amph I) is a nerve terminal-enriched protein which participates in synaptic vesicle endocytosis by binding to dynamin GTPase via its Src homology 3 (SH3) domain and targeting dynamin to the neck of clathrin-coated pits. Amph I also interacts with a variety of proteins other than dynamin and may thus serve as an adapter molecule regulating the endocytic process [1,2]. Many proteins implicated in synaptic vesicle trafficking appear to be present in multiple isoforms or splice variants [3]. Multiple splice variants of amphiphysin II (amph II), an isoform of amph I, have been reported [4–6]. These variants may be important to differentiate their molecular partners [7]. Some of the variants show a tissue-specific expression, implying that they have distinct roles in the vesicular recycling process unique to each cell type.

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[1] Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers: CT2, AF498092; CT3, AF498093; CT4, AF498094; CT5, AF498095; NC1, AF498096; NT2, AF498097.

Abbreviations: amph I, amphiphysin I; RT-PCR, reverse transcription polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase

Amph I is highly expressed in nervous tissues including retina and some other tissues such as testis, lung, and adrenal gland [8–11]. A variant of amph I lacking 42 amino acid residues was found recently in a breast cancer tissue [9]. This is the only splice variant of amph I described so far. In the present study, we identify novel amph I variants that are transcribed specifically in retina from the authentic amph I gene on human chromosome 7.

2. Materials and methods

2.1. Tissue samples

Human retinal tissue was collected from patients under surgery and frozen immediately. Experiments were undertaken with the understanding and written consent of each subject in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). Fresh rat tissues were obtained from killed animals (adult Wistar rats) and quick-frozen in liquid nitrogen. All experiments with animals, including rabbits for immunization, were carried out in accordance with the NIH Guidelines regarding the care and use of animals for experimental procedures.

2.2. Reverse transcription polymerase chain reaction (RT-PCR) and cDNA cloning

The PolyATtract® System 1000 and Access RT-PCR System (both from Promega) were used for mRNA purification and RT-PCR, respectively. From 40 mg of tissue samples, mRNA was isolated according to the manufacturer's protocol. The reaction mixture for RT-PCR (50 μl) contained the reaction buffer, 5 U each of AMV reverse transcriptase/Tfl DNA polymerase, 0.2 mM dNTPs, 1 μM primers and 10 ng of mRNA isolated from various tissues. Resulting PCR products were separated by agarose gel electrophoresis, purified using DEAE-cellulose paper, blunt-ended and ligated into the *Eco*RV site of a zero-background cloning vector pZErO 2.1 (Invitrogen). After transforming *Escherichia coli* TOP10F′ competent cells, recombinant plasmids were purified and sequenced on both strands with appropriate primers and a premixed cycle sequencing reagent using ABI Prism™ 373 sequencer (Perkin-Elmer).

The following oligonucleotides were used as primers for RT-PCR amplifications. F1 (5′-ATGGCCGACATCAAGACGGG-3′), F2 (5′-ATGTCTCAGACATTGCCCTGG-3′), F3 (5′-CAGGTGGAAATGAACAACTG-3′), F4 (5′-GAGCGCCCAGAATGAGGT-3′), rF4 (5′-AGCTCCCAGAGCGAGGT-3′), F5 (5′-AACTGGGTGACCAGCACG-3′), rF6 (5′-GAGGAAACTCCTAACAGTGG-3′), R1 (5′-CCAGGGCAATGTCTGAGACAT-3′), R2 (5′-CTAATCTAAGCGTCGGGTGAAG-3′), rR2 (5′-CTATTCTAGGTGTCGTGTGAAG-3′), R3 (5′-CTCTATGACCACCGAAGGA-3′), R4 (5′-GGTGATGGTGTCTTTGCAAT-3′), rR5 (5′-ATGACCTTCCTTGAGCAGG-3′). Approximate locations of these primers are shown in Figs. 1 and 3B. Primers based on rat sequences are designated by 'r'.

2.3. Northern blot analysis

Total RNA extracted from various rat tissues using the acid gua-

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nidinium–phenol–chloroform method was electrophoresed in 1% agarose/2.2 M formaldehyde gels and transferred with 0.05 M NaOH onto Hybond-N^+ membrane (Amersham). The rat insert B1 region was amplified by RT-PCR using rat retinal RNA and primer sets rF6/rR5. The resulting product of 384 bp was purified, ^{32}P-labeled, and then used as a hybridization probe.

2.4. Western blot analysis

Frozen tissues were dissolved in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer, boiled for 2 min, and subjected to 6% SDS–PAGE. Western blotting was performed according to a standard procedure. Briefly, separated proteins in the gel were transferred electrophoretically to nitrocellulose membranes. Blots were incubated successively with primary antibodies, peroxidase-labeled anti-immunoglobulin secondary antibodies, and then the peroxidase activity was visualized with 0.05% 4-methoxy-1-naphthol and 0.015% hydrogen peroxide.

2.5. Preparation of antibody against insert B1

The human insert B1 (174 amino acids) was expressed in bacteria as a glutathione *S*-transferase (GST) fusion protein. To prepare the expression construct, amph Ir cDNA (CT2) was amplified by PCR with primers 5′-CCGGATCCCGACTGACTCTTCAGAAAGTC-3′ and 5′-CCGAATTCCTTTTGACACGCCTCCACAC-3′ that were designed to amplify the entire region of insert B1 with terminal restriction sites for in-frame insertion into the pGEX-3X vector (*Bam*HI and *Eco*RI sites are underlined). The recombinant plasmid was used to transform *E. coli* strain JM109 and verified by DNA sequencing. The GST insert B1 fusion protein was produced in the same host and purified on glutathione-Sepharose 4B according to the manufacturer's instructions (Pharmacia).

The purified protein was emulsified with Freund's complete adjuvant and injected subcutaneously into two rabbits. Antibody titers were monitored by immunoblotting on serum samples collected after several booster injections. The pooled antiserum with high titer was affinity-purified by successive adsorptions to nitrocellulose membrane strips blotted with GST or GST insert B1 fusion protein (antigen). The GST membrane removed antibodies reactive to GST and insert B1-specific antibodies were eluted from the second membrane.

3. Results

3.1. Presence of multiple mRNA variants of amph I in retina

Since multiple bands migrating more slowly than amph I had been detected on Western blots of retina tissue probed with anti-amph I antibodies (shown in Fig. 5A), we suspected that novel amph I variants with additional domains are expressed in retina. To see if this is the case, RNA isolated from human retina was subjected to RT-PCR amplifications with two primer sets that target the 5′-half (F1/R1) or 3′-half (F2/R2) of the canonical amph I mRNA (GenBank accession number U07616, see Fig. 1A for primer positions). The PCR products were cloned into a vector and sequenced. Two out of eight 5′-half clones showed a sequence identical to the authentic amph I cDNA (designated NT1 in Fig. 1B). The other clones, however, contained a nine nucleotide insertion after the second letter of the triplet encoding Pro249 (NT2). The insertion introduces an addition of three amino acids but no frame shifting downstream (Fig. 3B). Similarly, the 3′-half clones consisted of a canonical amph I (CT1) and a novel variant clone CT2 that contained a 174 amino acid insert (insert B1) after Pro394 and a 42 amino acid deletion between Leu424 and Ile467 (Fig. 1B). The deleted segment was identical to the one deleted in the 'non-neuronal form' of amph I we reported previously [9].

To further analyze mRNA variants harboring the insert B1, a forward primer F3 was designed within the insert and RT-PCR was performed using the primer F3 and a reverse primer R3. In the resulting cDNA clones, additional inserts were

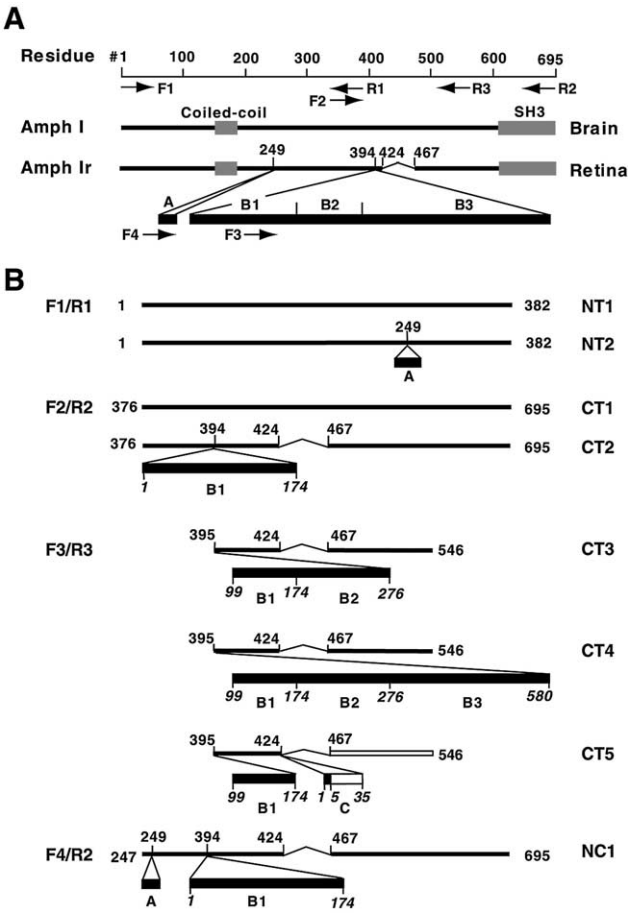

Fig. 1. Amph I variants expressed in human retina. Numbers represent the amino acid residue number for canonical amph I (GenBank accession U07616). Thick labeled bars designate the newly found inserts. Residue numbers for the insert are italicized. A: Schematic representation of a retina-specific amph I variant (amph Ir) as compared to amph I. The coiled-coil and SH3 domains are shown by shaded boxes. Approximate locations of PCR primers are indicated by arrows. B: Summary of amph I variants identified in human retina by RT-PCR-based cloning. Primer sets used for amplification are shown on the left and clone names on the right. The blank bar in CT5 denotes the untranslated region created by an in-frame stop codon within insert C.

identified: insert B2 in CT3, inserts B2 and B3 in CT4, and insert C in CT5 (Fig. 1B). Clones CT3 and CT4 had uninterrupted open reading frames, whereas CT5 would encode a truncated protein due to a termination signal TGA within insert C as the sixth codon following Leu424. All the clones had the 42 amino acid deletion after Leu424.

Next, we assessed whether mRNA species containing both inserts, A and B, are expressed in retina by RT-PCR with the primer pair F4/R2, where the 3′ nine nucleotide sequence of F4 was identical to that of insert A. All the cDNA clones obtained from this amplification contained the inserts A and B1, and lacked the 42 amino acid region following Leu424 (NC1), suggesting that mRNA of this sequence organization is the major amph I variant in retina. The results are consistent with the view that in addition to the canonical amph I the retina expresses a splice variant, termed amph Ir, with novel inserts at two sites (after Pro249 and Pro 394) and a 42 amino acid deletion after Leu424 (Fig. 1A). Amph Ir mRNA appears to consist of three molecular species differing from each other

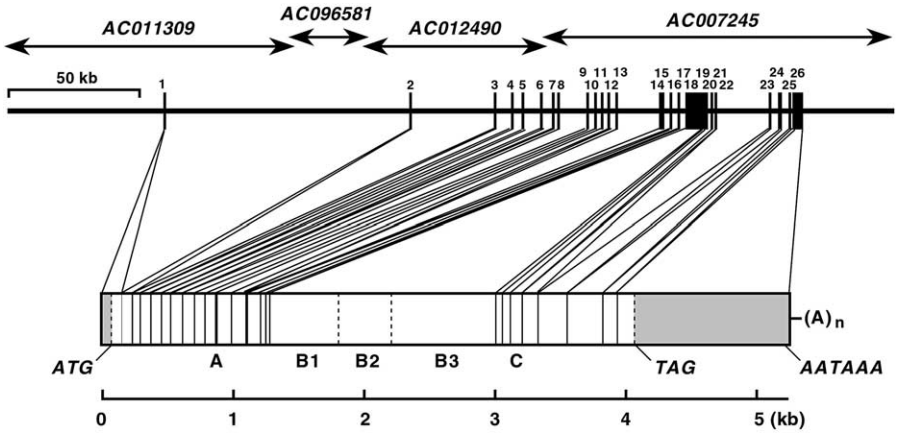

Fig. 2. Exon–intron organization of the human amph I gene deduced from the transcript analysis. The contiguous BAC clones are shown at the top and predicted exons are shown underneath. The coding sequence for amph I appears on the minus strand of the BAC sequence. A hypothetical mRNA composed of all the predicted exons is also shown at the bottom. Exons 11, 18, and 21 encode the amph Ir inserts A, B, and C, respectively. Positions for translation initiation, termination, and polyadenylation signals are indicated. The shaded area in the first and last exons stands for untranslated regions.

in the length of insert B. Although transcripts with B1+B2 (as in CT3) and B1+B2+B3 (CT4) were detectable, these forms are less abundant than the one with the B1 region only (CT2 and NC1).

3.2. Genomic organization of the amph I gene

Further evidence for the presence of amph Ir variants was obtained from the analysis of genomic sequences. The human amph I gene (AMPH) is located on chromosome 7p13–p14 [12]. BLAST search with the human amph I cDNA sequence in the GenBank database extracted four overlapping BAC clones assigned to chromosome 7 (AC011309, AC096581, AC012490, and AC007245) that covered the entire region of the amph I gene spanning about 250 kb (Fig. 2). Additional searches for potential exons in this region using the FGENESH algorithm revealed 26 putative exons. The splice donor and acceptor sequences at the exon–intron boundaries were all classical, including GT and AG dinucleotides on intron ends (not shown). Out of 26 putative exons, 23 were present in the amph I variant clones listed in Fig. 1. These cDNA sequences matched exactly the genomic sequence except for a few nucleotide positions, and all the exon boundaries were precisely spliced. The deleted segment in amph Ir (between amino acids 424 and 467) corresponds to exon 22. Exons 6 and 14 may be a false prediction because they were not present in either amph I or amph Ir. Exons 11 and 21 encoded insert A and insert C, respectively. Insert B (B1+B2+B3) was encoded by a single large exon (exon 18). Junctional sequences at B1/B2 (AG/GTAGA) and B2/B3 (AG/GTAAT) were very similar to the splice donor consensus sequence AG/GTAAG. Therefore, the shorter forms of amph Ir message are most likely to be generated by splicing events occurring at these alternative donor sites. The last exon (exon 26) contained the termination codon TAG at nucleotides 109–111.

3.3. Tissue specificity of amph Ir mRNA expression in rat

Detection of insert A in transcripts was improved by designing a PCR amplification that targets a short region spanning the insertion site. Amplification of human retina RNA with the primer pair F5/R4 will bring about two RT-PCR products of 91 bp and 100 bp, the latter being derived from amph Ir. The result showed, however, that in addition to the predicted products (b1 and b2) a slowly migrating fragment (b3) was produced (Fig. 3A, lane 1). Strangely, cloning of the total PCR product yielded plasmids with inserts of either b1 or b2, but not b3. When isolated b1 and b2 fragments were simply mixed, denatured and annealed before electrophoresis, fragment b3 was generated (lane 5), indicating that b3 is the

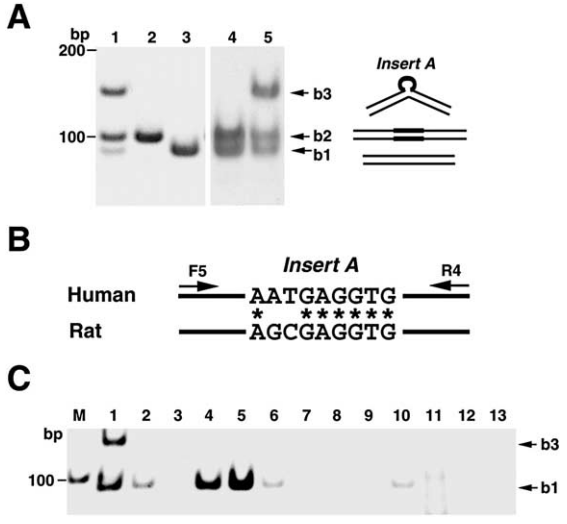

Fig. 3. RT-PCR analysis of insert A-containing mRNA in human and rat tissues. A: DNA fragments amplified with a primer pair F5/R4 flanking the insertion site on human retinal RNA were separated by polyacrylamide gel electrophoresis (lane 1). Amplification after cloning of the products (lanes 2 and 3). Lane 4, mixture of b1 and b2; lane 5, the mixture after denaturation–renaturation. Shown on the right are probable duplex structures of the fragments. B: Alignment of insert A sequences from human and rat retina. The same primer set (F5/R4) successfully amplified the corresponding region (b2) in both species, although the F5 primer had three mismatches against the rat sequence. C: RT-PCR products from various rat tissues. Fragment b2 of 100 bp is hardly visible here because of the lower abundance of amph Ir in rat retina. Band b3 serves as a diagnostic marker for co-expression of amph I and amph Ir in a particular tissue. M, 100 bp size marker; lanes 1, retina; 2, testis; 3, adrenal gland; 4, cerebral cortex; 5, cerebellum; 6, pons; 7, medulla oblongata; 8, skeletal muscle; 9, heart; 10, lung; 11, liver; 12, spleen; 13, kidney.

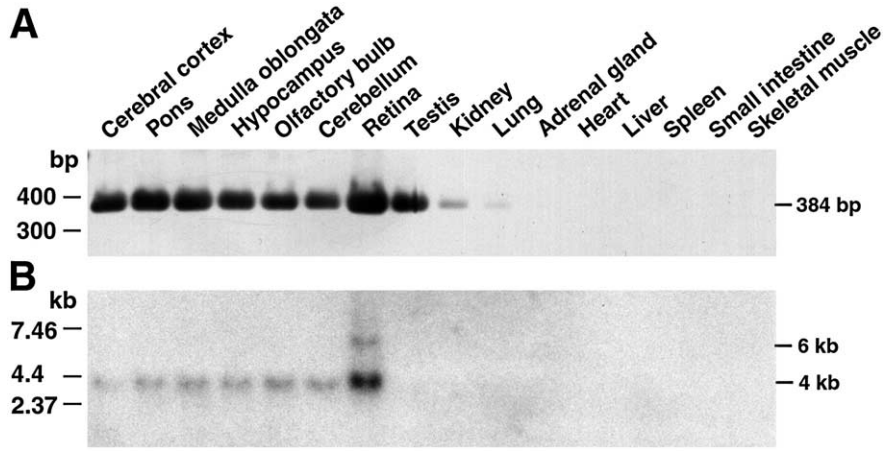

Fig. 4. Analysis of insert B-containing mRNA in rat tissues. A: Total RNA samples from rat tissues indicated at the top were subjected to RT-PCR using rat insert B1-specific primers (rF6/rR5). B: The same RNA samples were subjected to Northern blot analysis using the ^{32}P-labeled probe prepared from the rF6/rR5 amplification product. Marker positions are labeled on the left.

heteroduplex formed between b1 and b2 fragments. The aberrant mobility of b3 in polyacrylamide gels is consistent with a bent structure with a single-stranded loop. The same result was obtained when rat retina RNA was amplified with primers F5/R4 (not shown). Sequences for b1 and b2 obtained from both species were determined and the insert A sequence is aligned in Fig. 3B. Thus, it is now clear that the amph I variant containing 9 bp insert A, although with some sequence variations, is also expressed in rat retina together with the prototype amph I. A series of rat tissues were then examined by RT-PCR to see whether amph Ir with insert A is expressed in other tissues (Fig. 3C). The shorter band representing amph I (b1) was detectable in nervous tissues including retina, as well as testis and lung. The heteroduplex band b3, however, was evident only in retina, suggesting retina-specific expression of insert A.

The rat counterpart of amph Ir containing both inserts A and B was next cloned from RT-PCR products amplified with a primer pair rF4/rR2 using rat retina mRNA as a template. Sequencing of the resulting clones revealed that a novel insert of 471 bp was placed at the expected site after nucleotide 1182 of rat amph I cDNA (Y13381). The insert shares 76% homology with the human insert B1, suggesting that this variant is the rat counterpart of human NC1. Since no sequences homologous to the human inserts B2 and B3 were found in these clones, the variant with inserts A and B1 appears to be the most abundant form also in rat retina. Tissue specificity of the insert B1 expression was then investigated by RT-PCR with RNA from various rat tissues using a primer pair rF6/rR5 which amplifies a 384 bp segment within the insert (Fig. 4A). In contrast to insert A, insert B1 was expressed not only in retina but also in other central nervous tissues, testis, kidney and lung. To determine the size of transcripts containing insert B1, Northern blot analysis was performed (Fig. 4B). In retina, a prominent band and a minor band were detected at approximately 4 kb and 6 kb, respectively. The 6 kb transcript was not present in any other tissues, whereas the 4 kb transcript appeared to be expressed also in central nervous tissues at a lower level. These results are consistent with the idea that the 4 kb and 6 kb transcripts encode the retina-specific amph Ir with inserts B1 and B1-B2-B3, respectively. The weaker signal at 4 kb in other nervous tissues can be accounted for by the variant with insert C (like CT5 in Fig. 1), which is indistinguishable in size from the B1-containing amph Ir. The notion was corroborated by the Western blot analysis shown below.

3.4. *Analysis of retina-specific amphiphysin I variants by Western blotting*

To determine the tissue distribution of amph I-related proteins, rat tissue samples were subjected to Western blotting using antibodies against human amph I or insert B1 protein expressed in bacteria (Fig. 5). The anti-amph I antibody detected the canonical amph I migrating at 128 kDa in the brain and retina, as well as testis and lung. The antibody also recognized several bands with less mobility around 160–210 kDa, designated 'large forms', that were present only in retina. Similar bands were discernible also in human retina. Since the epitope of this antibody resides in the C-terminal region [9], the large forms appear to share the C-terminal domain of amph I. As expected, immunoblotting with anti-insert B1 antibody failed to detect the canonical amph I in all the tissues, but the large forms in retina were recognized by the antibody. These results indicated that the large forms most likely represent the retina-specific amph I variants containing insert B1 and B1 plus B2/B3. Apparent molecular size estimated from the blot showed that the ~160 kDa and ~210 kDa bands roughly correspond to amph Ir with insert B1 and B1-B2-B3, respectively. Another interesting observation is that insert B1 antibody, but not amph I antibody, recognizes a protein migrating at around 110 kDa in retina and in other tissues where amph I is expressed (asterisk in Fig. 5). When the data for cDNA cloning and transcript analysis are taken together, it is strongly suggested that the 110 kDa protein is encoded by the 4 kb transcript shown in Fig. 4. In summary, we conclude that amph I variants with novel insertions are expressed specifically in retina, and that a truncated protein lacking the SH3 domain is co-expressed with amph I in various tissues.

4. Discussion

All the insertion sequences newly found in retinal cDNAs were represented as hitherto unidentified exons in the genomic sequence of the human amph I gene. Analysis of exon bound-

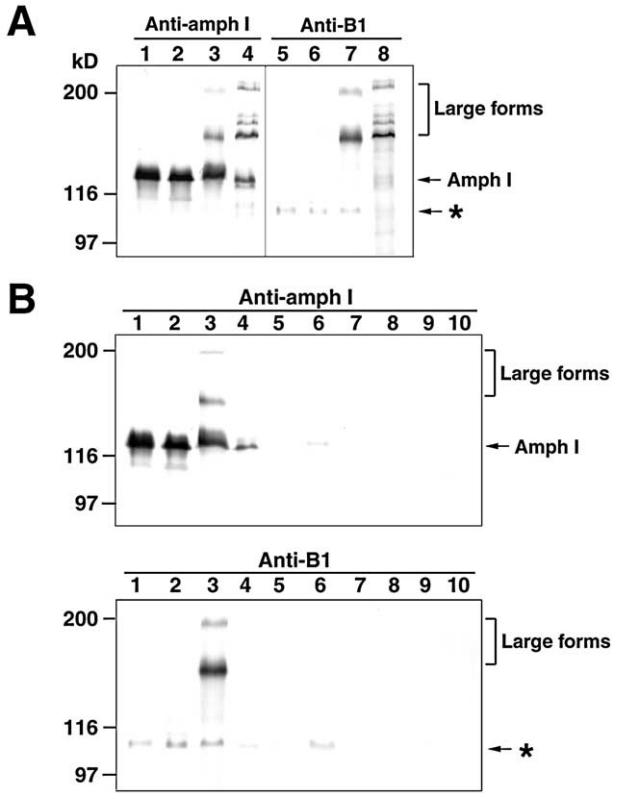

Fig. 5. Distribution of amph I-related proteins in rat tissues as assessed by immunoblotting. Antibodies used were IgG purified from a patient with paraneoplastic neuropathy (anti-amph I) and the affinity-purified IgG from a rabbit immunized with recombinant insert B1 protein (anti-B1). A: Presence of amph Ir (large forms) in both rat and human retina. Lanes 1 and 5, rat cerebral cortex; lanes 2 and 6, rat cerebellum; lanes 3 and 7, rat retina; lanes 4 and 8, human retina. B: Rat tissue proteins applied to the lanes were 1, cerebral cortex; 2, cerebellum; 3, retina; 4, testis; 5, kidney; 6, lung; 7, heart; 8, liver; 9, spleen; 10, skeletal muscle. Migration of marker proteins is shown on the left.

aries revealed that in 21 out of 25 junctional sites the point of intron insertion occurs at the codon boundary. The frequency (21/25 = 84%) is significantly higher than the statistical expectation for mammalian genes (44.3%) [13]. We made a similar observation previously in amph II transcripts [5]. There is a general preference for intron insertion at a codon boundary, as opposed to the middle of a codon, that has been ascribed to a consequence of gene evolution through the exon shuffling mechanism [14].

In correlation to the domain structure of amph I protein [1], the three insertion sites for inserts A, B, and C are situated right next to the proline-rich, clathrin binding, and hydrophobic domains, respectively. The exon–intron organization of the amph II gene is similar to that of the amph I gene although the gene size of amph II is much smaller [6]. Analysis of homologous exons between the two genes indicated that exon 11 of the amph I gene, the coding exon for insert A, corresponds to exon 10 of amph II. Interestingly, as insert A is specific to amph I variants expressed in retina, the corresponding exon encodes an NLS-like segment specific to the amph II variant expressed in skeletal muscle (alternatively called BIN1). Therefore, this small exon may be a signature exon for tissue-specific expression of amphiphysin variants. The internal coding exon 18, which encodes insert B, is unusually large and extremely rare in the mammalian genome [15]. Like the other domains in the amph I protein, this domain may well provide a site for interaction with unidentified protein partner(s). However, attempts to predict interacting proteins were unsuccessful because the deduced amino acid sequence of insert B showed little homology to known proteins.

The presence of a transcript containing insert C, which is encoded by exon 21, was an unexpected finding since it directs a truncated translation product due to an in-frame termination signal in the insert. A protein of predicted size (~110 kDa) was indeed detected by anti-insert B1 antibody (Fig. 5). The similar tissue distribution pattern of the 110 kDa protein and amph I may suggest that they form a heterodimer in these tissues. The cellular function of this protein should be different from that of amph I as it lacks the C-terminal region including the SH3 domain which is essential for the recruitment of dynamin to the site of endocytosis. To our knowledge, this is the first example of SH3-less amphiphysin-related protein in higher eukaryotes. In this context, the protein may be a functional homologue of the yeast amph I-like protein Rvs161 lacking the SH3 domain that was suggested to be involved in the control of the actin cytoskeleton [16].

The mammalian retina has a characteristic multi-layered structure composed of known cellular constituents. Localization of amph Ir protein in retina would provide a significant clue to its physiological function. To this end, we have immunostained rat retina with the anti-insert B1 antibody. As expected, the antibody stained the two synaptic layers termed outer and inner plexiform layer (data not shown). Amph I shows a similar distribution as stained with conventional antibodies [10,17]. In addition to conventional synapses, retina contains a morphologically distinctive synapse called ribbon synapses. The ribbon synapse is considered to be specialized for the continuous exocytotic release of neurotransmitters in certain sensory neurons including photoreceptors and bipolar cells of retina [18]. It is reasonable then to assume that ribbon synapses also possess a unique vesicle-recycling machinery and amph Ir may be involved as its essential component. Attempts to determine the precise location of amph Ir in retinal synapses are in progress.

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