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Calcium channel activity of purified human synexin and structure of the human synexin gene

(cDNA/membrane fusion)

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ABSTRACT **Synexin is a calcium-dependent membrane binding protein that not only fuses membranes but also acts as a voltage-dependent calcium channel. We have isolated and sequenced a set of overlapping cDNA clones for human synexin. The derived amino acid sequence of synexin reveals strong homology in the C-terminal domain with a previously identified class of calcium-dependent membrane binding proteins. These include endonexin II, lipocortin I, calpactin I heavy chain (p36), protein II, and calelectrin 67K. The M_r 51,000 synexin molecule can be divided into a unique, highly hydrophobic N-terminal domain of 167 amino acids and a conserved C-terminal region of 299 amino acids. The latter domain is composed of alternating hydrophobic and hydrophilic segments. Analysis of the entire structure reveals possible insights into such diverse properties as voltage-sensitive calcium channel activity, ion selectivity, affinity for phospholipids, and membrane fusion.**

Membrane fusion is an important and general process in biology, which has proved remarkably difficult to analyze in biochemical terms (1). One fruitful approach to this problem has been to study proteins such as synexin, which fuse certain natural and artificial membranes in a calcium-dependent manner (2–11). Recently, bovine synexin has been shown to generate classical capacitative gating currents in isolated acidic phospholipid bilayers (12) and to exhibit exquisitely selective, voltage-sensitive calcium channel activity in similar membranes (13). Therefore, synexin not only binds to specific phospholipids but also enters into and spans the bilayer. On this basis we have suggested that synexin might fuse membranes by entering and spanning both fusion partners simultaneously, thereby providing a bilayer-destabilizing hydrophobic bridge across which phospholipids from either side of the membrane might cross and mix (14).

This mechanism for membrane fusion could explain many aspects of synexin action and may be useful for thinking about other types of membrane fusion. We now report the successful cloning and sequencing of human synexin‡ and have deduced from analysis of the sequence that synexin is homologous with a class of calcium-dependent membrane binding proteins that include endonexin II, lipocortin I, calpactin I heavy chain (p36), protein II, and calelectrin 67K. The structure also provides insights into possible mechanisms for channel activity and membrane fusion.

MATERIALS AND METHODS

Purification of Human Synexin and Determination of Peptide Sequences. Initial isolations of bovine and human synexin were performed using a protocol developed for the bovine protein involving ammonium sulfate precipitation, Ultrogel chromatography, and chromatofocusing (2, 15). Tryptic fragments were prepared from 200-μg samples of purified synexin by heat denaturation in 8 M urea, reduction and alkylation in dithiothreitol and iodoacetamide, and digestion in L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (wt/wt = 1:25) for 24 hr at 37°C in a buffer containing 20 mM Tris acetate (pH 8.3) and 2 M urea. The resulting tryptic peptides were separated and repurified by reverse-phase HPLC and then sequenced on an ABI gas-phase sequencing apparatus.

Isolation of Human Synexin Clones. Oligonucleotide probes were prepared based on codon utilization frequency for most amino acids (16) and multiple deoxynucleotides plus deoxyinosine for highly redundant amino acids (arginine, leucine, and serine). For example, the D89 probe [dTAGTCGCCI-(CG)(AT)GGTGTCGCCAGCAATCATGGTGCC] corresponded to a portion of the amino acid sequence of the last human peptide in Fig. 2 and contained sequences complementary to the mRNA. This probe was treated with polynucleotide kinase following acrylamide gel purification (Applied Biosystems user bulletin issue no. 13) and used to screen human liver (Savio Woo; ref. 17) and lung (Clontech) λgt11 libraries using the Benton–Davis plaque hybridization technique (18). Nitrocellulose filters were hybridized at 42°C and washed at 48°C. The latter temperature was calculated to correspond to 75% homology of probe to target sequence (18, 19). cDNA inserts of the three positive recombinants or restriction fragments of the largest cDNA (L4a) were subcloned into M13 for DNA sequencing with M13- or synexin-specific primers (20). Specific oligonucleotide or cDNA probes were used to screen additional cDNA libraries: retina from Jeremy Nathans (Genentech) (Fig. 2); B-cell, adrenal, and lung from Clontech; and fibroblast from H. Okayama (National Institutes of Health) (data not shown) (20).

Single Channel Current Measurements. Calcium channel activity of human synexin was measured by methods previously described (refs. 13, 21; Fig. 1 *B* and *C*). Briefly, bilayers of phosphatidylserine (PtdSer) or phosphatidylinositol (PtdIns) (Avanti Polar Lipids) were prepared at the tips of patch pipets by a double dip method. Upon forming the bilayer the open tip resistance rose from 15–20 MΩ to 3–5 GΩ, and channel activity was acquired only after addition of synexin to the bath. The compositions of the various solutions are stated either in the legend to Fig. 1 or in the text.

RESULTS

Purification of Human Synexin. Initial studies of human synexin in crude fractions from liver revealed calcium-

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Abbreviations: PtdSer, phosphatidylserine; PtdIns, phosphatidylinositol.

‡The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04543).

dependent aggregating activity with bovine chromaffin granules. We purified this activity using methods previously devised by us for bovine synexin and found human synexin to be a single band of M_r 51,000 after SDS/PAGE and also to react with a goat anti-bovine synexin antiserum (Fig. 1*A*). Comparison with purified bovine synexin revealed human synexin to be slightly larger than the bovine protein (M_r 47,000) and to lack the minor M_r 52,000 immunoreactive species (u synexin), which may be a precursor since both have virtually the same peptide map (22).

Calcium Channel Activity of Human Synexin. Human synexin (*ca.* 3 nM) was incorporated into a PtdSer bilayer in the presence of 1 mM $CaCl_2$, and the calcium concentration was lowered to *ca.* 15 μM by addition of $MgSO_4$. A continuous recording of synexin channels in a PtdSer bilayer at a pipet potential of −30 mV is shown in Fig. 1*B*, where the magnitude of the unitary event is −1.9 ± 0.1 pA. Channel closings tended to occur in bursts under these conditions. Furthermore, human synexin was highly selective for calcium, much

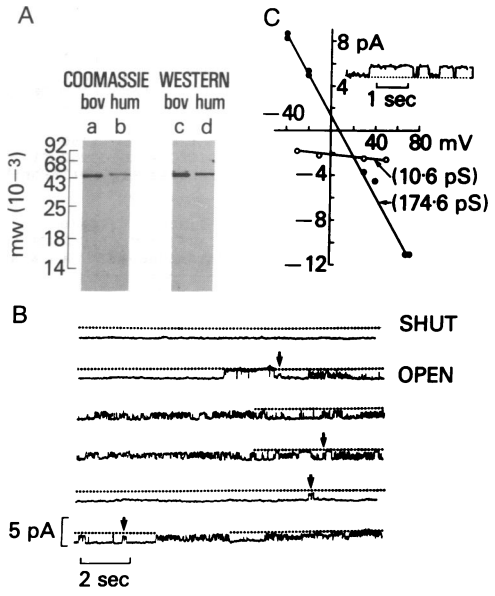


FIG. 1. (*A*) Physical and immunological comparison of bovine and human synexin: Coomassie blue staining and Western blot analysis. Samples of purified bovine (5 μg) and human (1 μg) synexin were run in parallel on 15% Laemmli gels in SDS. One set (left pair) was stained with Coomassie blue. The other set (right pair) was transblotted onto nitrocellulose filters overnight at 0.1 A under otherwise standard conditions, incubated with a goat anti-bovine synexin polyclonal antibody (1:200 in 5% dry milk in phosphate-buffered saline), and detected with rabbit anti-goat IgG-horseradish peroxidase (Vector Laboratories). Molecular weights are shown as 10^{-3}. (*B*) Synexin channel activity in the presence of a large $[Ca^{2+}]$ gradient. Six segments are shown from a continuous record of single human synexin channel currents. The pipet solution is 200 mM tetramethylammonium Pipes/25 mM CaHepes, pH 7.4. The chamber solution is 200 mM tetramethylammonium Pipes/1 mM CaHepes/2.5 mM $MgSO_4$, pH 6.5. The $[Ca^{2+}]$ in the chamber was estimated to be *ca.* 15 μM. Human synexin was added to the chamber in the presence of 1 mM $CaCl_2$ to a final concentration of 3 nM prior to the addition of $MgSO_4$. Dotted lines represent the closed state, and arrows indicate incomplete channel closures. The pipet potential is −30 mV (chamber potential is the reference). Fractional open time, P_o, is 0.68. (*C*) Single channel current–voltage relationships for human synexin in the presence of high and low calcium concentration gradients. The vertical axis represents the size of the single channel current and the abscissa represents the pipet potential. ○, Measurements made with 25 mM CaHepes in the pipet and 14.6 μM CaHepes in the chamber. ●, Measurements made with 25 mM CaHepes in the pipet and 43.8 mM CaHepes in the bath. The insert is a segment of the record showing single channel currents made at a pipet potential of −20 mV. The dotted line is the closed state, and the vertical calibration is 5 pA.

less selective for either Ba^{2+} or Mg^{2+}, and relatively insensitive to conventional calcium channel blockers such as cadmium (2 mM) or nitrendipine (100 μM). However, human synexin channels were quite sensitive to La^{3+} (0.2 mM). Fig. 1*C* shows two single channel current–voltage relationships obtained in the presence of low (15 μM) or high (43.8 mM) $[Ca^{2+}]_i$ (where "i" refers to inside) in the chamber bath. Similar data were obtained for parallel experiments using PtdIns bilayers instead of PtdSer bilayers (data not shown).

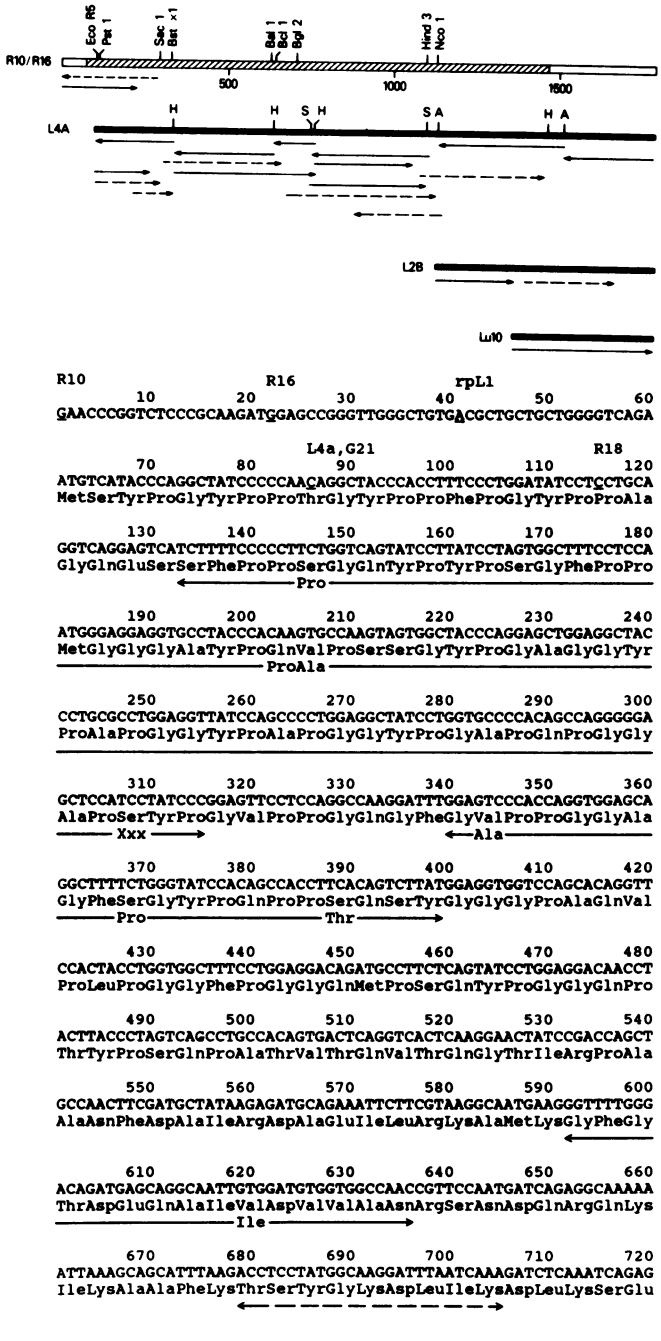
Nucleotide and Derived Amino Acid Sequence. A nucleotide probe based on amino acid sequence of human synexin was used to identify two clones from a liver library and one from a lung library. The larger liver clone contained sequences from base 86 to the poly(A) signal at base 1753 (Fig. 2). However, this cDNA also had an additional 565 base pairs (bp) at the 5′ end beginning at the same place as a splice junction of one of the synexin genomic clones (Fig. 2, G21) and presumed to be an intron. Therefore, additional screenings of one million recombinants per library were performed to isolate other cDNAs. Two clones from a retinal library (Fig. 2, R10 and R16) and one synthesized with a specific oligonucleotide (Fig. 2, rpL1) contained more 5′ sequences and the presumed initiator codon at position 61. Six additional clones, each slightly shorter than the R10 clone, from three other libraries confirmed the sequence from base 38 to approximately base 200 but revealed unexplained sequence variability 5′ to this region (data not shown). Fig. 2 *Upper Left* contains a partial restriction map and the strategy for nucleotide sequencing.

The synexin cDNA was confirmed by the fact that the derived protein sequence was identical with four human peptide sequences and very similar to 10 bovine peptides (Fig. 2). Furthermore, the molecular weight and amino acid composition of this predicted protein sequence correlate well with the results obtained with purified liver synexin (Fig. 1*A* and data not shown). We assume for the present that the first ATG at position 61 is the initiation codon, because the sizes of the mRNAs correspond well with the observed cDNA lengths. Northern analysis of human liver mRNA resulted in the detection of hybridizing bands of 2.4 and 2.0 kilobases (kb) (20). The larger major band could correspond to the composite cDNA with a poly(A) tail of about 2 kb plus 336 bp of additional sequence, which was recently found in the 3′ noncoding region of a cDNA (data not shown). On the other hand, the Kozak consensus sequence is only modestly conserved and the blocked N-terminal amino acid of synexin has not been determined.

DISCUSSION

The cDNA encoding human synexin has been cloned and the amino acid sequence deduced from the cDNA reveals that synexin has extensive homology with a previously identified family of calcium-dependent membrane binding proteins (24). These include endonexin II (25, 26), calpactin I heavy chain (27–30), lipocortin I (31), protein II (32), and calelectrin 67K (33). In general, proteins in this group contain a unique and relatively short hydrophilic N-terminal sequence, followed by a conserved C-terminal domain comprising four imperfect repeats.

Synexin differs from these proteins by having a longer (167 amino acids) and more hydrophobic N-terminal region. This segment is also highly enriched in glycine, tyrosine, and proline and is largely β-turn and β-sheet. For example, a characteristic GYP motif occurs 8 times and is predicted to be β-turns. Variations in this motif occur 7 times as GXP, where X is usually hydrophobic (F, A, A, V, V, F, Q), and 6 times as XYP, where X is usually neutral (S, Q, A, S, Q, T). Furthermore, GG, PP, and PG doublets occur 10, 8, and 13 times, respectively. The significance of these structures



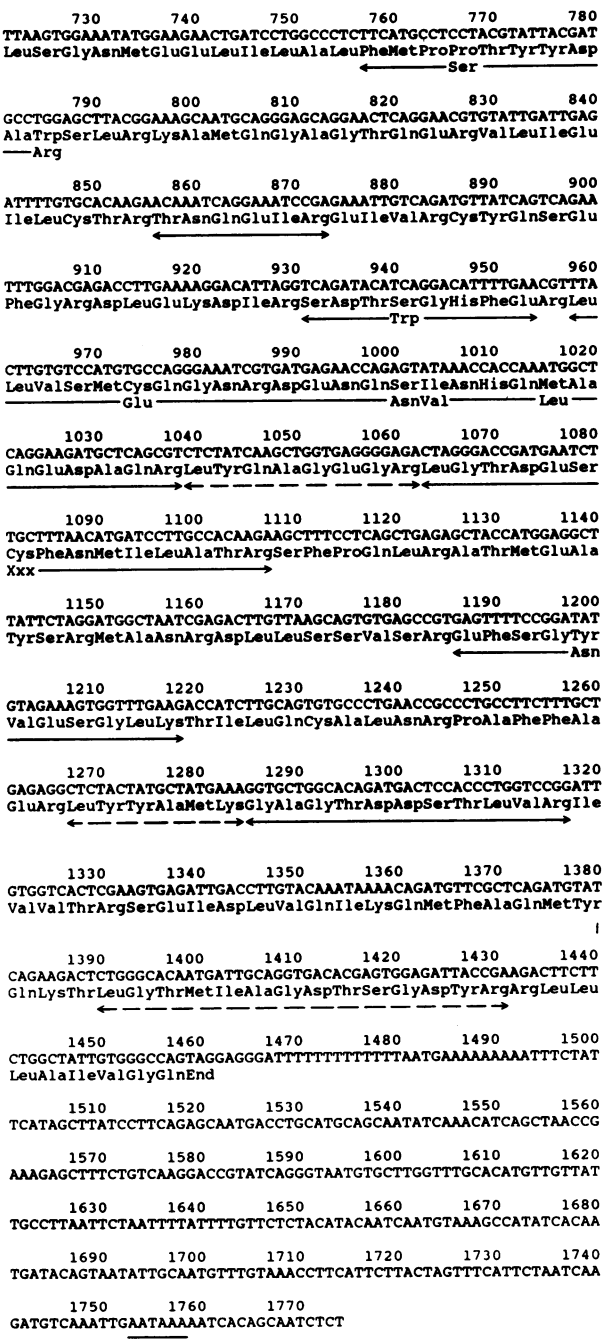


FIG. 2. (*Upper Left*) Strategy for sequencing cDNA clones encoding human synexin. The top box represents the composite cDNA clone with a partial restriction map above and the size in base pairs given below. The hatched area denotes the coding region that is flanked by 5′ and 3′ noncoding sequences. Most of the nucleotide data were obtained with the L4a cDNA or its restriction fragments, which after subcloning in M13 were sequenced with specific oligonucleotides (----→) or the universal primer (——→). R10, R16, and R18 are retinal cDNAs; L4A and L2B are liver cDNAs; Lu10 is a lung cDNA. (*Lower Left* and *Right*) Nucleotide and predicted amino acid sequence of human synexin. Similarities of peptide sequences determined from purified bovine (←——→) or human (←----→) synexin tryptic fragments are flanked by arrows below the derived synexin sequence. Where bovine amino acids differ from the derived human sequence the bovine residue is shown within the arrowheads. Notations over specific underlined bases at the 5′ end of the sequence indicate the 5′ end points of various clones. These are retinal cDNAs (R10, R16, R18), liver cDNA specifically primed with an oligonucleotide (rpL1), liver cDNA (L4a), and a human genomic clone (G21). The underlined sequence starting at base 1753 is the presumed poly(A) site. The two most N-terminal peptide sequences were previously reported (23).

may be related to the fact that β-turns, GG pairs, and PG pairs expose backbone carbonyls (summarized in ref. 34), thus enhancing the polar character of a particular conformation and also stabilizing hydrophobic side chains in energetically favored clusters (34). These facts may be highly relevant to our eventual understanding of the structure and function of the hydrophobic N-terminal segment.

The C-terminal domain of synexin contains four imperfect repeats, which are indicated as repeat I (amino acids 168–239), repeat II (amino acids 240–322), repeat III (amino acids 323–399), and repeat IV (amino acids 400–466) (Fig. 3). The lengths, order, and homology of these repeats in synexin are similar to the other proteins. We have arranged the amino acid sequence of several homologous proteins from this gene family to be coincident with the human synexin sequence, leaving blank spaces where the amino acids are identical to synexin. An asterisk (*) was placed above the synexin sequence when a specific amino acid was conserved in all of

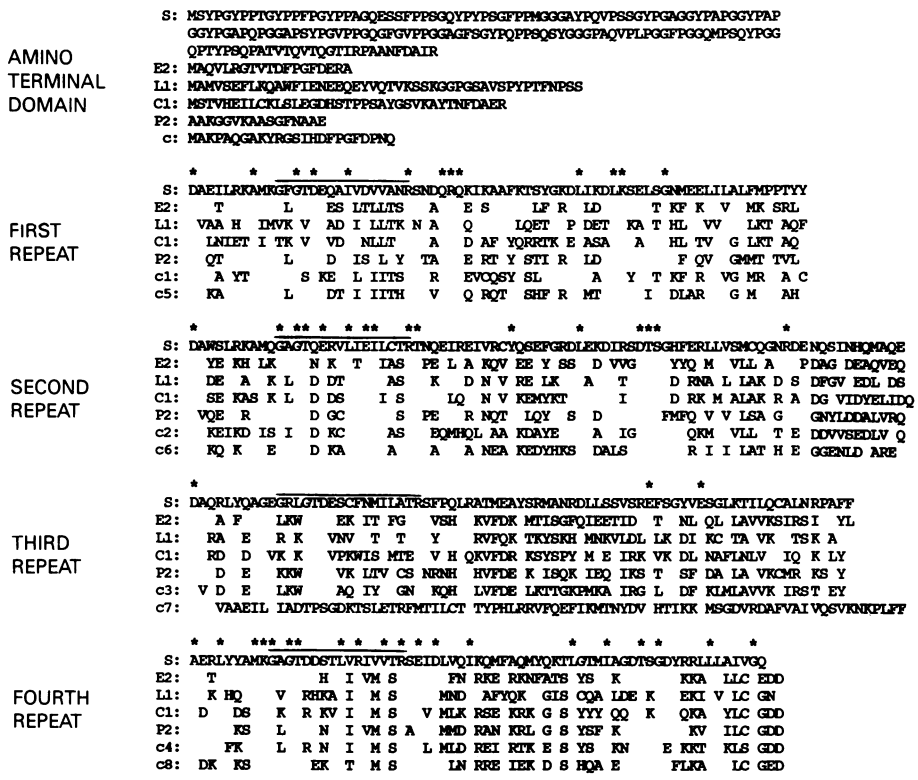


FIG. 3. Comparison of human synexin with related human proteins and porcine protein II. The predicted amino acid sequence of synexin (S) is entered above each sequence segment and compared with endonexin II (E2, ref. 27), lipocortin I (L1, ref. 30), calpactin I heavy chain (C1, ref. 28), protein II (P2, ref. 31), and calelectrin 67K (c in the N-terminal segment and c1–c8 in the C-terminal segments, ref. 32). Most of these proteins can be separated into a unique N-terminal domain, followed by a C-terminal domain consisting of four repeats. The exception is calelectrin 67K, which has the tetrad repeat duplicated. The sequence for calelectrin 67K shown above lacks the connecting segment (amino acids 326–367) between c4 and c5 and appears unique to this protein. Asterisks above the synexin sequence indicate amino acid identity within each repeat. Blank spaces under the synexin sequence represent amino acid similarity of the other proteins with synexin. Amino acid differences are shown by the presence of the alternate amino acids under the synexin sequence.

the proteins. As previously noted, there is also a 16-member core repeat (23, 35) within all regions (overlined in Fig. 3), which contains invariant glycine and arginine. The function of these highly conserved regions is not known, although the strongly anionic character of many of the 16-member core repeats has led some investigators to speculate that these specific regions might be calcium binding sites (35).

The channel-forming properties of synexin lead us to examine the predicted secondary structure for helical regions believed to be typical of conventional membrane channels (36, 37), and none was amphipathic. However, only 4 of 18 well-defined hydrophobic segments displayed α-helical character (38). Indeed, most hydrophobic segments were predicted to be either β-pleated sheet or β-turn. However, very few examples were found for alternating polar character, indicative of an amphipathic β-structure. Instead, the basic structural motif in the C-terminal tetrad appeared to be alternating lengthy hydrophilic and hydrophobic segments.

As an alternative approach to understand how synexin could form a channel, we considered whether the hydrophobic segments in the synexin sequence might provide the required hydrophobic surface facing the lipids (34, 38), allowing synexin to be stable within the low dielectric medium of the membrane. The remaining hydrophilic segments might then be segregated into the core of the intramembranous synexin molecule, coincidentally providing a charged pathway for ions to be conducted across the membrane.

A minimal test of this hypothesis is shown in the modified hydrophobicity plot in Fig. 4*B*. The sides of the box (I, II, III, and IV) represent the four tetrad repeats with the axis of the hydrophobicity plot in Fig. 4*A* forming the lines. Each amino acid residue is placed inside the box if in a hydrophilic segment or outside the box if in a hydrophobic segment. We minimized any conformational assumptions by simply placing the residues in each hydrophilic or hydrophobic segment adjacent to one another and perpendicular to the line. Loops of amino acids cross the hydrophobicity axis at transition points defined by the hydrophobicity plot in Fig. 4*A*. For consistency, the 167-amino acid hydrophobic N-terminal segment is placed outside the box in the hydrophobic domain. The structure in Fig. 4*B* is therefore an explicit two-dimensional model of a synexin molecule as it might occur when viewed face-on within the plane of a membrane bilayer.

Close scrutiny of this model actually reveals some possible physical and chemical bases for a number of properties exhibited by synexin when inside a membrane. Certainly the hydrophobic collar around the protein could mediate placement of the protein in the membrane. Furthermore, the specificity of synexin for acidic phospholipids could be related to the net positive charge (+6) of the hydrophobic exterior. Also implicit in the model is the possibility that the hydrophilic core could provide the conducting pathway for ions.

The synexin channel is highly selective for calcium, is voltage gated, and also responds to applied voltage by moving within the bilayer. With respect to ion selectivity, the model predicts that the net charge in the hydrophilic core is −7. Such a net charge would indeed be appropriate to attract cations and repel anions (41, 42). Consistently, the distribution of charge both in the core and in the hydrophobic exterior is asymmetric, thus providing a possible structural basis for voltage gating.

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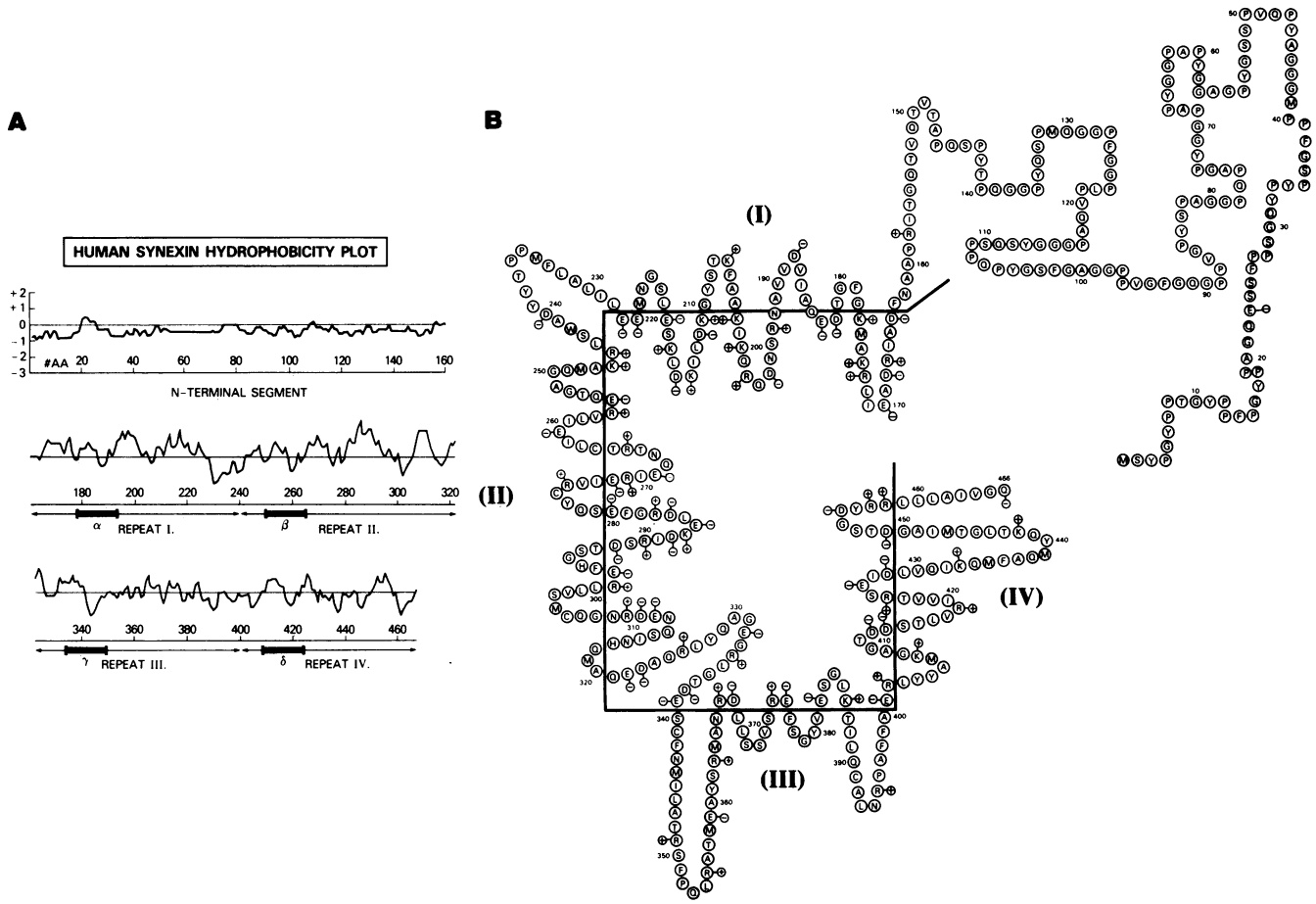

FIG. 4. (*A*) Hydrophobicity plot of human synexin. Lengthy hydrophobic domains (negative values using the algorithm of Hopp and Woods, ref. 39) are stippled. Hydrophilic domains (positive values) are left blank. The location of each major repeat is marked off by double-headed arrows. The respective highly conserved core repeats are marked by heavy black lines and are marked α, β, γ, and δ. The secondary structure was analyzed using the algorithm of Garnier *et al.* (40). The N-terminal segment is predicted to be entirely β-sheet or β-turn as described in the text. (*B*) Two-dimensional (face-on-view) model of the synexin calcium channel inserted into a membrane. The hydrophobicity plot in *A* serves as the basis of this model, in which the four major repeats (I, II, III, and IV) in the C-terminal region form the sides of a box and the highly hydrophobic N-terminal segment presumably protrudes into the membrane. Amino acid stretches of hydrophobic character in the repeat regions that were above the line in the hydrophobicity plot are now placed outside the lines of the box and likely interact with the hydrophobic elements of the membrane. Conversely, the hydrophilic amino acid areas were placed inside of the lines. The inside of the box has a net charge of −7 and is consistent with calcium ion specificity of the channel.

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