

10. O. P. Hamill *et al.*, *Pfluegers Arch.* **391**, 85 (1981).
11. We obtained this estimate from the reversal potential of the K^+ channel current-voltage plots observed in cell-attached patches, using electrodes filled with 140 mM KCl. It is unlikely that the intracellular K^+ concentration is as high as 140 mM, so the reversal potential will underestimate the actual membrane potential, which is likely to be even more negative.
12. A. Saroli, *Naturwissenschaften* **71**, 428 (1984).
13. R. G. Murray, A. Murray, S. Fujimoto, *J. Ultrastruct. Res.* **27**, 444 (1969).
14. J. C. Kinnamon, B. A. Taylor, R. J. Delay, S. D. Roper, *J. Comp. Neurol.* **235**, 48 (1985).
15. R. Y. Tsien *et al.*, *Cell Calcium* **6**, 145 (1985).
16. S. S. Schiffman, A. J. Hopfinger, R. H. Mazur, in *The Receptors*, P. M. Conn, Ed. (Academic Press, New York, 1986), vol. 4, pp. 315–377.
17. R. W. Tsien *et al.*, *Annu. Rev. Biophys. Biophys. Chem.* **16**, 265 (1987).
18. M. Kuno and P. Gardner, *Nature* **326**, 301 (1987).
19. In addition to releasing Ca^{2+} from internal stores, second messengers may increase ion conductances. This may account for the changes in transmembrane potential reported by several investigators (20). However, the low resting membrane potentials and input impedance reported in many of those studies, compared to those reported here, suggests that damage to the plasma membrane at the electrode penetration site may complicate the interpretation of their results. Further experiments will be necessary to resolve these issues.
20. T. Sato and L. M. Beidler, *Comp. Biochem. Physiol. A* **73**, 1 (1982); *ibid.* **75**, 131 (1983); M. Ozeki, *J. Gen. Physiol.* **58**, 688 (1971).
21. J. B. Harborne, *Introduction to Ecological Biochemistry* (Academic Press, New York, 1982).
22. M. J. Berridge, *Annu. Rev. Biochem.* **56**, 159 (1987).
23. C. E. Semrad and E. B. Chang, *Am. J. Physiol.* **252**, C315 (1987).
24. H. S. Chase and S. M. E. Wong, *ibid.* **254**, F374 (1988).
25. A. Farbman, *J. Ultrastruct. Res.* **12**, 328 (1965).
26. J. Dodd and T. M. Jessell, *J. Neurosci.* **5**, 3278 (1985).
27. We thank S. Siegelbaum, H. S. Chase, and D. Landry for helpful discussions and for allowing us to use their equipment during the early phases of this work; we thank A. Finkelstein for helpful comments on this manuscript. M.H.A. is the recipient of an American Heart Association clinician-scientist award. Supported in part by Public Health Service grants DK 34742 and DK 20999 (to Q.A.) and by grant NS22993 (to J.D.).

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Identification of Synaptophysin as a Hexameric Channel Protein of the Synaptic Vesicle Membrane

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The quaternary structure and functional properties of synaptophysin, a major integral membrane protein of small presynaptic vesicles, were investigated. Cross-linking and sedimentation studies indicate that synaptophysin is a hexameric homo-oligomer, which in electron micrographs exhibits structural features common to channel-forming proteins. On reconstitution into planar lipid bilayers, purified synaptophysin displays voltage-sensitive channel activity with an average conductance of about 150 picosiemens. Because specific channels and fusion pores have been implicated in vesicular uptake and release of secretory compounds, synaptophysin may have a role in these processes.

NEUROTRANSMITTER RELEASE FROM presynaptic nerve terminals is thought to involve specialized vesicular compartments, synaptic vesicles, for both storage and exocytosis of transmitter molecules (1). Despite extensive investigation, the molecular mechanisms of accumulation and release of synaptic vesicle contents remain obscure. Morphological (2) and recent electrophysiological (3, 4) studies on other se-

cretory systems, however, have provided evidence for the involvement of defined channels and "fusion pores" in these processes. In particular, a proteinaceous pore of diameter and conductance similar to that of hexameric gap-junction proteins has been inferred to initiate exocytosis of vesicle contents (4).

Synaptophysin (p38) is a major integral protein of the synaptic vesicle membrane (5, 6). It is expressed in central and peripheral neurons as well as in neuroendocrine cells (7, 8) and represents a major cytoplasmic Ca^{2+} binding site of synaptic vesicle preparations from rat brain (9). On the basis of analysis of its primary structure deduced from the cDNA sequence (10–12), it has been proposed that synaptophysin spans the vesicle membrane four times, with both its NH_2 - and $COOH$ -termini located on the cytoplasmic surface. This transmembrane topology is reminiscent of that of hexameric

gap-junction proteins (13), raising speculations that homo-oligomeric forms of synaptophysin (14) also may form a transmembrane channel (10, 11).

Upon SDS-polyacrylamide gel electrophoresis (PAGE), brain synaptophysin exhibits a relative molecular mass of 38 kD (5, 6), which is reduced to 34 kD on deglycosylation (8, 9). Cross-linking of purified synaptophysin preparations isolated by a new chromatofocusing method (15) resulted in five adducts of the 38-kD polypeptide of apparent molecular masses of 72 kD, 110 kD, 145 kD, 180 kD, and 220 kD, all ± 5 kD (Fig. 1A) (16). On immunoblots, all adducts were recognized by the monoclonal antibody SY38 (Fig. 1A) (17), which reacts with the cytoplasmic $COOH$ -terminal region of synaptophysin (5, 10). Cleavage of the oligomers obtained with the reversible cross-linking reagent *N*-sulfo-succinimidyl(4-azidophenyl)-1,3'-dithiopropionate (SSADP) reduced their apparent molecular masses to 38 kD (Fig. 1B), showing that all adducts comprised only synaptophysin monomers. These data indicate that synaptophysin forms a hexameric complex in the synaptic vesicle membrane.

Consistent with a hexameric structure of synaptophysin is its molecular weight calculated from hydrodynamic properties (18). Sedimentation on H_2O and D_2O sucrose density gradients and gel exclusion chromatography yielded a Stokes radius of 6.5 nm and a sedimentation coefficient ($S_{20,w}$) of 8.9S (Table 1). The molecular mass of 230 kD estimated from these values (19) is similar to that determined by SDS-PAGE for the extensively cross-linked protein and that predicted for a synaptophysin hexamer (228 kD).

Ultrastructural analysis of negatively stained preparations of synaptophysin that had been solubilized in Triton X-100 and purified revealed a largely homogeneous population of particles of mean diameter of 7.8 nm (Fig. 2). In regions of shallow staining, a number of these randomly oriented particles exhibited a "rosette-like" structure with a central 1- to 2-nm density of heavy metal deposition, apparently resulting from filling of a central depression with uranyl salt (Fig. 2, inset). This appearance is reminiscent of the negative staining pictures of the 8- to 8.5-nm particles of hepatic gap junction protein preparations (20). The structure also resembles the 9- to 10-nm "doughnut-shaped" particles or rosettes reported for detergent-solubilized preparations of the Na^+ channel protein (21) and the nicotinic acetylcholine receptor (22) from fish electric organ. Similar 8-nm particles have also been described for a delipidized protein preparation proposed to me-

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mediate acetylcholine release through the presynaptic plasma membrane and termed "mediatophore" (23). These ultrastructural observations are consistent with synaptophysin having a molecular mass of about 200 to 250 kD. Hence, in addition to its predicted transmembrane topology, synaptophysin also shares ultrastructural features with the elementary membrane unit (or "hemi-channel") of gap junctions, the connexon (20). This similarity prompted us to investigate a possible channel or pore activity of this protein.

When purified synaptophysin was reconstituted into liposomes, fusion of the latter to planar lipid bilayers (24) induced channels of an average conductance of 154 ± 11

pS (Fig. 3A) (25). The open probability of the channels increased with positive voltage, whereas at negative potentials no channel activity was observed (Fig. 3C). In parallel experiments, liposomes prepared without synaptophysin did not display any channel activity. Fusion of purified synaptic vesicle preparations from rat brain, however, also produced channels of comparable conductance. The channels observed with reconstituted synaptophysin preparations were unlikely to result from contaminating protein because addition of the specific monoclonal antibody to synaptophysin, SY38 (5), produced a consistent alteration of channel properties (Fig. 3B). Whereas the elementary conductance of the channel decreased

from 154 ± 11 to 101 ± 4 pS, the mean channel opening time was prolonged, and fewer channel opening events (about 40% of control) were seen. Control experiments confirmed the specificity of this effect: (i) a monoclonal antibody specific for the chloride channel-forming postsynaptic glycine receptor did not affect channel activity; and (ii) in bilayers containing gramicidin channels, the SY38 antibody had no effect. Consequently, we conclude that synaptophysin is a hexameric protein capable of forming transmembrane channels.

These results demonstrate that the structural similarities between synaptophysin and gap junction channel proteins reflect common functional properties of these membrane proteins. Specifically, the conductance values for synaptophysin are in the same range as those reported for coupled cells and reconstituted gap junction protein preparations (about 100 to 200 pS) (26) and considerably larger than those of tetrameric and pentameric channel proteins (27). It should, however, be noted that there are also marked differences between synaptophysin and gap junction proteins. First, the amino acid sequences of liver gap junction protein (13) and synaptophysin (10–12) exhibit little homology (13 to 15% identical residues, depending on alignment). Second, no gap junction channel shows the voltage dependence of opening found here for reconstituted synaptophysin. Third, in contrast to synaptophysin, gap junction proteins are not

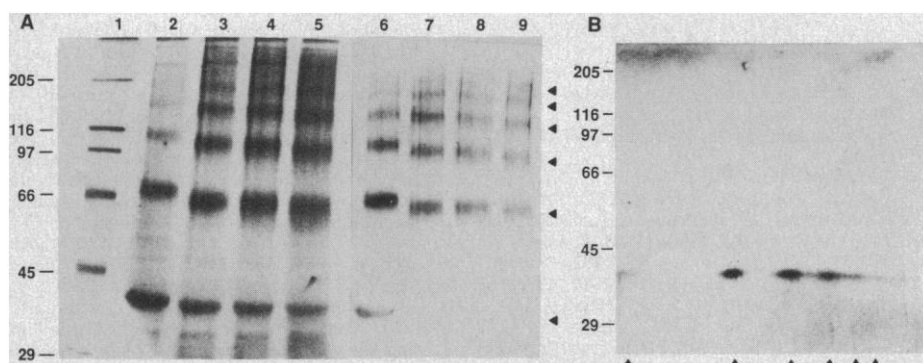


Fig. 1. Cross-linking of synaptophysin. (A) Purified synaptophysin was subjected to SDS-PAGE under nonreducing conditions either without (lanes 2 and 6) or with (lanes 3 to 5 and 7 to 9) prior treatment in the dark with 0.5 mM SSADP, followed by exposure to one (lanes 3 and 7), three (lanes 4 and 8), or ten (lanes 5 and 9) flashes of light. Lanes 1 to 5 were silver-stained. Lanes 6 to 9 were transferred to nitrocellulose, and oligomers of synaptophysin were visualized by reaction with monoclonal antibody SY38. (The monomeric form of synaptophysin is poorly visible on the immunoblot because of prolonged blotting times required for efficient transfer of high molecular weight adducts). Triangles on the right indicate the different immunoreactive adducts of synaptophysin. Molecular masses (in kilodaltons) of marker proteins (lane 1) are on the left. (B) Purified synaptophysin was cross-linked with SSADP as in (A), and the resulting adducts were separated by SDS-PAGE under nonreducing conditions. The entire lane was cut out, incubated with 100 mM DTT for 45 min at room temperature, and placed on top of a second gel. After electrophoresis, silver staining showed six spots of molecular mass 38 kD (indicated by triangles at the bottom). Molecular masses (in kilodaltons) of marker proteins are shown on the left.

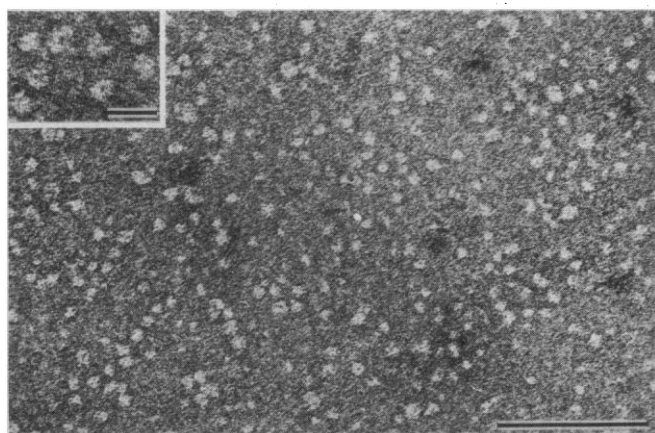


Fig. 2. Electron microscopy of purified synaptophysin. A drop of a Triton X-100-solubilized synaptophysin solution was placed on a freshly glow-discharged carbon-coated grid. The volume of the drop was reduced by soaking from the margin and was restored by addition of 1% glutaraldehyde (in 40 mM sodium cacodylate buffer, pH 7.2). After 30 s, the fixative was removed by repeated rinsing in distilled water. The specimens were then negatively stained in 2% aqueous uranyl acetate and examined in a Siemens 101 electron microscope. The preparation consists of roundish particles of mean diameter 7.8 nm (range: 5.5 to 8.6), some of which reveal a central hollow region filled with uranyl acetate (inset). Scale bars represent 100 nm and 200 nm (inset), respectively.

Table 1. Physical characteristics of synaptophysin. Synaptophysin was solubilized as described (15) except that 10 mM sodium phosphate, pH 7.4, 150 mM KCl, and 1.5% (w/v) Triton X-100 were used. Gel-exclusion chromatography was performed on Sephacryl S-300 equilibrated with 10 mM sodium phosphate, pH 7.4, and 150 mM KCl containing 0.1% (w/v) Triton X-100. Both crude and purified synaptophysin preparations gave the same elution profile. Sucrose gradient centrifugation in H₂O and D₂O was as described (9), with the same buffer as for gel-exclusion chromatography. The partial specific volume of the protein was calculated as 0.732 ml/g from the predicted amino acid sequence (10). The data are the mean \pm SD of four (Stokes radius) and of two ($S_{20,w}$) independent experiments.

Parameter	$\bar{x} \pm \text{SD}$
Stokes radius (nm)	6.51 ± 0.21
Partial specific volume (ml/g)	0.76 ± 0.02
Sedimentation coefficient ($S_{20,w}$)	8.92 ± 0.19
Total molecular mass (kD)	276 ± 23
Frictional ratio	1.49 ± 0.05
Weight fraction of protein (g/g)	0.835 ± 0.096
Molecular mass of protein (kD)	230 ± 8

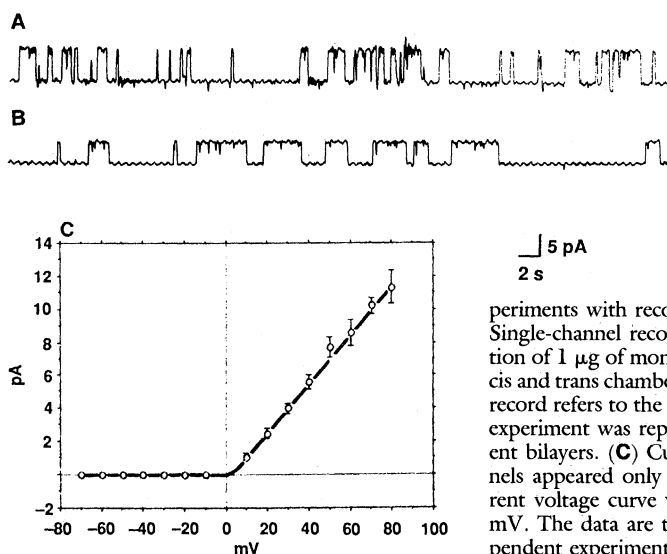


Fig. 3. Channel activity of reconstituted synaptophysin. **(A)** Single-channel current records at +50 mV holding potential in symmetrical bath solutions. Polarity refers to the cis chamber. Openings of the channel are shown as upward deflections. Channels were obtained in 14 of 34 experiments with reconstituted synaptophysin. **(B)** Single-channel recording at +50 mV after addition of 1 μ g of monoclonal antibody SY38 to the cis and trans chambers and stirring for 5 min. This record refers to the same bilayer as trace (A). The experiment was repeated three times with different bilayers. **(C)** Current-voltage relation. Channels appeared only at positive voltages; the current-voltage curve was linear between 0 and 80 mV. The data are the mean \pm SD of four independent experiments.

glycosylated. These differences may reflect specialized functions of these transmembrane channels.

The channel-forming properties of synaptophysin suggest that this protein may translocate low molecular weight compounds across the vesicle membrane. In analogy to the Ca^{2+} -regulated channels of plant vacuoles (28), synaptophysin may allow solute or metabolite exchange between cytoplasmic and vesicular compartments. Alternatively, synaptophysin may be implicated in transport processes across two apposed lipid bilayers, as is the case for gap junction proteins. For example, homophilic binding between synaptophysin molecules could facilitate equilibration of vesicular contents prior to quantal release (29). A more tempting hypothesis emerges from the discovery of fusion pore formation as the initial event of exocytosis (3, 4). Correspondingly, synaptophysin might couple to a channel protein (vesicle-docking protein) of the presynaptic membrane. Concurrent formation of a pore connecting the synaptic vesicle interior and the synaptic cleft may eventually result in neurotransmitter release or vesicle-plasma membrane fusion (30). Transmembrane channels similar to the mediatothore isolated from *Torpedo* presynaptic membranes (23) are possible candidates for the hypothesized vesicle-docking proteins.

REFERENCES AND NOTES

- B. Ceccarelli and W. P. Hurlbut, *Physiol. Rev.* **60**, 396 (1980); L. F. Reichardt and R. B. Kelly, *Annu. Rev. Biochem.* **52**, 871 (1983).
- D. E. Chandler and J. E. Heuser, *J. Cell Biol.* **86**, 666 (1980).
- J. M. Fernandez, E. Neher, B. D. Gomperts, *Nature* **312**, 453 (1984); J. Zimmerberg, M. Curran, F. S. Cohen, M. Brodwick, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1585 (1987); L. J. Breckenridge and W. Almers, *ibid.*, p. 1945.
- L. J. Breckenridge and W. Almers, *Nature* **328**, 814 (1987).
- B. Wiedenmann and W. W. Franke, *Cell* **41**, 1017 (1985).
- R. Jahn, W. Schiebler, C. Ouimet, P. Greengard, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4137 (1985).
- B. Wiedenmann, W. W. Franke, C. Kuhn, R. Moll, V. E. Gould, *ibid.* **83**, 3500 (1986); P. Knaus, H. Betz, H. Rehm, *J. Neurochem.* **47**, 1302 (1986).
- F. Navone *et al.*, *J. Cell Biol.* **103**, 2511 (1986).
- H. Rehm *et al.*, *EMBO J.* **5**, 535 (1986).
- R. E. Leube *et al.*, *ibid.* **6**, 3261 (1987).
- T. C. Südhof, F. Lottspeich, P. Greengard, E. Mehl, R. Jahn, *Science* **238**, 1142 (1987).
- K. M. Buckley, E. Floor, R. B. Kelly, *J. Cell Biol.* **105**, 2447 (1987).
- D. L. Paul, *ibid.* **103**, 123 (1986); N. M. Kumar and N. B. Gilula, *ibid.*, p. 767.
- Previous cross-linking experiments on synaptic vesicles and purified synaptophysin preparations, in which glutaraldehyde, copper(II) α -phenanthroline, or dimethylsuberimidate (DMS) was used, had produced di-, tri-, and tetrameric adducts of the 38-kD monomer (9).
- Synaptic vesicles were prepared according to published methods [W. B. Huttner, W. Schiebler, P. Greengard, P. De Camilli, *J. Cell Biol.* **96**, 1374 (1983)], except that the gel filtration step was omitted. Synaptic vesicles (3 mg of protein per milliliter) in 25 mM histidine-HCl, pH 6.2, were solubilized in the presence of 1.5% (w/v) Triton X-100 for 1 hour at 4°C and centrifuged for 1 hour at 100,000g. The supernatant was applied to a chromatofocusing column equilibrated with 25 mM histidine-HCl, pH 6.2, containing 0.1% (w/v) Triton X-100 and eluted with Polybuffer 74-HCl, pH 4.0, containing the same concentration of detergent. Fractions were analyzed by a dot assay for the detection of synaptophysin with monoclonal antibody SY38 (H. Rehm, in preparation). Synaptophysin eluted in almost pure form (as judged by silver staining of SDS gels under reducing conditions) as a broad peak at pH 4.3. Peak fractions were concentrated by dialysis against 10% polyethylene glycol 20,000 (w/v), 10 mM sodium phosphate, pH 7.4, 150 mM KCl, and 0.1% (w/v) Triton X-100 and subsequently separated from Polybuffer by gel exclusion chromatography on Sephacryl S-300.
- The cross-linkers DMS, dimethyl-3,3'-dithiobispropionimidate (DTBP), and 3,3'-dithiobis(sulfosuccinimidyl propionate) (DTSSP) gave identical results. It is unlikely that intermolecular cross-linking contributed to adduct formation, because the patterns of immunoreactive cross-linking products were indistinguishable when different protein concentrations (10 to 100 μ g/ml) were used [J. Hajdu, F. Bartha, P. Friedrich, *Eur. J. Biochem.* **68**, 373 (1976)]. Also, adduct formation was not caused by intermolecular disulfide bonding as reduction of synaptophysin with dithiothreitol (DTT), and subsequent alkylation with iodoacetamide did not result in altered adduct patterns on cross-linking with DMS.
- Under the conditions used in this study, cross-linking of synaptic vesicle preparations produced up to pentameric immunoreactive adducts.
- S. Clarke, *J. Biol. Chem.* **250**, 5459 (1975); J. E. Sadler *et al.*, *ibid.* **254**, 4434 (1979).
- The value of 230 kD is significantly higher than that determined with detergent-phospholipid extracts of synaptic vesicle preparations (9). This discrepancy probably is due to disproportionately high phospholipid binding by the hydrophobic (9-12) synaptophysin molecule under the sedimentation conditions (phospholipid-detergent buffer) used earlier.
- P. N. T. Unwin and G. Zampighi, *Nature* **283**, 545 (1980).
- M. H. Ellisman *et al.*, *J. Cell Biol.* **97**, 1834 (1983).
- F. Bon *et al.*, *J. Mol. Biol.* **176**, 205 (1984); A. Brisson and P. N. T. Unwin, *Nature* **315**, 474 (1985).
- N. Morel, M. Israel, B. Lesbats, S. Birman, R. Manaranche, *Ann. N.Y. Acad. Sci.* **493**, 151 (1987).
- Synaptophysin was purified from synaptic vesicles as described (15), except that solubilization was carried out in the presence of 6% (w/v) *N*-octyl- β -D-glucoside and 0.1 mM DTT and that all buffers in the following purification procedure contained 1% (w/v) of the same detergent, 0.1 mM DTT, and dipalmitoyl phosphatidylcholine (0.1 mg/ml). After chromatofocusing, 25 mM potassium phosphate, pH 7.4, was used as the buffer. Synaptophysin was reconstituted into phospholipid vesicles according to published procedures [L. T. Mimms, G. Zampighi, Y. Nozaki, C. Tanford, J. A. Reynolds, *Biochemistry* **20**, 833 (1981)]. Briefly, 10 mg of dipalmitoyl phosphatidylcholine (Sigma), or 5 mg of dipalmitoyl phosphatidylcholine (Sigma) mixed with 5 mg of diphytanoyl lecithin (Avanti Polar Lipids), were dried and dissolved in a solution containing 60 μ g of synaptophysin and 45 mg of octylglucoside in a total volume of 500 μ l of 25 mM tris-Hepes, pH 7.4, 150 mM KCl, 0.1 mM DTT, and 0.02% (w/v) azide (buffer A). After extensive dialysis at 4°C against eight 1-liter changes of buffer A, formation of vesicles (mean diameter, 50 to 60 nm) was confirmed by electron microscopy of negatively stained samples. Vesicles were frozen on liquid nitrogen and stored at -70°C. Planar bilayers painted across a hole with 1-mm diameter consisted of 1- α -dioleylethanolamine: 1- α -diphytanoyl lecithin at 4:1 (Avanti Polar Lipids) dissolved to a concentration of 1.5% (w/v) in decane. The buffers consisted of 6 ml of 25 mM Hepes, pH 7.4, 150 mM KCl, and 1 mM CaCl_2 in the cis chamber and 6 ml of 25 mM Hepes, pH 7.4, 50 mM KCl, and 1 mM CaCl_2 in the trans chamber, thus establishing an osmotic gradient across the planar lipid bilayer [C. Miller and E. Racker, *J. Membrane Biol.* **30**, 283 (1976)]. The intravesicular KCl concentration was brought to 250 mM by mixing with an appropriate amount of 4M KCl and freeze-thawing twice in liquid nitrogen. The samples were then bath-sonicated for 10 s, and 2 μ l of the resulting suspension were added to the cis side of a planar lipid bilayer. After stirring for 30 min, a holding potential of 50 mV (cis side-positive) was applied. After a fusion event, the osmotic gradient was abolished by addition of an appropriate amount of 4M KCl solution to the trans chamber. Signals were low-pass filtered at 10 or 33 Hz and stored on magnetic tape.
- In about 20% of our channel records, we also saw multiple values ($n = 2$ to 6 or 7) of this single conductance unit.
- J. Neyton and A. Trautmann, *Nature* **317**, 331 (1985); G. A. Zampighi, J. E. Hall, M. Kreman, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 8468 (1985); D. C. Spray *et al.*, *ibid.* **83**, 5494 (1986); R. D. Veenstra and R. L. DeHaan, *Science* **233**, 972 (1986); J. D.-E. Young, Z. A. Cohn, N. B. Gilula, *Cell* **48**, 733 (1987); J. Chow and S. H. Young, *Dev. Biol.* **122**, 332 (1987).
- J.-P. Changeux, A. Devillers-Thiéry, P. Chemouilli, *Science* **225**, 1335 (1984); N. Unwin, *Nature* **323**, 12 (1986); J. Bormann, O. P. Hamill, B. Sakmann,

- J. Physiol.* **385**, 243 (1987); D. Langosch, L. Thomas, H. Betz, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
28. R. Hedrich and E. Neher, *Nature* **329**, 833 (1987).
29. B. Katz, *The Release of Neural Transmitter Substances* (Thomas, Springfield, IL, 1969), The Sherrington Lectures, vol. 10, pp. 1–60.
30. As the cytoplasmic region of synaptophysin binds Ca^{2+} (9), coupling could be triggered by a depolarization-induced Ca^{2+} influx [B. Katz and R. Miledi, *J. Physiol. (London)* **203**, 689 (1969); T. Pozzan, G. Gatti, N. Dozio, L. M. Vicentini, J. Meldolesi, *J. Cell Biol.* **99**, 628 (1984)]. Alternatively, Ca^{2+} may affect the channel activity of synaptophysin. However, we have not been able to demonstrate a direct

effect of Ca^{2+} on reconstituted synaptophysin preparations.

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The Presence of Fibroblast Growth Factor in the Frog Egg: Its Role as a Natural Mesoderm Inducer

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A complementary DNA clone corresponding to a 4.2-kilobase transcript that is present in the *Xenopus* oocyte and newly transcribed in the neurula stages of development has been isolated. This messenger RNA encodes a 155-amino acid protein that is 84% identical to the human basic fibroblast growth factor (bFGF). When expressed in *Escherichia coli* and purified, the *Xenopus* FGF induced mesoderm in animal cell blastomeres as measured by muscle actin expression. Immunoblots with an antibody to a *Xenopus* FGF peptide show that the oocyte and early embryo contain a store of the FGF polypeptide at high enough concentrations to induce mesoderm. The presence of FGF in the oocyte, together with the apparent lack of a secretory signal sequence in the protein, suggest that the regulation of mesoderm induction may involve novel mechanisms that occur after the translation of FGF.

MESODERM IN VERTEBRATE EMBRYOS is induced in the ectoderm by signals emanating from the underlying endoderm (1). Such inductive signals are probably responsible for much of the tissue patterning in vertebrate organisms. Although these inductive interactions are well documented by transplantation and ablation experiments, the specific signals have been very difficult to characterize. Homologs of several growth factors may be the signaling molecules; basic and acidic fibroblast growth factors (bFGF and aFGF, respectively), transforming growth factor- β 2 (TGF- β 2), and a partially purified low molecular weight growth factor are potent inducers of mesoderm in *Xenopus* (2–4). At physiological concentrations these growth factors induce the synthesis of muscle gene products and the formation of morphologically identifiable mesodermal tissues in ectodermal cells of the animal cap. Transcripts encoding molecules related to FGF and TGF- β could be found in the early embryo

(3, 5). One of these transcripts encodes a protein (Vg-1) that is a member of the TGF- β superfamily but is distinct from either TGF- β 1, which can enhance the FGF-mediated induction of mesoderm (3), or TGF- β 2. Early embryonic transcripts also encode a peptide that is very similar to a COOH-terminal portion of the mammalian bFGFs used in the mesoderm induction

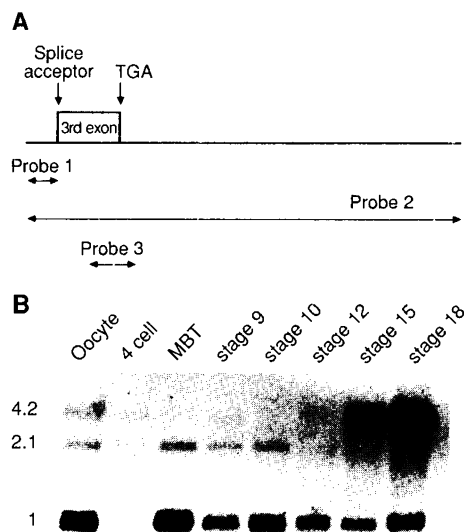
assays. Complementary DNA (cDNA) clones containing FGF sequences, however, all appeared to be copies of unspliced messages, so it was unclear if the encoded protein sequence was indeed translated as part of a complete FGF-like molecule (3).

Below we describe a cDNA clone, corresponding to a 4.2-kb transcript present in the *Xenopus* oocyte and at later stages, encoding a 155-residue protein that is closely related to human bFGF and that can induce the synthesis of muscle actin mRNA in animal cap explants. In addition, we show that the unfertilized egg contains an FGF polypeptide, suggesting that the regulation of induction may involve post-translational events.

We previously isolated a *Xenopus* oocyte cDNA that contained sequences similar to the human and bovine bFGF genes (3). This clone hybridizes to a 1-kb RNA that is present in the oocyte and in the midblastula to neurula stages. Although this RNA is large enough to encode a protein the size of bFGF, it lacks sequences similar to the first and second exons of human bFGF (6) and contains only a short open reading frame encoding a polypeptide domain homologous to the third exon of mammalian bFGFs [Fig. 1A, (3)]. It is unlikely that this RNA could encode a truncated form of FGF, since there are no in-frame potential translation initiation (ATG) codons upstream of the small coding region. In RNA blot analysis, a probe derived from the presumed noncoding sequences 5' of the FGF third exon (Fig. 1A, probe 1) hybridized to the 1-kb mRNA, as expected if the cDNAs represent copies of this mRNA. The existence of this major noncoding RNA is intriguing and is currently the subject of further study.

With a probe corresponding to the entire

Fig. 1. (A) DNA probes used for RNA blots and screening of the cDNA libraries. The 1.3-kb cDNA isolated previously (3) is shown, with the potential splice acceptor site and the open reading frame region homologous to the third exon of the mammalian bFGFs indicated. The TGA signifies the stop codon, coincident with the site of translation termination in human and bovine bFGF (6, 9). Probe 1 is a 105-bp Mnl I fragment. Probe 2, which consists of the entire cDNA clone, is a 1.3-kb Eco R1 fragment. Probe 3 is a 175-bp Pvu II–Ssp I fragment. **(B)** Analysis of RNA from various stages of *Xenopus* development. Polyadenylated RNA was isolated from 50 oocytes or embryos at different stages (22). The RNA was separated on a 1% formaldehyde-agarose gel and blotted onto Hybond-N (Amersham). The membrane was hybridized as previously described (3) with probe 3 labeled with ^{32}P (3000 Ci/mmol), and the filter was washed and exposed for 2 weeks. The sizes of the three transcripts (4.2, 2.1 and 1 kb) were determined relative to λ Hind III markers loaded in a parallel lane on the gel and separately hybridized to a λ DNA probe.



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