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A Synaptic Vesicle Protein with a Novel Cytoplasmic Domain and Four Transmembrane Regions

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Complementary DNA and genomic clones were isolated and sequenced corresponding to rat and human synaptophysin (p38), a major integral membrane protein of synaptic vesicles. The deduced amino acid sequences indicate an evolutionarily highly conserved protein that spans the membrane four times. Both amino and carboxyl termini face the cytoplasm, with the latter containing ten copies of a tyrosine-rich pentapeptide repeat. The structure of synaptophysin suggests that the protein may function as a channel in the synaptic vesicle membrane, with the carboxyl terminus serving as a binding site for cellular factors.

SYNAPTIC VESICLES PLAY A CRUCIAL role in neurotransmission. At the synapse, neurotransmitters are stored in synaptic vesicles and released by synaptic vesicle exocytosis. Although these functions must, to a large extent, be mediated by proteins in the synaptic vesicle membrane, the primary structure of none of these proteins has been characterized. Monoclonal antibodies have been used to identify an integral synaptic vesicle membrane protein named synaptophysin or p38 which constitutes 6 to 8 percent of the synaptic vesicle membrane protein but less than 0.1 percent of total brain protein (*1*). In addition to being a constituent of synaptic vesicles, synaptophysin is also found in a subset of brain coated vesicles, presumably as a result of vesicle recycling after exocytosis (*1, 2*). As one approach to the study of the molecular basis of neurotransmitter release, we undertook the molecular characterization of this protein using recombinant DNA and biochemical techniques and present here the primary structure of this integral synaptic vesicle membrane protein.

Synaptophysin was purified from rat brain cortex by monoclonal antibody affinity chromatography (*1*). Purified protein was proteolytically cleaved by trypsin and chymotrypsin, and peptides isolated by high-performance liquid chromatography were subjected to amino acid sequence analysis (*3*). A nonredundant oligonucleotide probe was designed from one of the three peptide sequences obtained [MATDPENIIKEMP (*4*), oligonucleotide sequence: ATGGCCACCGACCCCGAGAACATCATCAAGGAGATGCC] and was used to screen rat brain and human retina complementary DNA (cDNA) libraries (*5*). Of the 16 positive rat and 12 positive human clones isolated from the libraries, the majority proved to encode synaptophysin based on sequence analysis. RNA blots hybridized with probes derived from these clones revealed a single 3.4-kb species in rat brain (Fig. 1) (*6*).

Two overlapping rat clones and the largest human clone were selected for complete DNA sequence analysis on both strands (*7*). In all of these cDNAs an open reading frame extended to the very 5′ end, indicating that they did not contain the beginning of the coding sequence. Multiple attempts at isolating cDNA clones that extended more 5′ were unsuccessful. We therefore isolated rat genomic clones for synaptophysin from a rat genomic DNA library and used a novel approach to identify the 5′ end of the synaptophysin gene (*8*). The 5′ end of the synaptophysin messenger RNA (mRNA) from total rat brain RNA was sequenced directly by primer-extension in the presence of dideoxy nucleotides. An oligonucleotide designed from the sequence obtained was used to isolate the 5′ exon from the genomic clone. From our determination of the complete nucleotide sequence (*7*) we have deduced the protein sequence shown in Fig. 2.

A plot of the mean side-chain hydrophobicity of the protein sequence of synaptophysin shows four 23 or 24 amino acid stretches of high average hydrophobicity, suggesting the presence of four transmembrane regions that are separated by short hydrophilic sequences (Fig. 2). The four transmembrane regions exhibit a limited sequence homology to each other and may have arisen by gene duplication events. Synaptophysin is known to be *N*-glycosylated and disulfide linked (*1*). The only possible *N*-glycosylation site (Asn-X-Ser or Asn-X-Thr) in the amino acid sequence is found at residues 53 to 55, between the first and second transmembrane regions. Similarly, cysteines are only found between the first and second and between the third and fourth membrane-spanning regions. Therefore, the hydrophilic protein loops between the first and second and between the third and fourth transmembrane regions must extend into the intravesicular space, while the NH_2-terminal and COOH-terminal sequences of synaptophysin face the cytoplasm (Fig. 3). Since the NH_2-terminus is cytoplasmic, no signal sequence is observed. The nucleotide and amino acid sequences of synaptophysin are not significantly homologous to any reported sequence in the available data banks (*9*).

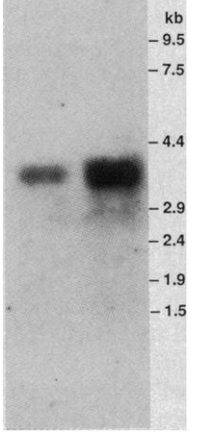

Fig. 1. RNA blot analysis of 5 μg and 20 μg of total RNA from rat brain hybridized with a single-stranded ^{32}P-labled DNA probe complementary to nucleotides 337 to 513 of the rat synaptophysin cDNA (*7*). Numbers on the right give the positions of RNA molecular size standards. The blot was exposed to film for 3 days at −70°C with an intensifying screen.

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The model of Fig. 2 predicts that synaptophysin has a cytoplasmic domain of 90 amino acids that contains ten copies of a pentapeptide repeat. These repeats can be aligned for maximum homology (Fig. 4). Nine of the ten repeats begin with a tyrosine. Their high glycine and proline content suggests that they form a rigid structure, which is not an α-helix. We have some experimental indications that this COOH-terminal domain forms the major immunogenic epitope of synatophysin which is recognized by several independently raised monoclonal antibodies. In agreement with the model in Fig. 2 this epitope faces the cytoplasm (*10*). Although glycine- and proline-rich sequences have also been found in other proteins (for example, synapsin I, collagen, and cytokeratins), none of these contain repeats punctuated by tyrosines. The size and unusual secondary structure of the cytoplasmic domain of synaptophysin raise the possibility that this domain may serve to link synaptophysin to extravesicular proteins during the lifetime of synaptic vesicles.

The composite synaptophysin sequences derived from the genomic and cDNA clones contain no in-frame stop codon 5′ to the proposed initiator methionine. Since the NH_2-terminus was blocked, no independent verification of the initiator methionine was achieved. However, the amino acid composition of synaptophysin calculated from the cDNA sequence and that determined experimentally agree very well, and the calculated and biochemically determined molecular weights are similar (33.4 kD and 34.0 kD, respectively) (*11*). Furthermore, the nucleotides surrounding the proposed initiator methionine exactly match the consensus sites for initiator codons (*12*). These findings suggest that the sequence is probably full length.

Comparison of the nucleotide and protein sequences derived from the human and rat synaptophysin clones reveals a high degree of conservation. The available amino acid sequences are 95% identical. The differences cluster in the intravesicular loops (90% identity), while the transmembrane regions and cytoplasmic portions are almost completely conserved (98% identity). All of the amino acid substitutions between the two sequences are conservative. Within the coding region, nucleotide sequence differences are mainly found in the third position of codons. The homology between the rat and human nucleotide sequences extends into the 3′ untranslated region. The cloned 3′ untranslated regions do not contain polyadenylate tails or polyadenylation consensus sequences, suggesting that the clones were derived by internal priming. Since RNA blots indicate a mRNA size of 3.4 kb for synaptophysin in rat brain (Fig. 1), a long 3′ untranslated region is probable. Nevertheless, the determined 3′ untranslated regions of the rat and human complementary clones are very homologous, including a 61-nucleotide stretch of identical sequence that contains an AT-rich sequence similar to those implicated in regulating mRNA stability (*7, 13*).

From the primary sequence of synaptophysin, a plausible model of the protein emerges (Fig. 2). Each synaptophysin subunit has four membrane-spanning regions with a rigid COOH-terminal domain extending into the cytoplasm. Previous biochemical studies with cross-linking reagents, sucrose-gradient centrifugation, and nonreducing gel electrophoresis indicate that several synaptophysin subunits are linked to each other by disulfide bonds or noncovalent forces (or both) to form a trimer or a

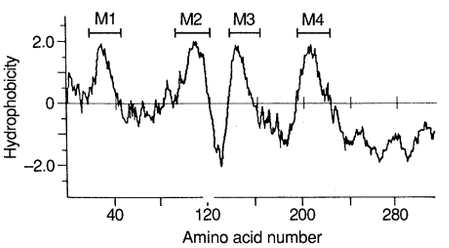

Fig. 3. Hydrophobicity plot of rat synaptophysin. The mean residue hydrophobicity was calculated by a computer program with the use of the algorithm of Kyte and Doolittle (*15*) with a 19–amino acid window and is displayed as a function of residue number. The positions of the transmembrane regions are drawn as horizontal lines labeled M1 to M4.

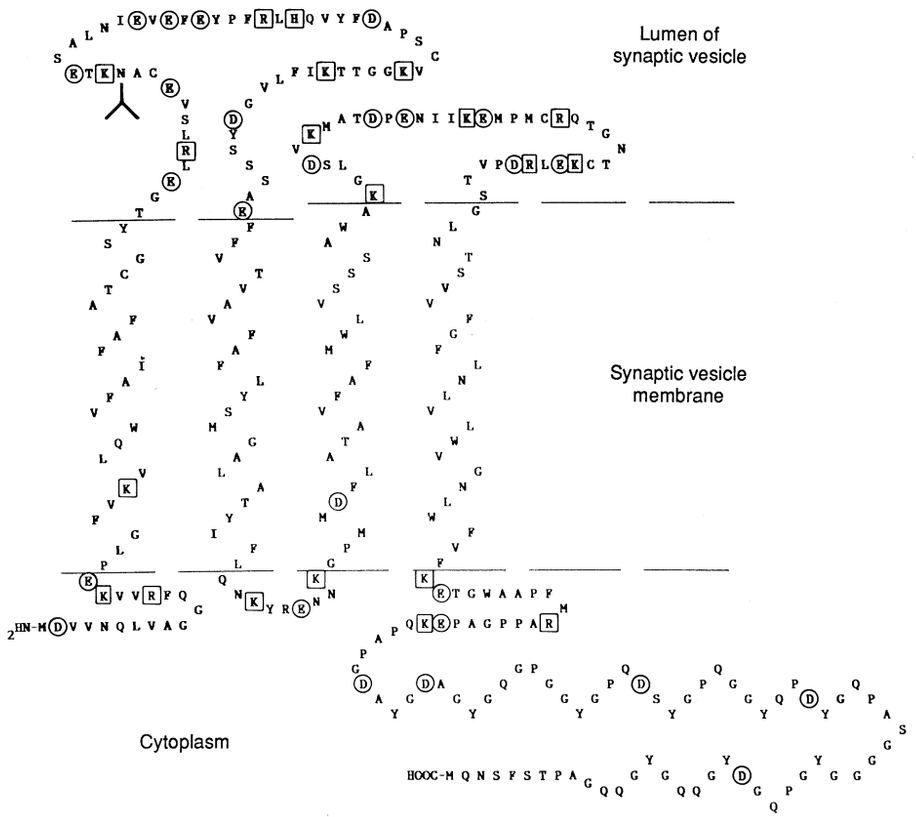

Fig. 2. Model of the transmembrane structure of synaptophysin. Each amino acid is shown in single-letter code (*4*), with positively charged amino acids boxed and negatively charged amino acids circled. The position of the single *N*-linked glycosylation site is indicated by a branched line.

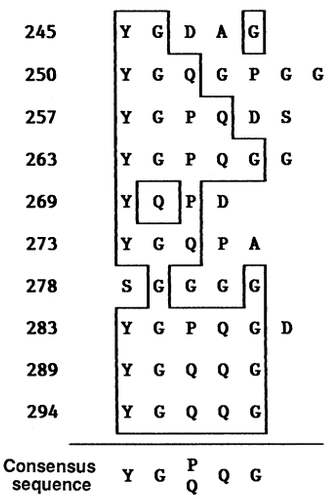

Fig. 4. Repeated sequence in the cytoplasmic domain of rat synaptophysin. The sequence repeats are aligned for maximum homology with the residue numbers given on the left.

tetramer. This association would result in the presence of 12 to 16 transmembrane regions per synaptophysin homomultimer, a structure very suggestive of a membrane channel. Remarkably, all of the transmembrane regions of synaptophysin are very hydrophobic, with only two containing single charged amino acids. This charge distribution decreases the possibility that synaptophysin is a channel for ions. The hydrophobicity plot and the proposed subunit structure of synaptophysin are very similar to those of the gap junction proteins (*14*).

In summary, the primary structure of synaptophysin suggests that the protein may form a synaptic vesicle–specific membrane channel, with a cytoplasmic domain instrumental in the interactions of synaptic vesicles with cytoplasmic factors.

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3. Since the NH_2-terminus of synaptophysin was blocked, tryptic and chymotryptic peptides, obtained from digests of 100 μg of synaptophysin, were separated by high-performance reversed-phase chromatography on a Vydac 219 TP 300 A phenyl column with a 0 to 70 percent (v/v) acetonitrile gradient in 0.1 percent (v/v) trifluoroacetic acid. One chymotryptic peptide [P1, NIEVEFEYPFR (*4*)] and two tryptic peptides [P2, MATDPENIIKEMP, and P3, APPGAPEKQPAPGD (*4*)] were analyzed by Edman degradation, and the phenylthiohydantoin derivatives were characterized by isocratic high-performance liquid chromatography [F. Lottspeich, *J. Chromatogr.* **326**, 321 (1985)].
4. In the one-letter amino acid code, the letters translate into the following amino acids: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; and Y, tyrosine.
5. The oligonucleotide was end-labeled (*16*) and used to screen 2×10^6 plaques of a rat brain cDNA library (RL1002, Clontech Lab. Inc.) and 1.5×10^6 plaques of a human retina cDNA library [J. Nathans, C. Thomas, D. S. Hogness, *Science* **232**, 193 (1986)] by standard procedures. After plaque purification, we obtained positive clones and subcloned them into pBR322, pGem 4-1 and M13 mp18 and mp19 vectors, and we analyzed them by the dideoxy sequencing method (*7*). All sequences were determined on both strands.
6. RNA blot analysis was performed (*17*) with single-stranded uniformly labeled DNA probes and with total RNA purified from brains obtained from adult male Wistar rats. The RNA size was determined by comparison with ribosomal RNA standards (Pharmacia) and RNA ladder standards (BRL).
7. T. C. Südhof, F. Lottspeich, P. Greengard, E. Mehl, R. Jahn, *Nucleic Acids Res.*, in press; this report contains the nucleotide and amino acid sequences of rat and human synaptophysin (the sequence will be supplied on request prior to publication).
8. Genomic Southern blots of rat DNA indicated the presence of a single synaptophysin gene. Plaques (2×10^5) of a rat genomic partial Eco RI library (RL1005a Clontech) were screened (*17*) with single-stranded ^{32}P-labeled probes corresponding to nucleotides 116 to 232 of the rat synaptophysin cDNA (*7*). Three identical hybridization-positive clones were isolated. A 3.8-kb Eco RI insert fragment from these was subcloned and analyzed by restriction enzyme mapping and sequencing. This analysis revealed an intron directly 5′ to the beginning of our cDNA clones. To localize the upstream exon, total RNA from rat brain was sequenced directly with an end-labeled oligonucleotide primer complementary to nucleotides 79 to 99 of the cDNA (*7*). RNA sequencing reactions were performed under the conditions of primer extension reactions (*16*), except that the deoxynucleoside triphosphates in the extension solution were exchanged for the appropriate dideoxy mixes. The reaction products were analyzed on sequencing gels, and the sequence was used to design an oligonucleotide to identify the exon in the genomic clone. The sequence of this exon confirmed and extended the RNA sequence. Primer extension analysis suggested a transcription start site 49 bases 5′ to the beginning of our clones. However, S1 nuclease mapping (*17*) with total rat brain RNA and end-labeled single-stranded probes, showed that the 5′ end of the primer extension product artifactually lies in the middle of a contiguous exon and does not represent the transcription start site.
9. Nucleotide sequence data banks (GenBank release 46; EMBL release 9.0) and protein sequence data banks (PSEQIP and NBRF, release 11) were searched for homologous sequences with standard programs. The cytoplasmic domain of synaptophysin was homologous to most glycine- or proline-rich proteins in the data banks simply because of amino acid frequencies. In addition, limited sequence homology was observed between the synaptophysin transmembrane regions and transmembrane regions of cytochrome c from several species. The biological significance of the latter observation remains to be established.
10. The characterized monoclonal antibodies to synaptophysin recognize a cytoplasmic epitope that is collagenase-sensitive, implicating the cytoplasmic tail with its high content of Gly-Pro bonds.
11. The molecular size of rat synaptophysin calculated from the deduced protein sequence is 33,404 daltons, while the experimentally determined value for the monomer of deglycosylated synaptophysin is 34,000 daltons (*1*). The amino acid composition of purified rat synaptophysin was analyzed after vapor-phase hydrolysis at 145°C for 1 hour in 6*N* HCl, 0.1 percent phenol by either the ninhydrin method (method A) or precolumn derivatization with *o*-phthalaldehyde (method B). The following values (molar percent) were obtained (amino acid [method A, method B; nucleotide sequence]): Asx [9.8, 9.4; 7.8]; Glx [9.3, 12.2; 10.5]; Ser [5.5, 6.5; 5.9]; Gly [13.1, 15.0; 12.4]; Thr [4.6, 5.4; 4.9]; His [0.6, 0.9; 0.3]; Ala [10.6, 10.3; 8.8]; Arg [3.1, 3.5; 2.3]; Tyr [5.6, 5.1; 5.2]; Val [7.5, 8.7; 8.1]; Met [1.6, 3.4; 3.3]; Ile [2.9, 2.6; 2.0]; Phe [7.4, 7.6; 7.8]; Leu [8.9, 9.3; 6.5]; Lys [4.3, 5.5; 4.2]; Pro [5.4, -; 6.5].
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Lymphotoxin Is an Important T Cell–Derived Growth Factor for Human B Cells

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Two different assays for B cell growth factors (BCGF) and an antibody against lymphotoxin were used to show that the presence of lymphotoxin in conditioned media derived from normal activated T cells and in a partially purified BCGF accounts for a substantial portion of their B cell growth-promoting activity. A competitive binding assay confirmed the presence of significant amounts of lymphotoxin in the partially purified BCGF. Recombinant lymphotoxin enhanced the proliferation of activated B cells and augmented B cell proliferation and immunoglobulin secretion induced by interleukin-2.

THE ROLE OF VARIOUS SOLUBLE FACtors in the regulation of human B lymphocyte proliferation is somewhat controversial. When activated in vitro with the polyclonal B cell mitogen *Staphylococcus aureus* Cowan I (SAC), human B lymphocytes undergo an initial round of proliferation (*1*); however, this proliferation is short-lived unless exogenous growth factors are added to the cultures. On the basis of this observation an assay was developed for the detection of human B cell growth factors (BCGF) (*2*). Nevertheless the nature of many of the factors that enhance B cell proliferation in this assay remain uncharacterized. Interleukin-2 (IL-2) is one of the growth factors contained in the supernatants conditioned by phytohemagglutinin (PHA)-activated T cells. Although IL-2 enhances the proliferation of SAC-activated B cells (*3*), an antibody to the IL-2 receptor inhibited only 20% to 30% of the BCGF activity present in a T cell supernatant conditioned by PHA-activated T cells (*4*). Recently, we showed that the monocyte product tumor necrosis factor-α (TNF-α), has

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