Tissue-specific Alternative Splicing Generates Two Synaptojanin Isoforms with Differential Membrane Binding Properties*

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Synaptojanin is an Src homology 3 domain-binding inositol 5-phosphatase that is thought to function in synaptic vesicle endocytosis. It is encoded by a cDNA with two open reading frames separated by an in-frame stop codon. The first open reading frame encodes a 145-kDa form of the protein, whereas a 170-kDa isoform appears to be composed of both open reading frames and contains additional Src homology 3 domain-binding consensus sequences. Here, we demonstrate that the two synaptojanin isoforms are generated by the alternative use of an exon containing the stop codon. Whereas the 145-kDa isoform is highly enriched in adult brain, the 170-kDa isoform is excluded from this tissue and has a widespread distribution in non-neuronal cells. Unlike the 145-kDa isoform, which can be removed from membranes by a low salt wash, the 170-kDa isoform remains membrane-associated, even in the presence of 1 M salt. Further, the 170-kDa form, but not the 145-kDa form, can be isolated from membranes as part of a large molecular weight complex. These properties may allow the 170-kDa isoform of synaptojanin to play a unique and perhaps more general role in endocytosis as compared with the 145-kDa isoform.

Synaptic vesicles are highly specialized organelles that neurons use to secrete non-peptide neurotransmitters at the synapse. After exocytosis, synaptic vesicle membranes are internalized and reused for neurotransmitter release by a process that likely involves clathrin-coated pits and vesicles (1). We have identified a 145-kDa protein, termed synaptojanin, which appears to function in synaptic vesicle endocytosis. Synaptojanin was identified, along with dynamin, as a major Src homology 3-domain-binding consensus sequence. Here, we demonstrate that the two synaptojanin isoforms are generated by the alternative use of an exon containing the stop codon. Whereas the 145-kDa isoform is highly enriched in adult brain, the 170-kDa isoform is excluded from this tissue and has a widespread distribution in non-neuronal cells. Unlike the 145-kDa isoform, which can be removed from membranes by a low salt wash, the 170-kDa isoform remains membrane-associated, even in the presence of 1 M salt. Further, the 170-kDa form, but not the 145-kDa form, can be isolated from membranes as part of a large molecular weight complex. These properties may allow the 170-kDa isoform of synaptojanin to play a unique and perhaps more general role in endocytosis as compared with the 145-kDa isoform.

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properties compared with the 145-kDa isoform. These differences may allow the two synaptojanin isoforms to play distinct functional roles in endocytosis.

EXPERIMENTAL PROCEDURES

SH3 Domain Purification of Synaptojanin Isoforms—Various tissues were homogenized at 1.5 (w/v) in buffer A (10 mM HEPES-OH, pH 7.4, 1 mM EDTA, 0.32 M sucrose, 0.83 mM benzamidine, 0.25 mM phenylmethylsulfonyl fluoride) using a polytron or a glass Teflon homogenizer. Triton X-100 was added to 1% final, and the extracts were incubated for 1 h at 4°C followed by maximal microcentrifugation for 15 min. Supernatants containing 2.7 mg of protein were diluted to 1 ml in buffer A with 1% Triton X-100 and were incubated overnight at 4°C with ~ 25 μg of GST/Grb2 pre-bound to glutathione-Sepharose. After incubation, the samples were washed 3 times in 1 ml of buffer A with 1% Triton X-100 by centrifugation, and bound proteins were eluted with SDS-PAGE sample buffer, transferred to nitrocellulose, and blotted with antibodies raised against the 145-kDa isoform of synaptojanin purified from adult rat brain (8) or anti-peptide antibodies specific to the 170-kDa synaptojanin isoform (9).

PCR Analysis—Poly(A) messenger RNA was prepared from 1 g each of adult rat brain, E18 rat brain, rat testis, and undifferentiated PC-12 cells using the Invitrogen FastTrack Kit. Aliquots of mRNA (3.0 μg) were reverse transcribed at 43°C for 1 h. using 37.5 units of avian myeloblastosis virus reverse transcriptase with random hexamers. The cDNA samples generated, in addition to a rat heart cDNA λ-gt 10 library (generously gift of Dr. Michael Tamkun, Vanderbilt University), which was extracted with phenol:chloroform, were used for PCR reactions with Vent Polymerase. Non-degenerate forward and reverse primers, corresponding to nucleotides 3961–3981 (encoding a BamHI site) and 4154–4173, respectively, of clone-9 (9) were used for PCR reactions with a 56°C annealing temperature. The PCR products, which were detected only in the presence of library and both oligonucleotides, were subcloned into Bluescript after digestion with BamHI and XhoI (the XhoI site was encoded by the oligonucleotide). Restriction digests revealed two size classes of inserts, both of which were sequenced using standard protocols. Rat genomic DNA (generous gift of Dr. Phil Barker, Montreal Neurological Institute), prepared as described (28) was used in PCR reactions with a 60°C annealing temperature with non-degenerate forward and reverse primers, corresponding to nucleotides 4003–4023 and 4154–4173, respectively, of clone-9 (9). The specific PCR product was subcloned into Bluescript after digestion with BamHI and XhoI (sites encoded by the oligonucleotides) and was sequenced at the ends using standard protocols.

Membrane Association—PC-12 cells (gift of Dr. Phil Barker, Montreal Neurological Institute) were maintained in culture at 37 °C in 7.5% CO2 in growth medium (Dulbecco’s modified Eagle’s medium supplemented with 1.25 mM pyruvic acid, 1.0 mM glutamine, 5% calf serum, 5% horse serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin). Undifferentiated PC-12 cells were homogenized at 1:5 (w/v) in buffer A in a glass-Teflon homogenizer. Homogenates were then passed three times through a 25G gauge needle and centrifuged at 2800 rpm in a Sorvall SS-34 rotor. The supernatant (S1) was centrifuged at 45,000 rpm for 30 min. The pellet was resuspended in 500 μl of buffer A, and equal aliquots of pellets and supernatants were separated on SDS-PAGE and processed for Western blot analysis.

Sucrose Density Gradient Centrifugation—Proteins of P2 fractions (approximately 10 mg) from E18 brains or PC-12 cells, prepared as described above, were solubilized by resuspension in buffer B (10 mM HEPES-OH, pH 7.4, 1% Triton X-100, 0.83 mM benzamidine, 0.25 mM phenylmethylsulfonyl fluoride) followed by incubation for 30 min at 4°C. Insoluble material was removed by centrifugation in a Beckman 50Ti rotor at 45,000 rpm for 30 min. Samples (4 μl) were loaded on a 40-ml 5–20% linear sucrose gradient prepared in buffer B and centrifuged in a Beckman VTi 50 rotor for 3.5 h. at 45,000 rpm with slow acceleration and no brake. Gradient fractions (2 ml) were removed from the bottom. S1 fractions (2 ml) from PC-12 cells and E18 brains, prepared as described above, were diluted 1:1 in 10 mM HEPES-OH, pH 7.4, 2% Triton X-100 and were loaded on 5–20% linear sucrose gradients prepared in buffer B. Gradients were centrifuged and fractionated as described above. Equal volumes of all gradient fractions (80 μl) were separated on SDS-PAGE and processed for Western blot analysis except for the detection of the 145-kDa synaptojanin isoform from membrane in which 500 μl of each fraction were incubated with ~ 1 μg of Grb2 conjugated to glutathione-Sepharose, and the bead samples were eluted with SDS-PAGE sample buffer and processed for Western blots.

Antibodies—Affinity purified antibodies against the 145-kDa synaptojanin isoform, the 170-kDa synaptojanin isoform, and dynamin were previously described (8–10).

RESULTS

Synaptojanin Isoform Expression in Developing Brain—In order to examine the developmental expression of the 170-kDa synaptojanin isoform, we enriched for SH3 domain-binding proteins from extracts made from rat E12 heads, as well as E16, E18, and adult brains by Grb2 affinity chromatography and blotted with an affinity purified polyclonal antibody that recognizes both the 145- and the 170-kDa synaptojanin isoforms (arrows). In the adult brain, expression of the 145-kDa synaptojanin isoform increases dramatically as compared with its expression in embryonic brain, whereas the 170-kDa isoform decreases to undetectable levels (Fig. 1).

Tissue Distribution of Synaptojanin Isoforms—Previously, we had demonstrated that the 145-kDa synaptojanin isoform was highly expressed in neurons (8). In an effort to examine the tissue distribution of the 170-kDa isoform, we enriched for SH3 domain-binding proteins from extracts of adult and E18 brain, undifferentiated PC-12 cells, and a variety of non-neuronal tissues by Grb2 affinity chromatography. Western blot analysis detected the 170-kDa synaptojanin isoform in virtually all of the tissues tested (Fig. 2A). Surprisingly, the 145-kDa synaptojanin isoform was also detected in a wide variety of non-neuronal tissues (Fig. 2A). The antibody detected the 145-kDa synaptojanin isoform in crude extracts of neuronal tissues but only detected the 145- and 170-kDa synaptojanin isoforms in non-neuronal tissues after enrichment by SH3 domain-affinity chromatography, which explains our previous inability to detect synaptojanin expression in non-neuronal tissues (8). Interestingly, the ratio of the 145- and 170-kDa isoforms varied depending on the tissue. For example, in adult brain only the 145-kDa synaptojanin isoform was detected (Figs. 1 and 2A), whereas in lung and heart, approximately equal levels of the two proteins were observed (Fig. 2A). The 170-kDa isoform appears to be expressed at higher levels than the 145-kDa isoform in testis and liver (Fig. 2A), whereas neither isoform was detected in skeletal muscle (data not shown). An antipeptide antibody, which recognizes the 170-kDa isoform of synaptojanin selectively recognizes the 170-kDa band expressed in non-neuronal tissues after SH3 domain-enrichment (Fig. 2B). Interestingly, a third protein of approximately 150
kDa, which is recognized by both antibodies, appears to be expressed in lung (Fig. 2, A and B).

**PCR Analysis of cDNA**—In order to explore the mechanism by which the 170-kDa synaptojanin isoform is generated, we performed PCR analysis on cDNA from a variety of tissues using primers that flanked the in-frame stop codon separating the two open reading frames (9). PCR products of the appropriate size (~200 bp), which were dependent on the presence of a DNA source and both primers (data not shown), were subcloned into Bluescript. Upon restriction digest of multiple isolated clones for each tissue, two size classes of inserts were detected (Fig. 3A). Sequencing of the larger inserts (long form) from each tissue in which they were detected revealed 100% homology with the adult rat brain cDNA encoding synaptojanin including the presence of the TAA stop codon at nucleotide position 4019 (9) (Fig. 3B). The smaller inserts (short form) were also 100% identical to the adult rat brain cDNA with the exception of a 27-nucleotide deletion from nucleotide positions 4000 to 4026, which removes 6 amino acids N-terminal to the stop codon and two amino acids C-terminal of the stop codon but which does not change the reading frame of the sequence (Fig. 3B). Thus, it appears that the two synaptojanin isoforms are produced by the alternative use of a 27-nucleotide exon that encodes a stop codon.

**PCR Analysis of Genomic DNA**—Further evidence in favor of an alternative splice mechanism in the production of synaptojanin isoforms was provided by PCR analysis of genomic DNA with primers that flanked the 3′ end of the proposed exon. PCR reactions yielded an approximately 1500-nucleotide DNA fragment that contained exonic sequences encoding synaptojanin. These exonic sequences flanked a sequence that is not found in synaptojanin cDNA nor in the Genbank® data base and that
conform to consensus splice sites (29, 30). The tojanin isolated from adult rat brain (9). The nucleotide number of the full-length cDNA clone encoding synaptojanin are in capital letters. The 5' and 3' ends of the proposed intron are in lowercase letters with the non-sequenced area indicated by periods. The bold letters correspond to the intron/exon boundaries that conform to consensus splice sites (29, 30). The numbers correspond to the nucleotide number of the full-length cDNA clone encoding synaptojanin isolated from adult rat brain (9).

appears to represent intronic sequence (Fig. 4). The exon/intron boundaries occur between nucleotides 4026 and 4027 as predicted from the analysis of cDNA (Fig. 3) and form good matches with consensus splice sites (29) including conforming to the “GT-AG” rule (30) (Fig. 4).

Membrane Association of Synaptojanin Isoforms—Because both the 145- and 170-kDa synaptojanin isoforms are present in soluble and particulate fractions (Ref. 8; data not shown), we decided to examine the membrane association of the two synaptojanin isoforms in PC-12 cells. In good agreement with experiments from adult brain (8), the membrane-associated fraction of the 145-kDa isoform from PC-12 cells is partially removed from the membrane by incubation in 0.2 M NaCl and is completely removed by incubation in 0.5 M NaCl (Fig. 5). In sharp contrast, the 170-kDa isoform completely resists extraction at 0.5 M NaCl and is only partially removed by 1.0 M NaCl (Fig. 5). The majority of the 170-kDa synaptojanin isoform is extracted from the membrane with pH 11.0 washes (data not shown).

Size Fractionation of Synaptojanin Isoforms—To further explore the nature of the differential membrane binding properties of the 145- and 170-kDa synaptojanin isoforms, we performed size fractionation of the proteins from Triton X-100 solubilized membranes and from cytosolic fractions using sucrose density gradients. Both the 145- and 170-kDa synaptojanin isoforms isolated from cytosolic fractions of PC-12 cells and E18 rat brains peaked in gradient fraction 11 corresponding to a native molecular mass of approximately 120 kDa as compared with globular protein standards (Fig. 6). The membrane-associated form of the 145-kDa isoform appeared to peak in fraction 9, indicative of a slightly larger native molecular mass (Fig. 6). Interestingly, the 170-kDa synaptojanin isoform isolated from membranes of both E18 brains and PC-12 cells ran as a broad peak between fractions 1 and 10 with a predicted molecular mass of between 140 and 300 kDa (Fig. 6). The increased migration of the 170-kDa synaptojanin isoform on the gradient is not due to an artifact mediated by the presence of Triton X-100 because the cytosolic fractions were run in the same concentration of this detergent. Thus, it appears that the membrane-associated 170-kDa synaptojanin isoform forms either homo-oligomers or hetero-oligomeric structures.

DISCUSSION

Synaptojanin is an inositol 5-phosphatase implicated in synaptic vesicle endocytosis (8–10). We had previously identified two isoforms of the protein with molecular masses of 145 and 170 kDa that appear to be generated from the same transcript. The data presented here suggest that both synaptojanin isoforms may play a more general role in clathrin-mediated endocytosis. Synaptic vesicle endocytosis appears to be similar to clathrin-mediated endocytosis used in all cells (31). For example, a number of proteins that function in clathrin-mediated endocytosis are enriched in nerve terminals including clathrin (6), AP2 (10), and dynamin (8). However, for each of these proteins, specialized neuronal isoforms exist that may determine the unique properties of synaptic vesicle endocytosis, particularly in terms of its rapid nature (32). Further, these proteins are all expressed at levels 10–50-fold higher in neuronal versus non-neuronal cells (32). For example, dynamin I (33) is highly expressed in neurons, where it functions in synaptic vesicle endocytosis. Related isoforms of the protein are expressed at lower levels in non-neuronal cells, where they are thought to function in general clathrin-mediated endocytosis (34–36). For synaptojanin, we have now demonstrated that both the 145- and 170-kDa isoforms have widespread tissue distributions, suggesting that the proteins have a general function in clathrin-mediated endocytosis. However, the 145-kDa isoform is expressed at much greater levels in neurons than in non-neuronal tissues. This would suggest that the 145-kDa isoform, as opposed to the 170-kDa isoform, which is expressed at comparable levels in all tissues, is more specialized for synaptic vesicle endocytosis. Consistent with this hypothesis, the two isoforms are expressed at similar levels early in neuronal development, but expression of the 145-kDa isoform increase dramatically with synaptic development, whereas the levels of the 170-kDa isoform decrease to below detectability in the adult nervous system.

It is conceivable that the proteins detected in non-neuronal cells may be due to neuronal innervation of the tissues, although several points argue against this interpretation. First, the ratio of the two isoforms varies from tissue to tissue, suggesting that the proteins are not coming from the same source. Second, the proteins are not detectable in skeletal muscle, which receives extensive motor neuron innervation. Finally, PCR analysis of cDNA generated from mRNA isolated from several non-neuronal tissues generates products that are identical in sequence to the cDNA for synaptojanin isolated from brain (see below).

Previously, the mechanism by which the 170-kDa isoform of synaptojanin was generated was not known, although it appeared to be derived from the same transcript as the 145-kDa isoform by suppression of an in-frame stop codon and the al-
ternative use of a second ORF (9). This suggestion was supported by several observations, including sequencing of a peptide fragment that indicated that the 145-kDa synaptojanin isoform ended on the glycine residue immediately before the in-frame stop codon (9). Possible mechanisms for the suppression of the stop codon to generate the 170-kDa isoform included read-through (37), RNA editing (38), and alternative splicing (39). To address this issue, we used oligonucleotide primers flanking the stop codon for PCR analysis of cDNA from a variety of tissues that express both isoforms as well as from adult brain which expresses only the 145-kDa isoform. Whereas all PCR products examined from adult brain contained the stop codon, products from heart and PC-12 cells fell into two size classes. The smaller class of PCR product had a 27-nucleotide deletion that removed the stop codon, suggesting that alternative splicing is responsible for the generation of the 170-kDa synaptojanin isoform. This idea is further supported by the isolation of a partial genomic clone, which contains the stop codon, products from heart and PC-12 cells fell

![Figure 6](https://example.com/fig6.png)

**Figure 6.** Size fractionation of synaptojanin isoforms on sucrose density gradients. Proteins from Triton X-100 solubilized membrane fractions (membrane) or high speed supernatant fractions (cytosol) from PC-12 cells or rat E18 brain were separated on 5–20% sucrose density gradients by centrifugation. Proteins from equal volumes of each gradient fraction (fractionated from the bottom) were separated on SDS-PAGE and processed for Western blot analysis with antibodies against the synaptojanin isoforms as indicated by the arrows on the right (see legend to Fig. 5). The migratory positions of globular protein standards are indicated at the top of the figure.

example, dynamin forms homo-oligomers in a manner that is at least partially dependent on its proline-rich C terminus (7, 40) and that is stimulated by association with membrane vesicles (40). Of interest, dynamin self-association is a critical regulator of its intrinsic GTPase activity (41). However, the increased resistance to salt extraction seen for the 170-kDa isoform suggests that it has an increased membrane affinity mediated by interactions with membrane-associated proteins. Further, the cytosolic pool of the 170-kDa synaptojanin isoform does not form any detectable large molecular mass complexes suggesting that oligomerization is not an inherent property of the protein. Thus, a future challenge will be to identify the protein(s) that interact uniquely with the 170-kDa synaptojanin isoform and not the 145-kDa isoform. Given the apparent differences in the functional role of the two isoforms in endocytosis, this information will be relevant to the nature of the mechanisms that regulate synaptic vesicle endocytosis versus general clathrin-mediated endocytosis.

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