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# Synaptophysin: molecular organization and mRNA expression as determined from cloned cDNA

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**Synaptophysin is a major glycoprotein of $M_r$ ~38 000 (in deglycosylated form: $M_r$ ~34 000) characteristic of a certain class of small (30–80 nm diameter) neurosecretory vesicles, including presynaptic vesicles, but also vesicles of various neuroendocrine cells of both neuronal and epithelial phenotype. Using synaptophysin-specific antibodies we have isolated cDNA clones from rat nervous tissue libraries, which identify an ~2.5-kb mRNA in rat and human cells, including neuroendocrine tumours, that contains a reading frame for a polypeptide of 307 amino acids with a total mol. wt of 33 312. The deduced amino acid sequence, which was partly confirmed by comparison with sequences of two tryptic peptides obtained from purified synaptophysin, revealed four hydrophobic regions of 24 amino acids each, which are characterized, according to conformation prediction analyses, by marked α-helicity. The sequence shows a single potential *N*-glycosylation site, which is assigned to the vesicle interior, and a carboxy-terminal tail of 89 amino acids which contains glycine-rich tetrapeptide repeats, the epitope of monoclonal antibody SY38, and a number of collagenase-sensitive sites accessible on the surface of the intact vesicles. These features suggest that the polypeptide spans the vesicle membrane four times, with both N and C termini located on the outer, i.e. cytoplasmic, surface of the vesicles.**

*Key words:* synaptophysin/neurotransmitter vesicles/neuroendocrine cells/membrane proteins/tumour diagnosis

## Introduction

Synaptophysin is a major integral membrane glycoprotein characteristic of a certain type of small (30–80 nm) neuroendocrine (NE) vesicles with an electron microscopically translucent content, originally isolated from — and identified in — presynaptic vesicles of neurons by the use of monoclonal antibodies (Jahn *et al.*, 1985; Wiedenmann and Franke, 1985). On SDS–PAGE the unfolded polypeptide chain appeared with $M_r$ values ranging from 38 000 to 40 000, depending on the cell type (Jahn *et al.*, 1985; Wiedenmann and Franke, 1985; Rehm *et al.*, 1986; Wiedenmann *et al.*, 1986), and it has been suggested that some of this variation may be due to different extents of glycosylation. On chemical deglycosylation and inhibition of glycosylation the $M_r$ value is significantly reduced to ~34 000 (Navone *et al.*, 1986; Rehm *et al.*, 1986). Cell biological and biochemical analyses have shown that synaptophysin can be readily extracted and solubilized with detergents whereupon it behaves as an oligomeric, rather hydrophobic molecule (Stokes radius: 7.3 nm) which can be cross-linked via disulphide bridges, and contains binding sites for $Ca^{2+}$ (Wiedenmann and Franke, 1985; Rehm *et al.*, 1986). The binding sites for both monoclonal antibody SY38 and $Ca^{2+}$ have been assigned to a protein domain exposed on the outer, i.e. cytoplasmic, surface. Furthermore, synaptophysin has been identified in a wide variety of normal (Wiedenmann and Franke, 1985; Navone *et al.*, 1986) and neoplastic (Franke *et al.*, 1986; Wiedenmann *et al.*, 1986, 1987a,b; Gould *et al.*, 1987) cells and tissues and is used as a broad-range diagnostic marker for NE tumours of both neuronal and epithelial origin.

Because of the potential importance of synaptophysin in the regulation of neurotransmitter storage and/or release as well as in cell typing and diagnosis, molecular information on this protein is desired. In this study, we report on the identification of cDNA clones specific for synaptophysin and show that they detect mRNAs encoding this protein in rat and human cells, including NE tumour cells. From the deduced amino acid sequence we predict that the synaptophysin molecule spans the vesicle membrane four times and possesses a cytoplasmic tail region of 89 amino acids characterized by a certain type of tetrapeptide repeats.

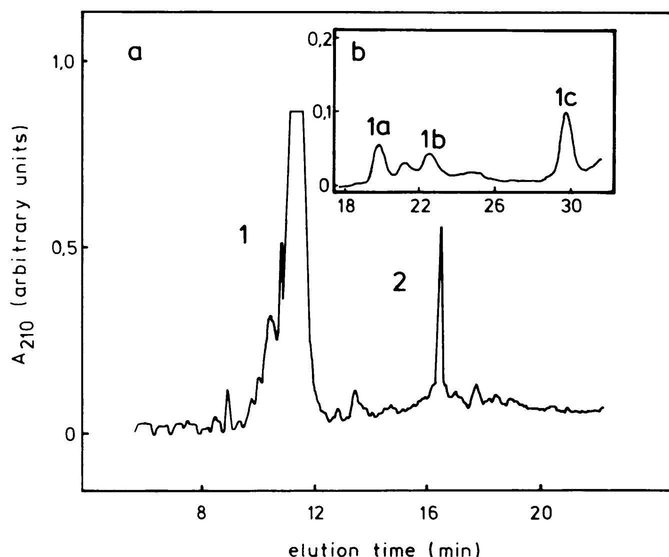


**Fig. 1.** H.p.l.c. of tryptic peptides of synaptophysin. (**a**) Peptides eluted from the Nucleosil C18 RP column using a 10–90% acetonitrile gradient as described in detail in Materials and methods. (**b**) Subsequent separation of peak 1 under the same conditions, but using an acetonitrile gradient from 17–22%. Only the relevant portion of the elution profile is shown. Upon gas-phase microsequencing, the same N-terminal sequence APPGAPEQKPAPGDAYGDAGYGQGP was found for peptides 1a, 1b and 1c, whereas the sequence LSVECAIKTESALNIEVEFE was obtained for peptide 2 (the underlined isoleucine differs from the amino acid sequence deduced from cDNA).

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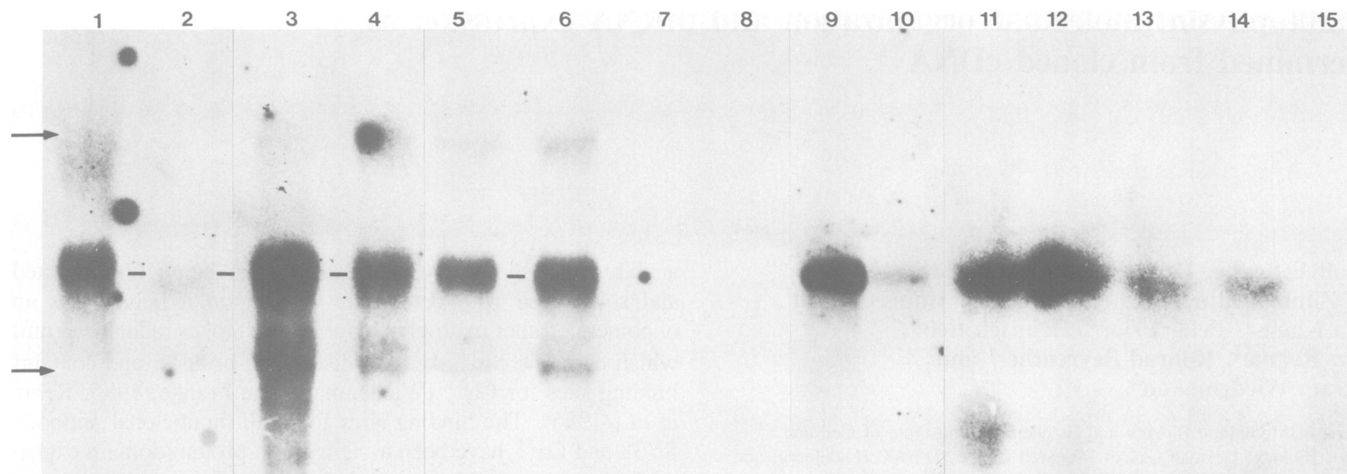


**Fig. 2.** Expression of synaptophysin in normal and neoplastic neuro-endocrine tissues and cell cultures from rat and man as shown by autoradiographs of Northern blot hybridization with clone pSR[1]. Ten micrograms total RNA (**lanes 1–9**), 20 μg total RNA (**lanes 11–15**) or 2 μg poly(A)$^+$-RNA (**lane 10**) were separated on formaldehyde/agarose gels. RNA was extracted from the spinal cord of the newborn and adult rat (**lanes 1** and **2**), rat cerebrum (**lane 3**), rat cerebellum (**lane 4**), the rat pheochromocytoma cell line PC12 grown in the absence or presence of nerve growth factor (**lanes 5** and **6** respectively), rat kidney (**lane 7**), rat liver (**lane 8**), total rat brain (**lane 9**), a human pheochromocytoma (**lane 10**), human cultured cells derived from small cell carcinomas of the lung (NCI-H69, **lane 11**; NCI-H82, **lane 12**; SCLC-22H, **lane 13**; and SCLC-16H, **lane 14**) and from a cell line established from an adenocarcinoma of the lung (ADLC 5M2, **lane 15**). Arrows denote the position of the 28S and 18S RNA. Bars demarcate the position of synaptophysin mRNA (~2.5 kb). Note that pSR[1] does not hybridize with mRNA from rat liver or kidney or human adenocarcinoma cell line ADLC-5M2.

## Results

### *Partial amino acid sequences of tryptic fragments of synaptophysin*

Synaptophysin, isolated according to Rehm *et al.* (1986) was further purified by preparative SDS–PAGE. The protein band was excised from the gel, and the protein was eluted, lyophilized and subjected to tryptic digestion. As shown in Figure 1 four tryptic fragments were isolated by h.p.l.c. Amino acid sequence analysis showed that three out of these four fragments contained identical sequences (see below) whereas the fourth was different. The native N-terminal amino acid was found to be blocked.

### *Isolation of cDNA clones*

A λgt11 expression library constructed from poly(A)$^+$-RNA of rat hypothalamus (Lechan *et al.*, 1986) was screened with monoclonal murine antibody SY38 and rabbit polyclonal antibodies (pSY38) specific for synaptophysin. Phages ($3 \times 10^5$) were plated. One clone, λSR[1], positive with both probes, was purified, and the ~1-kb insert was subcloned into M13 mp18 and pUC8 before sequencing (pSR[1]). The established nucleotide sequence of this clone contains an uninterrupted reading frame of 150 amino acids terminating with a methionine. Comparison of this partial sequence with the amino acid sequence information available from the tryptic peptides showed that the entire sequence of peptide 1 (Figure 1) is located within this reading frame (see also Figure 3). Therefore, this clone encodes the carboxy terminus of synaptophysin, confirming the observation that SY38 is specific for the cytoplasmic carboxy-terminal portion of the protein (see also Wiedenmann and Franke, 1985).

Of a λgt10 library prepared from poly(A)$^+$-RNA of rat spinal cord (Grenningloh *et al.*, 1987) $1.2 \times 10^6$ p.f.u. were subsequently screened with several nucleotide probes: (i) an *in vitro* transcribed riboprobe of *Sal*I-truncated 'bluescribe' vector containing the *Eco*RI-limited insert of λSR[1]; (ii) the gel-purified insert of pSR[1] labelled with [$\alpha$-$^{32}$P]dATP using random oligonucleotide primers in a Klenow reaction; and (iii) a synthetic 54-mer oligonucleotide labelled in a polynucleotide kinase reaction. This oligonucleotide sequence was derived from the amino acid sequence of peptide 2 considering codon usage and nucleotide frequency (Lathe, 1983). Later, the sequence GTG GAG TGT GCC ATC AAG ACA GAG AGT GCC CTG AAC ATC GAG GTG GAG TTT GAG turned out to be 87% identical to the corresponding cDNA (cf. Figure 3). Clones positive with all three hybridization probes were purified. One clone (λSR[2]) extended further into the 5′ direction and contained the sequence coding for peptide 2. A putative N-terminal methionine was located close to the cloning site of the phage insert. Another clone (λSR[3]) obtained from this screening extended the sequence in the 3′ direction. Sequencing of these isolated clones allowed the establishment of an uninterrupted reading frame consistent with all available information on synaptophsyin (Figure 3).

### *Expression of synaptophysin*

Clone pSR[1] was used to study the expression of synaptophysin in various tissues, tumours and cell lines by Northern blot hybridization (Figure 2). The radioactively labelled insert of pSR[1] reacted specifically with a mRNA of ~2.5 kb length in rat as well as in man (Figure 2, lanes 9 and 10). In accordance with previous biochemical and immunocytochemical data, neuronal tissues such as the cerebrum, cerebellum and spinal cord were found to contain synaptophysin mRNA wheras no hybridization signal was obtained in kidney and liver (Figure 2, lanes 1–4, 7–9). Interestingly, the level of synaptophysin mRNA in spinal cord appeared to be dramatically reduced in the adult rat as compared with newborn animals. A weak signal was also obtained with adrenal gland (data not shown), confirming its neuronal differentiation. PC12 cells, the protoytpe of a NE cell line (Greene and Tischler, 1976) producing synaptophysin-containing vesicles (Franke *et al.*, 1986), were also strongly positive with the synaptophysin cDNA probe. Somewhat unexpectedly, however, the level of synaptophysin mRNA in PC12 cells was not significantly increased after stimulation of the cells with nerve growth factor (Figure 2, lanes 5 and 6).

The rat clone pSR[1] also proved useful for studies of the expression of synaptophysin mRNA in cells and tissues. Typical NE tumours such as pheochromocytomas were clearly positive

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   1 GCAGCAGCAATGGACGTGGTGAATCAGCTGGTGGCTGGGGGTCAGTTCCGGGTGGTCAAGGAGCCCCTTGGCTTCGTGAAGGTGCTGCAG
   1             M  D  V  V  N  Q  L  V  A  G  G  Q  F  R  V  V  K  E  P  L  G  F  V  K  V  L  Q

  91 TGGGTCTTTGCCATCTTCGCCTTTGCTACGTGTGGCAGCTACACCGGGGAGCTTCGGCTGAGCGTGGAGTGTGCCAACAAGACGGAGAGT
  28  W  V  F  A  I  F  A  F  A  T  C  G  S  Y  T  G  E  L  R  L  S  V  E  C  A  N  K  T  E  S

 181 GCCCTCAACATCGAAGTTGAATTCGAGTACCCCTTCAGGCTGCACCAAGTGTACTTTGATGCACCCTCCTGCGTCAAAGGGGGCACTACC
  58  A  L  N  I  E  V  E  F  E  Y  P  F  R  L  H  Q  V  Y  F  D  A  P  S  C  V  K  G  G  T  T

 271 AAGATCTTCCTGGTTGGGGACTACTCCTCGTCGGCTGAATTCTTTGTCACCGTGGCTGTGTTTGCCTTCCTCTACTCCATGGGGGCCCTG
  88  K  I  F  L  V  G  D  Y  S  S  S  A  E  F  F  V  T  V  A  V  F  A  F  L  Y  S  M  G  A  L

 361 GCCACCTACATCTTCCTGCAGAACAAGTACCGAGAGAACAACAAAGGGCCTATGATGGACTTTCTGGCTACAGCCGTGTTCGCTTTCATG
 118  A  T  Y  I  F  L  Q  N  K  Y  R  E  N  N  K  G  P  M  M  D  F  L  A  T  A  V  F  A  F  M

 451 TGGCTAGTTAGTTCATCAGCCTGGGCCAAAGGCCTGTCCGATGTGAAGATGGCCACGGACCCAGAGAACATTATCAAGGAGATGCCCATG
 148  W  L  V  S  S  S  A  W  A  K  G  L  S  D  V  K  M  A  T  D  P  E  N  I  I  K  E  M  P  M

 541 TGCCGCCAGACAGGGAACACATGCAAGGAACTGAGGGACCCTGTGACTTCAGGACTCAACACCTCAGTGGTGTTTGGCTTCCTGAACCTG
 178  C  R  Q  T  G  N  T  C  K  E  L  R  D  P  V  T  S  G  L  N  T  S  V  V  F  G  F  L  N  L

 631 GTGCTCTGGGTTGGCAACTTATGGTTCGTGTTCAAGGAGACAGGCTGGGCAGCCCCATTCATGCGCGCACCTCCAGGCGCCCCGGAAAAG
 208  V  L  W  V  G  N  L  W  F  V  F  K  E  T  G  W  A  A  P  F  M  R  A  P  P  G  A  P  E  K

 721 CAACCAGCACCTGGCGATGCCTACGGCGATGCGGGCTACGGGCAGGGCCCCGGAGGCTATGGGCCCCAAGACTCCTACGGGCCTCAGGGT
 238  Q  P  A  P  G  D  A  Y  G  D  A  G  Y  G  Q  G  P  G  G  Y  G  P  Q  D  S  Y  G  P  Q  G

 811 GGTTATCAACCCGATTACGGGCAGCCAGCCAGCGGTGGCGGTGGCTACGGGCCTCAGGGCGACTATGGGCAGCAAGGCTATGGCCAACAG
 268  G  Y  Q  P  D  Y  G  Q  P  A  S  G  G  G  G  Y  G  P  Q  G  D  Y  G  Q  Q  G  Y  G  Q  Q

 901 GGTGCGCCCACCTCCTTCTCCAATCAGATGTAATCTGGTCAGTGAAGTTCATGAAGATCCCACGGGTGGGCAAGAGCTCAAGAGAAGGCC
 298  G  A  P  T  S  F  S  N  Q  M  *

 991 TGCCCCCCTTTTCCCATCCCCATATCCTAGGCCTCCACCCCTCAACCCAGGAGACCCTAACTGTCTTTGCTGTTTATATATATATATATT

1081 ATATATAAATATCTATTTATCTGTCTGAGCCCTACATTCACCCACTTCTCCATGCACTAGAGGCCCAGTCCTGAATGGGCTCCTCCCCAA

1171 CCCTGACCTTGCATTCCTCAGCCCCTATCTGTTCCCCAGCCCTGTCCCTTGAGGTAAGGGGCTCTAGAAAGGGGACAGGAAGGGAACCAG

1261 ACCTTGGCTGCATGGAGTGGGTTGGTGTGACTTTCTCTCCTTCCTCCTCTCCCTCTGCCCCTCCTAACTCTGGCCTTGGTCCTCCAGCAT

1351 CACCTGAACTTCAGAAGCTCTCGAATGGAAATCTGACCCCAAGAGTAGAGCAGTAGACTGAGTGGAGGAGGCTTGGGTGAAACGGGCAGA

1441 GAGGAGATAACCTCTGTAGAGAGAGGACTAGTCAGCCAAGAGTTGAATTCCAGACATACTGGATGTGCAGTCTAAAAGGAAAGTGGTATC

1531 CTACCGCATTCTGCAATGGGGCTTTAAGTGACCAGAGAGAGTGGTTCTAGGAGGGGTGTGGCTTAAACACAGCGGGCTCCAGAGTGGGCA

1621 GGTTTGGGTTGGATCCAGCATCTCTAGAAGGGGTGTGTCTTGGAACATTCAGGAGTTTGAGCTTGATTCCAAAGAGCTTGAGCAAGGGTA

1711 GAAGTGGGGTTCAGGGATGATCTAAGCTTGAGATGAGGTCCTGCTTAGGATTCCAGACAAGCATATGAAGAGATGTGTGGTGGTCTCAGA

1801 AGAGGAAACCCATTCTGTAATGGGGCTGAGAAGAAAACACGTTTTCAATTGAGTGGAGTTTGAGAGGGTGCAGGAGGCCATGGTTAGGAG

1891 CTATGAGGTGAGTTTTGCTAAAAGCAGGAGGGCGTACTCC...
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**Fig. 3.** Nucleotide sequence combined from cDNAs encoding synaptophysin and the derived amino acid sequence. The sequence was established from overlapping fragments of clones pSR[1], pSR[2] and pSR[3] (see Results). The sequences of the tryptic fragments peptide 1 and peptide 2 (see Figure 1) are underlined. Clone pSR[1] contains nucleotides 477–1490, pSR[2] nucleotides 1–965 and pSR[3] nucleotides 33–1930. Clone pSR[1] has been sequenced with both the Maxam and Gilbert (1980) technique and the procedure of Sanger *et al.* (1977), whereas the two other clones were sequenced in both strands but only with the Maxam and Gilbert techniques.

with this probe (Figure 2, lane 10). Moreover, various carcinomas of NE differentiation also contained this mRNA, including small cell carcinomas of the lung (data not shown). Most remarkably, several types of human small cell carcinoma-derived cell lines could be characterized by the presence of synaptophysin mRNA. The 'classic' small cell lung carcinoma cell lines NCI-H69 and SCLC-22H, which express cytokeratins, as well as the 'variant' small cell carcinoma cell lines NCI-H82 and SCLC-16H, which lack cytokeratins as inferred from immunostaining, gel electrophoresis and Northern blotting (R.E.Leube *et al.*, in preparation; Broers *et al.*, 1985, 1986), all contained a mRNA of 2.5 kb hybridizing with pSR[1] (Figure 2, lanes 11–14). In these cases, the presence of the protein was confirmed by immunoblotting and immunocytochemistry (data not shown). Several kinds of non-NE tumour cells were negative, such as an adenocarcinoma-derived cell line of the lung (Figure 2, lane 15) or a urothelial carcinoma-derived cell line (not shown).

### *cDNA nucleotide and deduced amino acid sequence of synaptophysin*

The 1930 nucleotides of cloned cDNA present an open reading frame which starts at the first AUG. The features of the AUG-surrounding sequence are in reasonable agreement with the consensus initiation site patterns proposed by Kozak (1986). The en-

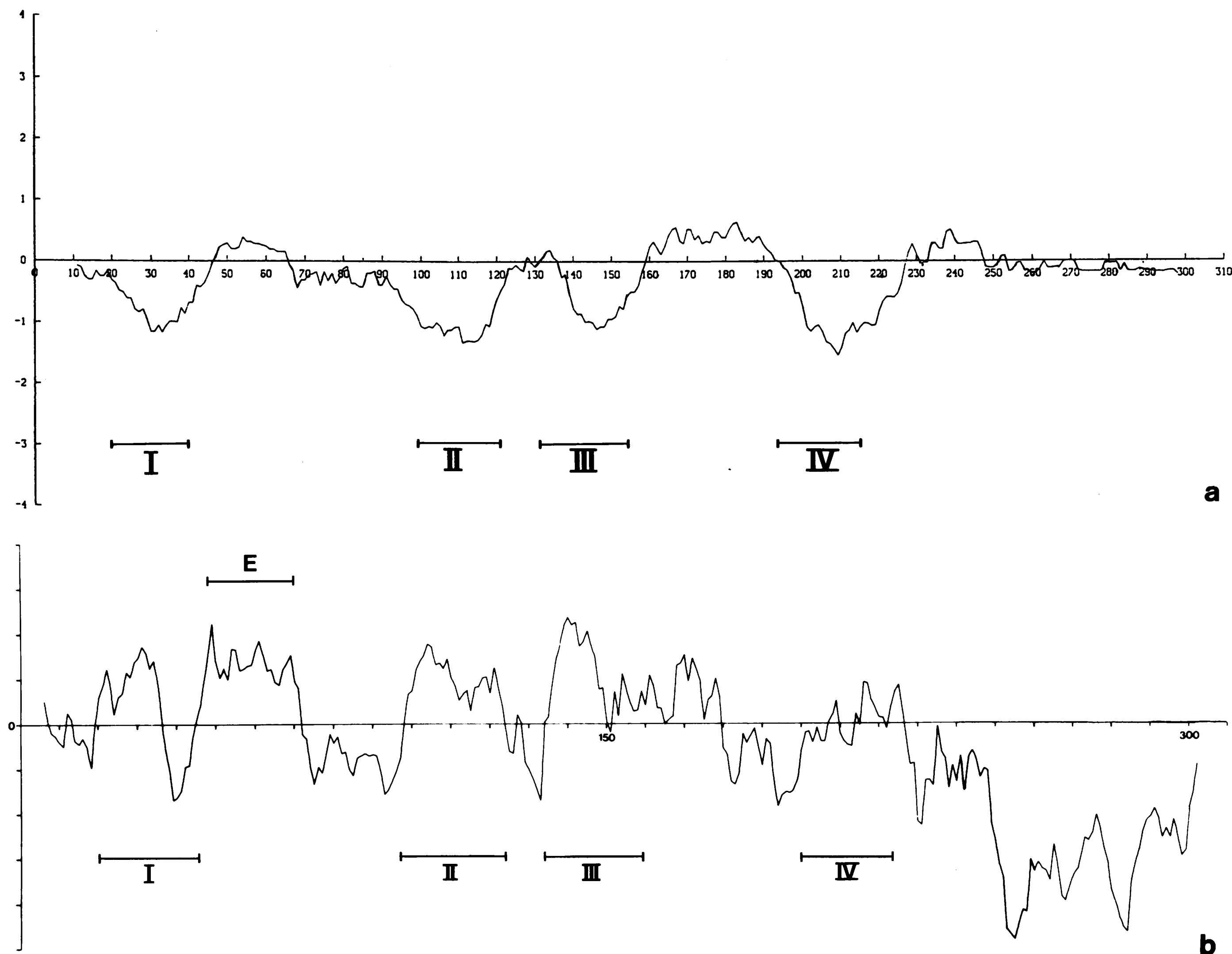


**Fig. 4.** Hydrophilicity plot and conformational predictions for synaptophysin. (**a**) Hydrophilicity plot according to Hopp and Woods (1981) (window size: 20 residues) revealing four major hydrophobic domains of 24–25 amino acids (I–IV) proposed to span the vesicle membrane. (**b**) Conformation prediction according to Garnier *et al.* (1978), showing only the probability of α-helix formation. The four putative membrane-spanning domains, characterized by their high hydrophobicity, are most likely present as α-helices (a window of five residues is shown here). The domain designated E, probably also α-helical, is located on the inner vesicle surface, equivalent to the external surface of the plasma membrane and contains the unique glycosylation site.

tire polypeptide encoded by this reading frame, including the initial methionine which may be lost post-translationally, contains 307 amino acids, corresponding to a total mol. wt of 33 312. This corresponds well with the $M_r$ value of 34 000 determined for non-glycosylated synaptophysin by SDS–PAGE (Navone *et al.*, 1986; Rehm *et al.*, 1986). The open reading frame is confirmed by comparison with the amino acid sequence of the two tryptic peptides representing residues 47–66 and 230–254 (Figure 3). Although the initiation at the AUG selected here has not been confirmed experimentally all available evidence is compatible with this interpretation. The synaptophysin mRNA also contains an exceedingly long 3′-non-coding portion, which is only partially represented by the clones shown in Figure 3 and contains a number of interesting features to be discussed elsewhere.

The hydrophilicity profile (Figure 4a) and secondary structure prediction (Figure 4b) analyses according to Garnier *et al.* (1978; Figure 4b) and Chou and Fasman (1978) revealed a number of characteristic domains within the synaptophysin molecule. Although we do not know the N-terminal amino acid of the protein, the absence of typical sequence features of signal peptides and their preferred cleavage sites (e.g. von Heijne, 1983; Perlman and Halvorson, 1983) suggests that the polypeptide is not synthesized as a cleavable precursor. Most conspicuously, the sequence exhibits four major hydrophobic domains, designated I–IV (Figures 4 and 5) of 24 residues each, which are flanked by clusters of charged amino acids. Three of these domains (I–III) correspond to regions with pronounced α-helical character (Figure 4b). The fourth hydrophobic domain (IV) may also be organized in an α-helix, although in this case regions with extended conformation have also been indicated in analyses according to Garnier *et al.* (1978). A fifth region predicted to be α-helical is designated E and corresponds to a hydrophilic domain (Figure 4b). These features are presented in Figure 5 in context with the amino acid sequence. We suggest that the N-terminal portion of 19 amino acids is located on the cytoplasmic side and that the domains I–IV are membrane-spanning α-helices with a length typical for membrane-traversing domains of established transmembrane proteins (see below). Two of these hydrophobic domains contain a single charged amino acid, i.e. a lysine in I and an aspartic acid in III, a feature also found in

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        M D V V N Q L V A G G Q
 13  F R V V K E P L G F V K V L Q W V F A I F A F A T C G S Y T G E L R L S V E C A N K T E S A L N I E V E F E Y P F R L H
 73  Q V Y F D A P S C V K G G T T K I F L V G D Y S S S A E F F V T V A V F A F L Y S M G A L A T Y I F L Q N K Y R E N N K
133  G P M M D F L A T A V F A F M W L V S S S A W A K G L S D V K M A T D P E N I I K E M P M C R Q T G N T C K E L R D P V
193  T S G L N T S V V F G F L N L V L W V G N L W F V F K E T G W A A P F M R A P P G A P E K Q P A P G D A Y G D A G Y G Q
253  G P G G Y G P Q D S Y G P Q G G Y Q P D Y G Q P A S G G G G Y G P Q G D Y G Q Q G Y G Q Q G A P T S F S N Q M
```

I II III IV

**Fig. 5.** Putative amino acid sequence and molecular organization of synaptophysin (one-letter code). The proposed four membrane-spanning domains (I–IV) are underlined. The N-glycosylation site is indicated by an arrow. Possible collagenase sensitive sites (GP) are overlined. Repetitive tetrapeptide motifs in the carboxy-terminal part are indicated by brackets.

certain transmembrane domains of other proteins (see Discussion).

The hydrophilic domain E contains the only possible N-glycosylation site (Figure 5) and is therefore assigned to the vesicle interior. The hydrophilic region between domains II and III is very short (seven amino acids) and positively charged and allows for only a short loop on the cytoplasmic side, i.e. the outer vesicle surface. In contrast, the hydrophilic domain located between III and IV is more extended, rich in charged amino acids and tentatively assigned to the internal surface of the vesicle.

The carboxy-terminal portion of 89 amino acids differs from the rest of the molecule in several respects (Figure 5). It is non-$\alpha$-helical and unusually rich in glycine (27%), proline (15%) and glutamine (14%). It contains nine repeats of a basic tetrapeptide GYGP which are either complete or modulated: G(A,S,D) YG(Q) P(D,Q). It also presents several cleavage sites for collagenase (Q,Y)GP and four successive collagen-type motifs with glycine as every third residue. This region also contains the epitope for monoclonal antibody SY38. Indeed, on treatment of frozen and freshly isolated intact synaptic vesicles with collagenase (Figure 6), the synaptophysin polypeptide was trimmed to two distinct fragments, one of $M_r$ ~33 000 which still contained the SY38 epitope (Figure 6) and a smaller one of $M_r$ ~28 000 which did not bind SY38 but contained epitopes recognized by synaptophysin antibodies present in a rabbit antiserum (Figure 6b). This suggests that the SY38 epitope is associated with the intercept located in between the site of the more rapid cleavage, resulting in the larger ($M_r$ ~33 000 fragment), and the second major cleavage site, resulting in the $M_r$ ~28 000 component. These findings, together with our previous immunoelectron microscopic localization data (Wiedenmann and Franke, 1985; Franke *et al.*, 1986), assignes the carboxy terminus to the outer, i.e. cytoplasmic, surface of this type of neuroendocrine vesicles.

Extensive computer search did not show any significant sequence homology with other proteins.

## Discussion

The small (30–80 nm), empty-looking neurosecretory vesicles containing synaptophysin are of widespread occurrence in NE cells of both neuronal and epithelial origin. The prototype of this vesicle class is the presynaptic vesicle of neurons which is a typical neurotransmitter storage vesicle often containing acetylcholine. In addition to its neurobiological importance, this kind of NE vesicle is also of general interest in membrane biology because it is one of the smallest vesicles known.

The present study has provided cDNA clones of a major integral membrane protein of this type of vesicle, i.e. synaptophysin, which allow the identification of its expression at the nucleic acid level and will be valuable in studies of the regulation of genes involved in NE differentiation and function. Furthermore, the cDNA-deduced amino acid sequence of synaptophysin now provides the first information on the primary structure and topology of a transmembrane protein of this type of vesicle, thus complementing the recent work on synapsin I, a peripheral cytoskeletal protein specifically associated with the surface of these vesicles (McCafferty and DeGennaro, 1986).

The amino acid sequence presented here confirms our earlier conclusion that synaptophysin is a transmembrane glycoprotein (Wiedenmann and Franke, 1985). As the N terminus of the native protein was blocked under our conditions and it is unclear whether clone $\lambda SR^2$ contains the actual 5′ end of mRNA, at present we cannot be certain about the true N terminus of synaptophysin. Assuming that the single N-glycosylation site of synaptophysin is located on the internal surface of the vesicle membrane and considering the fact that the SY38 epitope is in the carboxy-terminal portion located on the outer vesicle surface (cf. Wiedenmann and Franke, 1985; Rehm *et al.*, 1986; this study), we propose that the polypeptide chain spans the membrane at least four times with both the N and the C termini on the cytoplasmic surface of the vesicle. This interpretation is also supported by the finding that collagenase cleaves carboxy-terminal synaptophysin portions from intact vesicles. The arrangement of four membrane-spanning regions of ~24 amino acids each, probably in $\alpha$-helical conformation, is reminiscent of the molecular topology of several other transmembrane proteins (for review see Wickner and Lodish, 1985). Examples include the similarly sized gap junction polypeptide which as a hexameric protein forms an intercellular channel (Kumar and Gilula, 1986; Paul, 1986; Unwin, 1986), and the small (mol. wt 16 700) human T-cell differentia-

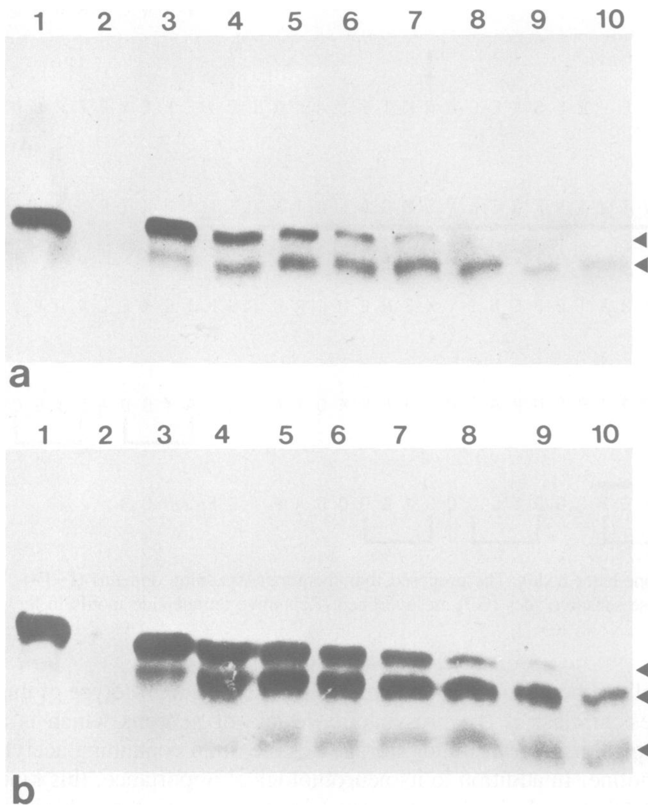


**Fig. 6.** Fragments of synaptophysin obtained by digestion of vesicles with collagenase III and detected by monoclonal antibody SY38 **(a)** and a rabbit synaptophysin antiserum **(b)** in immunoblots after SDS–PAGE, using the alkaline phosphatase detection method. Synaptic vesicles (150 $\mu$g in 100 $\mu$l) were digested with 10 units of purified collagenase III at 30°C for 15 s **(lane 3)**, 2.5 min **(lane 4)**, 5 min **(lane 5)**, 7.5 min **(lane 6)**, 10 min **(lane 7)**, 15 min **(lane 8)**, 20 min **(lane 9)** and 30 min **(lane 10)**. Reference **lane 1** shows untreated synaptic vesicles, **lane 2** collagenase III alone. The upper arrow denotes the position of intact synaptophysin whereas the proteolytic fragments of $M_r$ ~33 000 and ~28 000 are indicated by the lower arrows in **(a)** and **(b)**. The synaptophysin antiserum reacts with a proteolytic fragment of $M_r$ ~28 00 (lowest arrow in **b**) which does not react with antibody SY38 **(a)**.

tion protein MAL (Alonso and Weissman, 1987). Four transmembrane $\alpha$-helices have also been reported for the acetylcholine receptor subunits (e.g. Noda *et al.*, 1983) (for review and discussion of a possible fifth membrane-spanning region see Stroud and Finer-Moore, 1985), although in this case the extended N-terminal segment has been assigned to the outer surface of the membrane. Clearly, in view of the limitations of algorithms currently used for the identification of hydrophobic membrane-spanning regions and for predictions of protein conformation (Kabsch and Sander, 1983; Eisenberg, 1984; Wallace *et al.*, 1986) the proposed assignments of $\alpha$-helical transmembrane regions must await confirmation by more direct experimental results.

Although we cannot be certain about the actual N terminus of the mature synaptophysin, the fact that the first 19 amino acid residues of the reading frame determined here are only moderately hydrophobic, contain four charged amino acids, and do not present sequence features typical of signal peptide leaders to be cleaved off upon translocation across the membrane (cf. von Heijne, 1983; Perlman and Halvorson, 1983), suggests that synaptophysin lacks such a signal leader. This has also been found for several other integral membrane proteins that span the membrane several times (e.g. Nathans and Hogness, 1983; Noda *et al.*, 1984; MacLennan *et al.*, 1985; Wickner and Lodish, 1985: Paul, 1986; Alonson and Weissman, 1987). Consequently, the chain arrangement proposed for synaptophysin differs from that of many typical integral membrane proteins in that its N terminus is located on the cytoplasmic side. This type of orientation, however, has also been proposed for various other transmembrane proteins (e.g. Hunziker *et al.*, 1986), particularly in those that repeatedly cross the membrane (e.g. Noda *et al.*, 1984; Kopito and Lodish, 1985; MacLennan *et al.*, 1985; Shull *et al.*, 1985; Brandl *et al.*, 1986; Hager *et al.*, 1986; Alonso and Weissman, 1987).

Two of the four hydrophobic domains contain a charged amino acid. This has also been seen in many integral membrane proteins with multiple membrane-traversing domains (e.g. Engelman and Zaccai, 1980; Engelman and Steitz, 1981; Nathans and Hogness, 1983; Noda *et al.*, 1983, 1984; Kopito and Lodish, 1985; MacLennan *et al.*, 1985; Shull *et al.*, 1985; Stroud and Finer-Moore, 1985; Brandl *et al.*, 1986; Diehl *et al.*, 1986; Alonso and Weissman, 1987; Hager *et al.*, 1986; Jingami *et al.*, 1987; Peralta *et al.*, 1987). It is commonly assumed that such polar groups present in transmembrane domains are involved in the formation of intramembranous amphiphatic compartments or in the stabilization of adjacent transmembrane $\alpha$-helices by ionic interaction.

The 89-amino acid carboxy-terminal tail region, which projects from the vesicle surface into the cytoplasm, contains the SY38 epitope, several sites susceptible to cleavage with pronase and collagenase and at least one $Ca^{2+}$-binding site (Wiedenmann and Franke, 1985; Rehm *et al.*, 1986; this study). However, computer search has not revealed a sequence homologous to any of the typical $Ca^{2+}$-binding sites known so far, indicating that the $Ca^{2+}$-binding site of synaptophysin is of a different kind. This region is also unusually rich in proline and glycine and contains a number of complete or modulated tetrapeptide repeats of the basic type GYGP, several collagen-type tripeptides (GXX) repeats and many XGP motifs that can be cleaved by collagenase. All these features are shared with synapsin I, a cytoplasmic protein located on the outer vesicle surface (Ueda and Greengard, 1977; McCafferty and DeGennaro, 1986). This similarity of structural features in two otherwise unrelated vesicle-associated proteins may facilitate their interaction, e.g. chain interactions similar to the triple helices of collagen.

The cross-hybridization of the rat synaptophysin cDNA with the corresponding, similarly sized mRNA of human NE cells, together with our previous demonstrations of a broad range of cross-reactivities of synaptophysin antibodies (Wiedenmann and Franke, 1985; Navone *et al.*, 1986), suggest that synaptophysin is a protein markedly conserved during mammalian evolution. Because of this cross-hybridization between rat and human synaptophyins, the cDNA probes described in this paper should also be valuable in studies of the expression of synaptophysin in the development and differentiation of NE cells and tissues. In addition, they will be useful as a broadly reactive differentiation marker in the diagnosis of NE tumours, including small cell carcinomas of the lung. The possibility of probing for synaptophysin mRNA by Northern blot analysis or *in situ* hybridization provides an important alternative method to the widely used immunocytochemical procedures in examinations of the expression of this marker, in particular as a control for possible false positive or negative immunocytochemical diagnosis.

## Materials and methods

*Cell cultures*

PC12 cells which were kindly provided by Dr H.Thoenen (Max-Planck Institute for Psychiatry, Martinsried, FRG) were grown in the presence or absence of nerve growth factor (for culture conditions see Franke *et al.*, 1986). The small cell carcinoma-derived human cell lines NCI-H69 and NCI-H82, originally established at the National Cancer Institute (Bethesda, MD, USA) were obtained from Dr G.Bepler (Department of Internal Medicine, University of Marburg, FRG) and grown in RPMI 1640 medium (Flow, Meckenheim, FRG) essentially as described (Broers *et al.*, 1985) supplemented with 10% fetal calf serum (Flow, Meckenheim, FRG). Cell lines SCLC-H22 and SCLC-H16, both derived from patients with small cell carcinomas of the lung, and cell line ADLC-5M2 from an adenocarcinoma of the lung were kindly provided by Dr G.Bepler and grown as described by Bepler *et al.* (1987a,b).

*Amino acid sequencing of isolated synaptophysin fragments*

Synaptophysin (~2 mg) was isolated from Triton X-100 extracts of synaptic vesicle preparations by chromatography on DEAE–Sepharose (Rehm *et al.*, 1986). The protein was further purified by SDS–PAGE on a 10% gel in the presence of sodium thioglycolate (Hunkapiller *et al.*, 1983). After visualization of protein bands by incubation of the gel in 4 M sodium acetate, the 38-kd band was excised from the gel and the protein was fixed in 10% methanol and 5% acetic acid. After five washes in distilled water, the gel pieces were minced, lyophilized and incubated in 10 ml of 50 mM ammonium bicarbonate, pH 8.0, containing 3 mM $CaCl_2$ and TPCK-treated trypsin (Worthington, Freehold, NJ, USA; ratio of trypsin:synaptophysin 1:50) at 37°C for 48 h. The eluate was collected, lyophilized, and the residue was dissolved in 70% acetic acid. After centrifugation the supernatant was lyophilized, re-dissolved in 70% acetic acid and subjected to h.p.l.c. on a Latek system equipped with a Nucleosil C18 RP column, using an acetonitrile gradient in 0.1% trifluoroacetic acid (Benedum *et al.*, 1986). Peptide-containing peaks were collected, concentrated by lyophilization and subjected to amino acid sequence analysis on an Applied Biosystems 470A gas-phase sequencer equipped with a 120A PTH analyser (Hewick *et al.*, 1981).

*Preparation of RNA*

Total RNA and poly(A)$^+$-RNA from various NE and non-NE tissues of rat and human, including several human tumours, and from cell cultures was prepared as described by Kreis *et al.* (1983) or Cathala *et al.* (1983).

*RNA blot analysis*

Total RNA or poly(A)$^+$-RNA were separated on a 1% formaldehyde/agarose gel (Lehrach *et al.*, 1977) and transferred to a nylon membrane for hybridization with electrophoretically purified cDNA, which was radioactively labelled either by nick-translation (Rigby *et al.*, 1977) or by random-priming (Feinberg and Vogelstein, 1983). The hybridization mix contained 5 × SCC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 5 × Denhardt's solution (1 × Denhardt's solution corresponds to 0.02% Ficoll, 0.02% bovine serum albumin and 0.02% polyvinylpyrrolidon), 1% SDS and 100 μg/ml yeast tRNA. Hybridization was carried out at 42°C. Washes were in 0.1 × SSC and 0.1% SDS at 50°C.

*Library screening and DNA sequencing*

A λgt11 library prepared from poly(A)$^+$-RNA of rat hypothalamus was generously provided by Dr R.Goodman (Tufts University, Boston, MA, USA; cf. Lechan *et al.*, 1986). A λgt10 library constructed from poly(A)$^+$-RNA from rat spinal cord was kindly obtained from Dr G.Grenningloh (ZMBH, Heidelberg, FRG; cf. Grenningloh *et al.*, 1987). Screening of the λgt11 library was performed according to standard protocols (Benton and Davis, 1977; Huynh *et al.*, 1985). Synaptophysin antibody SY38 (Wiedenmann and Franke, 1985) and a rabbit antiserum specific for synaptophysin (Knaus *et al.*, 1986) were used for the initial screening. For the secondary screening, cDNA probes, riboprobes and a synthetic oligonucleotide were used. In the case of the cDNA probes, washes were performed in 0.1 × SSC and 0.1% SDS for 2 h at 65°C. Riboprobes (*in vitro* transcribed *Sal*I-truncated transcription plasmids; for labelling details see Leube *et al.*, 1986) were incubated with the membrane-bound phage DNA at 53°C. Non-specific binding was removed with RNase A (50 μg/ml) and with two washes of 30 min in 0.1 × SSC and 0.1% SDS at 68°C. A 54-mer oligonucleotide (see Results) was synthesized by phosphoramidite chemistry (Sinha *et al.*, 1984) on an Applied Biosystems 380A DNA synthesizer (Applied Biosystems, Foster City, CA, USA) and purified by PAGE; 10 ng were $^{32}$P-labelled with polynucleotide kinase, passed through a Sep-Pak C18 Cartridge (Waters Associates, Milford, MA, USA) and hybridized at lowered formamide concentration (20%). Washes were 3 × 10 min in 2 × SSC/0.1% SDS at room temperature before autoradiographic exposure.

Positive clones were plaque purified. Phage DNA was isolated as described by Leube *et al.* (1986). However, the PEG-precipitated phage pellet was dissolved in SM buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 mM $MgSO_4$) and loaded onto a CsCl step gradient (Maniatis *et al.*, 1982). The collected phage band was dialysed against TE buffer (10 mM Tris–HCl, pH 7.5, 0.1 mM EDTA), and proteins were extracted with phenol before digestion with restriction enzymes. Inserts were excised and subcloned into M13 (Messing, 1983), pUC8 (Vieira and Messing, 1982), Bluescribe or Bluescript (both Stratagene, San Diego, CA, USA). The nucleotide sequence was determined by chemical cleavage (Maxam and Gilbert, 1980) and dideoxy chain termination (Sanger *et al.*, 1977).

*Electrophoresis and immunoblotting*

Synaptic vesicles from rat brain were purified according to Huttner *et al.* (1983), except that gel permeation chromatography was omitted. One-hundred microlitres of synaptic vesicles (concentration: 1.5 mg protein/ml) in 20 mM Hepes buffer, pH 7.5, 10 mM $CaCl_2$ were incubated with 10 units of purified collagenase type III (Sigma, Munich, FRG) at 30°C for various periods of time (see Results). The reaction was stopped by adding an equal volume of SDS–PAGE sample buffer (Laemmli, 1970) to each aliquot. The samples were boiled immediately thereafter and subjected to SDS–PAGE, followed by immunoblotting (Towbin *et al.*, 1979) using monoclonal antibody SY38 (Wiedenmann and Franke, 1985) or rabbit antiserum pSY38 (Knaus *et al.*, 1986).

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