Alternative Splicing of Human Synexin mRNA in Brain, Cardiac, and Skeletal Muscle Alters the Unique N-terminal Domain*

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Several synexin (annexin VII) mRNAs have been identified by screening a human fibroblast cDNA library. One type of message contained an alternatively spliced cassette exon, predicting two isoforms of synexin differing in the N-terminal domain. Polymerase chain reaction analysis of synexin mRNA from various fetal and adult tissues, from human and monkey, revealed that the alternative splicing event is tissue-regulated; synexin mRNA containing the cassette exon is prevalent in brain, heart, and skeletal muscle. This is supported by Western blot analysis showing that muscle synexin (annexin VIIb) is larger than synexin from lung (annexin VIIa). The muscle and lung isoforms have the same molecular mass as the recombinant synexins expressed in Escherichia coli using cDNAs containing or lacking the cassette exon, respectively. The difference in size is consistent with the molecular masses predicted from the proteins encoded by the alternatively spliced synexin mRNAs. Another type of synexin mRNA contained a longer 3′-noncoding region generated by the selection of an alternate poly(A) signal. Northern analysis of human fibroblast RNA showed the presence of two bands (2.0- and 2.4-kilobase) when hybridized with a synexin cDNA probe (7). The role of the C-terminal conserved repeats appears to include the binding of calcium and phospholipids (11, 13) as well as ion conductance (8), whereas the N terminus is thought to be important in conferring functional specificity to each particular annexin (11–13).

Previous studies on synexin have indicated that polymorphisms might exist at both the protein and mRNA level. Bovine adrenal medulla and liver contain a major synexin variant of 47 kDa and a minor one of 51 kDa (4, 14). On the other hand, the 51-kDa protein is the only synexin found in bovine skeletal muscle (14). More recently, Northern blot analysis of human liver poly(A) RNA showed the presence of two bands when hybridized with a synexin cDNA probe (7).

In the present report we have attempted to resolve the issue of possible synexin polymorphisms by sequencing human synexin cDNA variants and by analyzing human and monkey tissues for the expression of different synexin messages. We have identified several different synexin mRNAs produced by alternative splicing of a cassette exon and by selection of alternate polyadenylation signals. In addition, we show that the alternative splicing is tissue-regulated, resulting in the inclusion of the cassette exon predominantly in synexin mRNA from brain, cardiac, and skeletal muscle. Furthermore, analysis of human tissues showed that human skeletal muscle expresses a larger form of synexin, the molecular mass being the same as the recombinant synexin expressed in Escherichia coli using cDNA containing the cassette exon. This is the first instance in which a member of the annexin gene family has been shown to exhibit tissue-regulated alternative splicing.

MATERIALS AND METHODS

Screening of Human Fibroblast cDNA Library—An Okayama human fibroblast cDNA library (Dr. H. Okayama, National Institutes of Health) was screened (15) with a labeled oligonucleotide, rp172 (5′AGCCATAGGATAAGGATAGCACAGAG). (Unless otherwise specified, the numbers of the primers used in this report correspond to the nucleotide position where the primer starts, according to the R10/R16 clone (7). Rp and p denote noncoding and coding strands, respectively.) DNA from colonies giving positive signals on duplicate filters were purified and sequenced directly or after subcloning BamHI fragments into M13mp18. Synexin- or M13-specific primers were used in reactions with Sequenase (U. S. Biochemical Corporation).

RNA Extraction and Northern Blot Hybridization—Human liver poly(A) RNA was prepared by the guanidine thiocyanate method and oligo(dT)-cellulose chromatography (16); total RNA was prepared from liver, heart, and skeletal muscle of fetal monkey (near end of pregnancy at 160 days of gestation) and from human skeletal muscle using RNAzol (Cinna/Biotecx Laboratories International Inc.,

tetrad repeat (or octad repeat for annexin VI) of about 70 amino acids each, but they are distinguished from each other by having unique N-terminal domains. In the case of synexin, this domain is extraordinarily long and highly hydrophobic (7). The role of the C-terminal conserved repeats appears to include the binding of calcium and phospholipids (11, 13) as well as ion conductance (8), whereas the N terminus is thought to be important in conferring functional specificity to each particular annexin (11–13).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EMBL Data Bank with accession number(s) J05732.

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Synexin mRNA Alters N-terminal Domain

Identification of Different Types of cDNAs Encoding Human Synexin—Three different types of synexin cDNA clones were isolated from a human fibroblast library (Fig. 1A). The first type (F4) was identical to the previously reported R10/R16 clone (7), from the 3' end (bp 1771) to the 5' end (bp 26). However, the sequence of both cDNAs diverged upstream of bp 26. The 5' end of the F4 clone was identical to genomic sequences (data not shown), suggesting colinearity of the F4 cDNA and genomic DNA in the 5' noncoding region. The second type (F8) contained a 66-bp insertion at position 495, whereas the remainder of the cDNA was identical to the R10/R16 clone from the 5' end (bp 47) to the 3' end (bp 1774). This insert is an open reading frame encoding for 22 amino acids within the N-terminal domain without changing the reading frame of synexin (Fig. 1B, upper and middle panels). The same 66-bp sequence is found in the synexin gene flanked by consensus splice junctions (Fig. 1B, lower panel). These data therefore suggest that this insert corresponds to an alternatively spliced cassette exon (19, 20). In the third type of clone (F6 and F14), the 3'-untranslated region was extended to the next polyadenylation signal at position 2095, ending with a poly(A) tail at position 2111 (Fig. 1C). Analysis of genomic DNA using the PCR technique revealed that both poly(A) signals are contained in the same exon (data not shown), suggesting that the selection of the poly(A) signal is not due to alternative splicing of synexin mRNA. Although a putative fourth type of cDNA containing both the cassette exon and the longer 3' end was not isolated, analysis of RNA from different tissues using the PCR technique revealed the existence of this type of message (data not shown). As shown in Fig. 1C, the site of poly(A) tail addition governed by the proximal polyadenylation signal was found to be heterogeneous among the different clones isolated, occurring at three distinct sites between nucleotides 1771 and 1794.

Confirmation of the Existence of Two mRNA Variants Differing at the 3' End—Northern analysis of human fibroblast mRNA, using a PstI/SspI fragment of synexin cDNA as a probe, revealed the presence of two bands of 2.4 and 2.0 kilobases (Fig. 2, left panel). The sizes of these bands correspond approximately to the size of the synexin clones with or without the additional 3' noncoding region, respectively, assuming that each message contains a poly(A) tail of 200–250 bases. When a duplicate Northern blot was hybridized to a probe made from the longer 3' end, only the upper 2.4-kilobase band was evident (Fig. 2, right panel). These data thus confirm the existence of two differently sized synexin mRNA molecules as a result of alternate polyadenylation signal selection.

Determination of Tissue-specific Regulation of Alternative Splicing of Synexin mRNA—Because alternative splicing plays a crucial role in development and tissue specificity (for review, see Refs. 19–22), we used the PCR technique to examine various fetal and adult tissues from human and monkey for the presence of alternatively spliced synexin mRNA. As shown in Fig. 3, these studies clearly showed that synexin mRNA containing the cassette exon is the major variant in brain (lanes 5 and 7), heart (lane 11), and skeletal muscle (lane 10), whereas synexin mRNA without this exon is more abundant in liver, lung, kidney, spleen, fibroblast cells, and placenta (lanes 6 and 9, 8, 12, 13, 14, and 16, respectively). Analysis of mRNA from different parts of the brain (frontal pole, dorsal lateral prefrontal cortex, hippocampus, caudate, visual cortex, and somatosensory cortex) all showed prevalence of mRNA containing the cassette exon, suggesting no regional difference in the alternative splicing pattern between

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**RESULTS**

**Identification of Different Types of cDNAs Encoding Human Synexin—** Three different types of synexin cDNA clones were isolated from a human fibroblast library (Fig. 1A). The first type (F4) was identical to the previously reported R10/R16 clone (7), from the 3' end (bp 1771) to the 5' end (bp 26). However, the sequence of both cDNAs diverged upstream of bp 26. The 5' end of the F4 clone was identical to genomic sequences (data not shown), suggesting colinearity of the F4 cDNA and genomic DNA in the 5' noncoding region. The second type (F8) contained a 66-bp insertion at position 495, whereas the remainder of the cDNA was identical to the R10/R16 clone from the 5' end (bp 47) to the 3' end (bp 1774). This insert is an open reading frame encoding for 22 amino acids within the N-terminal domain without changing the reading frame of synexin (Fig. 1B, upper and middle panels). The same 66-bp sequence is found in the synexin gene flanked by consensus splice junctions (Fig. 1B, lower panel). These data therefore suggest that this insert corresponds to an alternatively spliced cassette exon (19, 20). In the third type of clone (F6 and F14), the 3'-untranslated region was extended to the next polyadenylation signal at position 2095, ending with a poly(A) tail at position 2111 (Fig. 1C). Analysis of genomic DNA using the PCR technique revealed that both poly(A) signals are contained in the same exon (data not shown), suggesting that the selection of the poly(A) signal is not due to alternative splicing of synexin mRNA. Although a putative fourth type of cDNA containing both the cassette exon and the longer 3' end was not isolated, analysis of RNA from different tissues using the PCR technique revealed the existence of this type of message (data not shown). As shown in Fig. 1C, the site of poly(A) tail addition governed by the proximal polyadenylation signal was found to be heterogeneous among the different clones isolated, occurring at three distinct sites between nucleotides 1771 and 1794.

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**Abbreviations used** are: PCR, polymerase chain reaction; EGTA, ethylenebis(oxyethylenenitriilo)tetraacetic acid; bp, base pair.
Alternative Splicing of Synexin mRNA Alters N-terminal Domain

FIG. 1. A, schematic representation of human synexin cDNAs. Coding and noncoding sequences of F4, F8, F6, and F14 human fibroblast cDNA clones are represented by wide and narrow boxes, respectively. The filled box represents the 66-bp cassette exon. B, DNA and the derived amino acid sequence of human synexin alternatively spliced cassette exon (middle row), cDNA flanking sequences (upper row), and intron flanking sequences (lower row). Middle row, acidic amino acids are underlined; asterisks indicate the differences in nucleotide sequences found in monkey, and the amino acid changes are in parentheses. Upper row, numbers above and below the sequence indicate the nucleotide and derived amino acid position, respectively, according to the R10/16 clone (7). Lower row, the intron junction is in agreement with the GT/AG rule. Consensus donor and acceptor splice sequences are underlined. C, sequence of the 3’ end of human synexin cDNA clones. Numbers above the arrows (1–4) correspond to clones F4, R10/16, F8, and F6/F14, respectively.

FIG. 2. Detection of synexin mRNA bands by Northern blot analysis of human fibroblast RNA. A, 10 and 5 μg of poly(A) RNA was probed with the cDNA probe (left panel) or the 3’ probe (right panel). Sizes of hybridizing bands are indicated in kilobases. B, diagram of synexin cDNA indicating the PstI/SspI fragment and the 3’ end PCR fragment (hatched box) that were used as probes for hybridization. Coding and noncoding regions are represented by a filled box and lines, respectively.

brain mRNAs (data not shown). Comparison of RNA from hearts and livers of adult and fetal monkeys (lanes 11 and 9, 2 and 1, respectively) and of RNA from adult and embryonic human fibroblast cells (lanes 14 and 15) revealed no detectable difference in the alternative splicing pattern. Southern blot hybridization with specific or common oligonucleotide probes (Fig. 4, middle and lower panels, respectively) showed that, although one variant of synexin mRNA predominates, both forms are found in every tissue examined.

The sequence of the amplified RNA from monkey muscle revealed that the alternatively spliced exon is highly conserved between monkey and human, except for two bases, which change an aspartate to a glutamate and a serine to a proline (Fig. 1B, middle panel). In addition, the entire sequence of fetal and adult PCR fragments (liver and lung, respectively) were found to be identical.

Determination of Tissue-specific Expression of Human Synexin Isoproteins—Comparison of partially purified synexin from human skeletal muscle and lung by Western blot analysis (Fig. 5, left panel) revealed that muscle contains a major synexin variant (lane 1) which is larger than synexin from lung (lane 2). The muscle and lung isoforms have the same molecular mass as the recombinant synexins expressed in E. coli (Fig. 5, right panel) using cDNAs containing (lane 3) or lacking (lane 4) the cassette exon, respectively. The size difference of approximately 2000 daltons is consistent with the difference in molecular mass predicted from the proteins encoded by the alternatively spliced synexin mRNAs. In addition, the smaller synexin isoform is also present in muscle in minor amounts, consistent with the results obtained at the mRNA level (Fig. 3, lane 10). Finally, partially purified human muscle synexin also aggregated bovine chromaffin granules in a calcium-dependent manner (data not shown), a characteristic property of synexin (1).

DISCUSSION

Synexin (annexin VII) polymorphisms at both mRNA and protein level have been suspected but poorly understood at
controls position 54 to 19.

The production of mRNA species of different lengths via the utilization of multiple poly(A) signals has been shown for many genes (for reviews, see Refs. 19–22). One previous instance in the annexin family is annexin VIII, which contains two poly(A) signals. However, only the distal one has been shown to be used in the clones so far isolated (23). Although selection of alternate poly(A) signals can often alter the order of splicing, resulting in the generation of various protein isoforms (19–22), analysis of synexin mRNAs from different tissues using the PCR technique revealed that both poly(A) signals are used independently of the splicing pattern (data not shown). Thus, the choice of poly(A) signal does not appear to influence the alternative splicing mechanism in the synexin gene.

Intriguingly, the position of the cleavage/polyadenylation site governed by the proximal poly(A) signal was found to be heterogeneous, occurring at three distinct sites between nucleotides 1771 and 1794 (Fig. 1C). Although pre-mRNA cleavage/polyadenylation usually occurs at a single site (24), several examples of naturally occurring 3' end heterogeneity have been reported for other genes (25–29).

We had considered the possibility that the alternative splicing of synexin mRNA might be regulated developmentally. However, no significant difference was found in the pattern of alternative splicing when we compared heart and liver RNA from monkey fetus (160 days, near end of pregnancy) or adult and RNA from human embryonic and adult fibroblast cell lines. Furthermore, PCR analysis of RNA from fetal tissues showed that these also contain the two poly(A) signals found in adult tissues (data not shown). Thus, there seems to be no developmental regulation of alternative splicing of the synexin gene at the different stages of the tissues used.

Both variants of alternatively spliced synexin mRNAs are found to some extent in every tissue examined. This is also observed at the protein level with synexin from human skeletal muscle (this report) and bovine liver, adrenal medulla (14), lung, and various regions of the brain. Whether each RNA form is present in every tissue but is required in different amounts or each cell type present in a given tissue expresses a different splicing variant remains to be solved. Recently, it was reported that another member of the annexin family, annexin VI, appears to undergo alternative splicing close to

\[ \text{H. B. Pollard and A. L. Burns, unpublished observations.} \]
the start of the seventh repeat of the C-terminal domain (30); however, no tissue or developmental regulation was shown.

The location of the 22 amino acids encoded by the 66-bp alternatively spliced exon is within the highly hydrophobic, unique N-terminal domain of synexin. The consequence of this inclusion may be important for the structure of the N terminus, because it results in the introduction of 3 acidic residues into an otherwise almost uncharged domain (7). The predicted amino acid sequence of this insert is conserved in monkey, except for two nucleotide substitutions which change an aspartate to a glutamate and a serine to a proline. Interestingly, the former change preserves the acidic nature of the amino acid at this position. In addition, the insert contains a Ser-Tyr-Pro sequence, a variation of the repetitive Gly-Tyr-Pro motif found in the N terminus of synexin (7, 31). Similar repetitive motifs have been reported for synaptophysin, RNA polymerase II, and several other proteins, and a group of related models were developed showing high content of consecutive helical B-turns (32).

The unique N-terminal domain of the annexins imparts distinct properties to each member of this family, and it has been proposed to act as a regulatory domain. Posttranslational modifications of the N terminus, such as phosphorylation, subunit association, or proteolysis have been shown to modulate the calcium and phospholipid sensitivity of annexin I and II (11–13). In the case of synexin, regulation of the C terminus could occur by posttranscriptional modification of the N terminus through alternative splicing. This may be analogous to the Shaker gene, where alternative splicing at the N terminus generates isoforms with different electrophysiological properties (33, 34). We are currently in the process of preparing isoform-specific antibodies to confirm unambiguously the relation between the two synexin isoforms and the two alternate spliced messages. We anticipate that comparison of synexin from human muscle with the smaller isoform containing (pTrCES) or lacking (pTrCFLS) the cassette exon were expressed in human skeletal muscle, and recombinant proteins expressed from vectors (11-13).

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**Addendum**—Partially purified human synexin samples from lung, skeletal muscle, and recombinant proteins expressed from vectors containing (pTrCES) or lacking (pTrCFLS) the cassette exon were reacted with two antisera after Western blotting. The goat antiserum raised against bovine liver synexin confirmed the results shown in Fig. 5, which identified bands from pTrCFLS and lung migrating faster than proteins in extracts from pTrCES and muscle. However, a new antisera raised in goats against 20 amino acids predicted from the exon sequence only reacted with the larger form of synexin. These data confirm that the alternatively spliced exon is actually utilized in human skeletal muscle to produce a larger form of synexin, which was suggested by the previous PCR and Western analyses.

**REFERENCES**