Human synexin (annexin VII) polymorphisms: tissue specificity and expression in *Escherichia coli*

A. Lee Burns,* Karin Magendzo,* Meera Srivastava,* Eduardo Rojas,* Claudio Parrra,* Milton de la Fuente,* Constance Cultraro,* Anat Shivan,* Tivka Vogel,* Judy Heldman,* Hung Caohuy,* Donatella Tombaccini* and Harvey B. Pollard*

*Laboratory of Cell Biology and Genetics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, U.S.A. and Cardiovascular Division, Food and Drug Administration, Bethesda, MD 20892, U.S.A.

**Introduction**

Synexin (annexin VII) is a cytosolic calcium binding protein, which was originally isolated from bovine adrenal medulla as part of a search for proteins involved in membrane fusion during exocytosis [1, 2]. Our earliest studies showed that chromaffin granules were aggregated in the presence of synexin and calcium, and that the aggregates became membrane upon the addition of arachidonic acid [3-5].

The fused structures resembled the vacuolar structures observed within secreting cells which are formed by simple fusion between granule and plasma membranes, and by compound fusion between membranes of secreted granules and more internal granules. Later, we found that synexin could fuse granule ghosts [6] without recourse to additional arachidonic acid. We and others [5] found similar results found with some composed of acidic phospholipids. Since synexin appeared to engineer the direct fusion of membranes widely considered to be relevant to exocytotic process, we continued efforts to understand the structural and biophysical basis for synexin action.

Using biophysical methods, we showed that when synexin attaches to a target membrane, substantial parts of the synexin molecule are inserted into the low dielectric substance of the bilayer [7]. The principle behind our experiment was the fact that the addition of protein into a membrane could raise the capacitance of the membrane, while no effect on capacitance could be expected from simple adherence of the protein to the exterior of the membrane. In addition, synexin could form calcium channels in the same membranes under specific conditions [8-11]. Depending on the calcium concentration, the chord conductance was as high as 175 pS. Synexin channels were highly selective for calcium, proved to be voltage sensitive, and were blocked by both lanthanum and phenothiazine drugs such as trifluoperazine and promethazine. By contrast, dihydropyridine calcium channel blocking drugs and cadmium failed to inhibit the synexin channels [9]. However, despite this detailed knowledge, we remain uncertain about the physiological role of synexin channels in biological systems. Nonetheless, the fact that channels are formed means that synexin not only inserts into, but actually spans the membrane. Thus, without understanding the meaning of the channel activity, we were nonetheless led to some insights as to the mechanism of membrane fusion induced by synexin.

We specifically proposed that synexin polymers [2] fused membranes by two entering neighbouring membranes simultaneously, forming a 'hydrophobic bridge' across which juxtaposed phospholipids crossed and mixed [10].

**When our most recent molecular studies revealed synexin to be a member of the annexin gene family [11], we considered annexin polymorphisms and conserved C-terminal tetrad repeats of about 70 amino acids each (except for annexin VI which has an octad repeat). The N-terminus of synexin (annexin VIIa, first isoform sequence derived from cDNA) is extraordinarily long (167 amino acids), highly hydrophobic with only two charged amino acids and contains a prevalent GlyTyrPro tripeptide motif [9]. Endonexin II (annexin V) with an N-terminal domain of 18 amino acids had little or no granule aggregation activity, but nonetheless formed excellent calcium channels [12, 13]. Both natural and recombinant endonexin II required calcium in order to enter into the membrane, and once there could also transport barium, lithium and caesium [13]. This activity profile contrasts with the synexin channel which is very specific for calcium [8, 9].**

We were thus led to conclude that the conserved tetrad repeat was likely to be the region most responsible for both phospholipid interactions and channel properties. It is possible that the fusion activity of synexin is conferred by the N-terminal domain or by the capacity to polymerize, a property lacking in endonexin II.

In principle, these structure-function questions could be answered by studying modified versions of synexin. For example, naturally occurring isoforms could be sequenced, and these defined differences could be correlated with functional changes. In this paper, we describe an isoform of synexin (annexin VIIb, second isoform sequence derived from cDNA) found primarily in cardiac and skeletal muscle and brain, which is due to alternative splicing of a cassette exon. In addition, we describe experiments with recombinant synexins and a chimera of synexin synthesized in *Escherichia coli* which further may help us to gain insight into the structural basis of various synexin activities.

**Synexin polymorphism in brain, cardiac and skeletal muscle**

Four synexin cDNAs from a human fibroblast library (gift of Dr H. Okayama, M.I.H.) were sequenced and classified into different groups depending on the presence or absence of a cassette exon and a second polyadenylation signal (K. Magendzo, A. Shirvan, H. B. Pollard & A. L. Burns, unpublished work). The first type (clone F4) is similar to the previously reported R10/R16 clone without the 66 bp insert and the second polyadenylation signal. The second type of cDNA (clone F6) contains a cassette exon inserted after bp 495 and the first polyadenylation signal at bp 1753. The 66 bp insert results in the addition of 22 amino acids, three of them charged, to the N-terminal domain, while the remainder of the synexin molecule is unchanged. The insert appears to be an alternatively spliced cassette exon, since it is flanked by consensus splice junction sequences in a genomic clone. In the third type (clones F6, F14), the cDNA lacks the cassette exon and utilizes the second polyadenylation signal at bp 2095, adding about 336 bp to the 3' non-coding end. Selection of two polyadenylation signals in liver RNA results in the production of 2.4 kb and 2.0 kb mRNAs, which are detectable with cDNA probes on Northern blots. Analysis of human genomic DNA using the polymerase chain reaction technique indicates that both signals are within the same exon and that polyadenylation site selection is not due to alternative splicing. Although the anticipated fourth type with both a cassette exon and the longer 3' end was not isolated from this library, analysis of RNA from different tissues using PCR revealed the existence of this type of message. Excluding these polymorphisms, other regions of the synexin cDNAs are remarkably similar in sequence from bp 26 to 2340, suggesting that these sequences could have emerged from an ancestor that already had membrane fusion properties. All annexins have unique N-terminal domains and conserved C-terminal tetrad repeats of...
Cloning artifacts in the latter phase may actually represent a cloning artifact in the latter phase. Since oligonucleotides to this region do not hybridize to synexin genomic clones (data not shown).

Pollard mRNA containing the cassette exon (i.e. clone F8) is the major variant in monkey heart, human brain and skeletal muscle, whereas synexin mRNA without the 66 bp insert (i.e. clones F4, F6 and F14) is more abundant in human liver, fibroblast and placenta, as well as monkey kidney liver, lung, and spleen. Comparison of heart and liver RNA from adult and fetal monkey, and of fibroblast RNA from adult and embryonic human cell lines revealed no difference in the alternative splicing pattern. Southern blot hybridization of the PCR products with oligonucleotide probes from cassette exon or cDNA sequences showed that one synexin mRNA predominates, even though both forms are found in all tissues (K. Magendzo, A. Shirvan, H. B. Pollard & A. L. Burns, unpublished work).

In addition, we compared expression of synexin isoforms in human skeletal muscle and lung using Western blot analysis to determine tissue specificity. Partially purified synexin from both tissues was separated on SDS/PAGE, blotted to nitrocellulose and detected with polyclonal antisera against bovine synexin. The predominant synexin from muscle is larger than synexin from lung by about 2000 kDa, which is consistent with the difference in molecular mass predicted for the proteins encoded by the two alternatively spliced mRNAs. This sample of muscle synexin has similar aggregating activity to that of both human lung synexin and the two bovine isoforms. The shorter synexin isoform prevalent in lung was also present in minor amounts in muscle, consistent with results from hybridization of PCR products derived from RNAs of different tissues (K. Magendzo, A. Shirvan, H. B. Pollard & A. L. Burns, unpublished work). From these data, we conclude that expansion of the hydrophobic N-terminal domain by 22 amino acids and emplacement of the former repeats of synexin, respectively. In the latter two cases, pTrcR4S and pTrcE11S are not precipitated with low salt concentration, suggesting that the hydrophobic N-terminal domain is responsible for the ability of recombinant and natural synexin precipitate in 20% ammonium sulphate.

Recombinant human synexin purified from E. coli was tested for its ability to aggregate chromaffin granules. As shown in Fig. 1(a), recombinant synexin indeed caused granules to aggregate, and furthermore, to do so in a manner dependent on the concentrations of both calcium and added protein. The calcium dependence for both natural and recombinant synexin proved similar, with $k_{1/2}$ values of

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\begin{array}{c|c|c|c}
\text{ Protein/ml } & 0 & 0.02 & 0.04 \\
\hline
\text{ Change in absorbance (A)$_{403}$} & 0 & 0.02 & 0.04 \\
\end{array}
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Expression of recombinant synexin isoforms and chimaera

Construction of the synexin expression plasmid (pTrcFLS) resulted from the ligation of three DNA fragments (pTrc99A digested with Neo I and Xba I; synexin cDNA digested with Xma I engineered at position 69 and Spe I; and a double stranded oligomer with CATGTCATAC and CGGGGTATGA having Neo I and Xma I compatible ends) (pTrc99A and E. coli RB 971 were gifts from Dr E. Amann, ref. [15]). Similar procedures were followed to make the pTrcR4S and the pTrcE11S plasmids, which encoded proteins containing only the four C-terminal repeats of synexin and a chimera with the N-terminus of endonexin II plus the C-terminus of synexin, respectively. In the latter two cases, pTrc99A Neo I/Xba I and synexin Xmn I/Spe I fragments were ligated to double stranded oligomers using sequence information from the respective cDNAs [9, 12]. Extracts of all three recombinant plasmids expressed in E. coli RB 971 contained proteins of the appropriate molecular weight and were immunoreactive with polyclonal anti-synexin antisera by Western analysis [15]. Recently, similar analysis of a pTrcCES plasmid containing synexin cDNA with the cassette exon was also positive by Western analysis using anti-synexin antisera. In addition, the E11S protein was also recognized with anti-endonexin II antisera (gift of Dr H. Haigler) on Western blots.

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Properties of human recombinant synexin

As a first step in the study of these proteins from E. coli, the recombinant synexin was assayed for chromaffin granule aggregation and calcium channel activities using previously described methods [9]. Synexin was easily purified by lysing E. coli containing the induced pTrcFLS construct in the presence of calcium, followed by elution of the synexin from the membrane fraction with EGTA, and precipitation of synexin in the EGTA supernatant fraction with 20% ammonium sulphate. The recombinant proteins encoded by pTrcR4S and pTrcE11S are not precipitated with low salt concentration, suggesting that the hydrophobic N-terminal domain is responsible for the ability of recombinant and natural synexin precipitate in 20% ammonium sulphate.

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Fig. 1. Granule aggregation properties of recombinant human synexin

(a) Time course for granule aggregation in the presence of different volumes of protein (42 µg/ml, stock solution); and
(b) dose-response curve for data in part (a) including the 150 µg/ml data point not shown in part (a).
approx. 200 μM (data not shown). As expected from experience with natural synexin, the recombinant protein was inactive in the presence of either magnesium or barium ions. The dependence of granule aggregation on synexin concentration (Fig. 1b) showed that the process had a threshold and could saturate at higher synexin concentrations with an apparent Kₘ of approx. 4 μg/ml (80 mM). The sigmoid curve shape and Kₘ values are in the same range as that which we previously reported for bovine liver synexin (approx. 40 nM, ref. [1]) and human lung synexin (data not shown).

In addition, we also found that recombinant synexin could aggregate and fuse artificial phosphatidylserine liposomes. As shown in Fig. 2(a), increasing concentrations of synexin caused liposomes to aggregate. Fusion, assessed by volume mixing using a DPX/ANTS system, proceeded apace (data not shown). A quantitative analysis of the dose dependence for liposome aggregation is shown in Fig. 2(b). From these data one can see that curve shape and potency are at least in the same range as for the granule aggregation experiment shown in Fig. 1(a).

Finally, we also found that recombinant human synexin could form calcium channels in phosphatidylserine bilayers formed at the tip of a patch pipette. An example of typical data is shown in Fig. 3, for which the term Mg/Ca indicates that the pipette contains magnesium, while the bath contains calcium. The pipette potential is negative, providing the electrical driving force to induce calcium ions to leave the bath through the conductive pathways provided by the recombinant synexin. Both natural and recombinant synexin channels were able to conduct calcium, but not magnesium, and the channel kinetics for both forms of synexin proved very similar.

Conclusions

The synexin molecule possesses various activities in vitro, which would appear to have great potential relevance to our understanding of important cellular processes, ranging from membrane fusion to ion channel conduction. Interestingly, many of these functions are shared with some but not all of the other members of the annexin family. And, as a consequence, the annexin gene family has become a source of naturally occurring structural variants, whose comparatively modified properties may be traced to specific changes in sequence. Indeed, from such an analytic approach we know that the C-terminal tetrad repeat domain is likely to be the region responsible for phospholipid binding and for ion channel activity. In addition, we can guess that the most highly conserved sequences in the annexin family, the endo-nexin folds, may be the locus of calcium binding. However, the problem with comparing sequence similarity and function is that any two annexin family members only share about 50% homology in the C-terminal domain. Thus, the very conserved amino acids are likely to be important for common functions, but this leaves the partially conserved much more difficult to interpret.

For analysis of synexin function, we have chosen to modify, delete or create specific sequences in synexin, in hopes of lessening the ambiguities necessarily implicit in the comparative analysis described above. The role of the unique N-terminal domain with 167 amino acids became available for analysis with our discovery that skeletal and cardiac muscle and brain have a C-terminal insert. Except for three acidic amino acids, inclusion of the exon maintained the /3-turn and /3-sheet motif characteristic of the whole N-terminal domain of synexin. Nonetheless, in the granule aggregation assay, the human muscle synexin containing information encoded by the cassette exon was as active as human lung synexin, lacking the insert. Pending other studies on natural and recombinant synexins containing the cassette exon, we might

Fig. 2. Phosphatidylserine liposome aggregation by recombinant human synexin

(a) Numbers given next to specific curves indicate protein concentration (μg/ml). Absorbance change in the vertical axis represents changes in turbidity of the liposome solution.
(b) Dose/response curve for aggregation of phosphatidylserine liposomes by human recombinant synexin. Rates are calculated from data in (a).

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Fig. 3. Calcium channel activity of human recombinant synexin

Details are given in the text, and methods are given in ref. [9].
conclude that membrane contact and granule aggregation seem not to be profoundly affected by this N-terminal polymorphism. Certainly, the function of this synexin isoform in brain and muscle remains to be understood.

While we were certainly fortunate to find a polymorphism for synexin in an interesting region of the molecule, we cannot anticipate more serendipitous findings like this for other regions. Certainly, site directed mutagenesis would necessarily be our approach of choice. However to pursue this pathway we would have to be successful in expressing an active, recombinant synexin molecule from a full length cDNA. As is clear from the results presented here, recombinant synexin possesses calcium dependent granule aggregating and liposome fusion activity, fully compatible with that of natural synexin from human sources. Furthermore, recombinant synexin also exhibits calcium channel activity, with selectivity and chord conductance quite similar to the natural material. Therefore, we can conclude that the infrastructure is now in place for our goal of preparing specific mutant synexins, designed to probe the structural bases of different synexin functions.


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