Cholesterol-dependent interaction of syncollin with the membrane of the pancreatic zymogen granule

Alois HODEL*, Seong J. AN*[1], Neal J. HANSEN*, Jared LAWRENCE*, Barbara WÄSLE*, Michael SCHRADER† and J. Michael EDWARDSON*[2]

*Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ, U.K., and †Institute for Cytobiology and Cytopathology, University of Marburg, Robert Koch Strasse 5, Marburg, Germany

Syncollin is a protein of the pancreatic zymogen granule that was isolated through its ability to bind to syntaxin. Despite this *in vitro* interaction, it is now clear that syncollin is present on the luminal side of the zymogen granule membrane. Here we show that there are two pools of syncollin within the zymogen granule: one free in the lumen and the other tightly associated with the granule membrane. When unheated or cross-linked samples of membrane-derived syncollin are analysed by SDS/PAGE, higher-order forms are seen in addition to the monomer, which has an apparent molecular mass of 16 kDa. Extraction of cholesterol from the granule membrane by treatment with methyl-β-cyclodextrin causes the detachment of syncollin, and this effect is enhanced at a high salt concentration. Purified syncollin is able to bind to brain liposomes at pH 5.0, but not at pH 11.0, a condition that also causes its extraction from granule membranes. Syncollin binds only poorly to dioleoyl phosphatidylcholine liposomes, but binding is dramatically enhanced by the inclusion of cholesterol. Finally, cholesterol can be co-immunoprecipitated with syncollin. We conclude that syncollin is able to interact directly with membrane lipids, and to insert into the granule membrane in a cholesterol-dependent manner. Membrane-associated syncollin apparently exists as a homo-oligomer, possibly consisting of six subunits, and its association with the membrane may be stabilized by electrostatic interactions with either other proteins or phospholipids.

Key words: exocrine pancreas, exocytosis, secretion, secretory granule.

INTRODUCTION

In the pancreatic acinar cell, digestive enzymes are packaged in a condensed (and predominantly inactive) form into zymogen granules [1]. Secretagogue stimulation of the cell causes an elevation of intracellular Ca^{2+} concentration, which in turn triggers the fusion of the granules with the apical domain of the plasma membrane [2,3]. Despite extensive study, the mechanism underlying zymogen granule exocytosis is still far from clear; however, some information is beginning to emerge about the proteins that mediate the exocytotic membrane-fusion event. For instance, it has been demonstrated recently that the soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) syntaxin 2, present on the apical plasma membrane, is essential for fusion between the zymogen granules and the plasma membrane, whereas syntaxin 3, on the granule membrane, mediates homotypic fusion between granules [4]. There is now considerable interest in identifying other proteins that contribute to the secretion of zymogens from the acinar cell.

Syncollin is a zymogen granule protein that was originally isolated through its ability to bind to syntaxin in a Ca^{2+}-sensitive manner [5]. This property, together with the observation that recombinant syncollin was able to inhibit Ca^{2+}-dependent fusion *in vitro* between zymogen granules and pancreatic plasma membranes, led to the proposal that syncollin was involved in the control of exocytotic membrane fusion in the pancreatic acinar cell. More recently, we have shown that syncollin has an N-terminal signal sequence that directs its translocation across the membrane of the endoplasmic reticulum [6]. In acinar cells, syncollin is found tightly attached to the luminal surface of the zymogen granule membrane. It resists salt-washing of the granule membranes, but is removed by treatment with sodium carbonate. These results led us to reconsider the likely function of syncollin, and in particular to question the significance of its interaction with syntaxin. It is known that the expression of syncollin depends upon feeding behaviour [7], suggesting that it is indeed involved in the secretion of digestive enzymes; however, its precise role is still unclear.

Here, we address the nature of the interaction of syncollin with the zymogen granule membrane. We report that there are two pools of syncollin: one free in the granule lumen and the other bound to the granule membrane. Syncollin associates with the granule membrane through an interaction with cholesterol, and as it does so it undergoes oligomerization. Finally, the association of syncollin with the granule membrane is apparently stabilized by electrostatic interactions with either other proteins or phospholipids.

MATERIALS AND METHODS

Antibodies

Antibodies used were as follows: 87.1, a mouse monoclonal antibody against recombinant syncollin [6]; anti-syncollin B, a polyclonal antibody also raised against recombinant syncollin

Abbreviations used: BS^3, bis(sulphosuccinimidyl) suberate; DOPC, dioleoyl phosphatidylcholine; GPI, glycosylphosphatidylinositol; HBS, Hepes-buffered saline; MβCD, methyl-β-cyclodextrin; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor.

[1] Present address: Vollum Institute, Oregon Health Sciences University, 3181 S.W. Sam Jackson Park Road L474, Portland, OR 97201-3098, U.S.A.
[2] To whom correspondence should be addressed (e-mail jme1000@cus.cam.ac.uk).

[8]; 69.1, a mouse monoclonal antibody against synaptobrevin 2 [9]; anti-amylase, a rabbit polyclonal antibody (Sigma, Poole, Dorset, U.K.); anti-GP-2, a rabbit polyclonal antibody against the appropriate band excised from an SDS/polyacrylamide gel of zymogen granule proteins, and 42.1, a mouse monoclonal antibody against rab3 [10].

SDS/PAGE and immunoblotting

Proteins were separated by SDS/PAGE and then, where appropriate, electrophoretically transferred to nitrocellulose (Schleicher & Schuell, Dassel, Germany) by semi-dry blotting. Blots were probed with primary antibodies at dilutions of 1:500–1:5000. Immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibodies (1:1000) and enhanced chemiluminescence (Pierce & Warriner, Chester, U.K.). Where appropriate, semi-quantitative analyses of proteins present on the immunoblots were carried out by densitometry using an ImageMaster system (Pharmacia Biotech, St. Albans, Herts., U.K.).

Preparation of rat pancreatic zymogen granules

Zymogen granules were isolated as described previously [11]. All buffers contained 1 mM EDTA and a protease-inhibitor cocktail (1 mM PMSF, 1 μg/ml pepstatin, 1 μg/ml antipain, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 17 μg/ml benzamidine and 50 μg/ml bacitracin). Granules were lysed by incubation for 1 h at 4 °C in 170 mM NaCl and 200 mM $NaHCO_3$ (pH 7.8; 1:3). Granule membranes and contents were separated by centrifugation at 100000 ***g*** for 1 h.

Purification of syncollin

All procedures were carried out at 4 °C. Zymogen granule membranes prepared freshly from five rats were resuspended in 200 μl of Hepes-buffered saline (50 mM Tris/HCl, pH 7.6/100 mM NaCl; HBS). To this suspension was added 400 μl of 0.3 M KCl, and the sample was incubated for 30 min, with end-over-end rotation. The washed membranes were collected by centrifugation at 21000 ***g*** for 20 min. The supernatant, containing proteins that had been loosely bound to the granule membranes, was discarded. The membrane pellet was resuspended in 1 ml of 0.1 M Na_2CO_3, and incubated for 30 min, again with rotation. The membranes were pelleted by centrifugation at 21000 ***g*** for 30 min. The supernatant was recovered and dialysed overnight against HBS, pH 7.6. The resulting precipitate was collected by centrifugation at 21000 ***g*** for 5 min. The pellet was dissolved in 100 μl of 0.5% taurodeoxycholate in HBS, pH 7.6. Any insoluble material was pelleted by centrifugation at 21000 ***g*** for 5 min. The protein concentration of the final sample was typically 20 μg/ml.

Chemical cross-linking of syncollin

Syncollin (200 ng) in HBS, pH 7.6, containing 0.5% taurodeoxycholate, was incubated with the cross-linker bis(sulphosuccinimidyl) suberate (BS^3, 4 mM; Pierce & Warriner) for 30 min at 4 °C. Cross-linking was terminated by addition of 100 mM Tris/HCl/100 mM glycine, pH 7.5. Pre-quenched BS^3 was used for control incubations. Samples were then analysed by SDS/PAGE and immunoblotting, as described above.

Cholesterol extraction with methyl-β-cyclodextrin (MβCD)

MβCD (Sigma) was dissolved in HBS, pH 7.6, and added at various concentrations to a suspension of zymogen granule membranes. The samples were incubated overnight at 4 °C, with agitation. Membranes and supernatant (containing extracted proteins) were separated by centrifugation at 21000 ***g*** for 5 min. Extracted proteins were precipitated with 10% trichloroacetic acid (10 min at 4 °C), and equivalent amounts of membranes and supernatant were then analysed by SDS/PAGE and immunoblotting.

Liposome preparation and syncollin binding

Lipids [either brain lipids or dioleoyl phosphatidylcholine (DOPC); Avanti Polar Lipids, Alabaster, AL, U.S.A.] were supplied in chloroform. The chloroform was evaporated from 2 mg of lipid under a stream of nitrogen, and the lipid was hydrated overnight at 4 °C in 500 μl of water. The lipid suspension was sonicated with a probe sonicator (tip amplitude 10 μm, for two bursts of 10 s) to produce liposomes. An equal volume of 2-fold-concentrated HBS, at the appropriate pH, was then added. Purified syncollin (10 μl, 200 ng of protein) was added in 0.5% taurodexycholate to 1 mg of liposomes in a 500 μl volume. The final detergent concentration was therefore 0.01%. Samples were incubated for 30 min at 4 °C, with agitation, and syncollin binding to the liposomes was then analysed by sucrose-density-gradient centrifugation.

Sucrose-density-gradient centrifugation

Syncollin binding to liposomes was determined by floatation of the liposomes on a discontinuous sucrose density gradient. Samples (500 μl) of liposomes after incubation with syncollin were added to 1.5 ml of 80% sucrose in the appropriate buffer. On to this suspension (now 60% sucrose) was layered 1.5 ml of 50% sucrose in buffer and 1.5 ml of buffer alone. The gradient was centrifuged at 115000 ***g*** for 2 h at 4 °C. Fractions (500 μl) were taken from the top of the gradient and analysed by SDS/PAGE and immunoblotting.

Phospholipid and cholesterol assays

The distribution of liposomes in sucrose density gradients was determined by phospholipid assay of the various fractions. To a 50 μl sample of each fraction (or the same volume of buffer solution, for blanks) was added 2 ml of 70% perchloric acid, and the samples were heated for 2 h at 200 °C. The samples were cooled, and 7 ml of water and 1 ml of 2.5% ammonium molybdate were added. After thorough mixing, 1 ml of 10% ascorbic acid was added, and the samples were boiled for 7 min. The absorbance of the samples at a wavelength of 820 nm was measured using a Unicam 5626 spectrometer (ATI Unicam, Cambridge, U.K.). Blank readings were subtracted from the readings for all samples.

The cholesterol contents of MβCD-treated and untreated membranes, and of the brain lipids, were determined using a cholesterol assay kit (Molecular Probes, Leiden, The Netherlands), according to the manufacturer's instructions.

Co-immunoprecipitation of cholesterol and syncollin

Samples of syncollin (0.5 μg in 100 μl of HBS, pH 7.6, containing 0.5% taurodeoxycholate and protease-inhibitor cocktail) or buffer alone were incubated with 1.25 μCi (2.8×10^6 d.p.m.) of [^{3}H]cholesterol (New England Nuclear, Hounslow, Middx, U.K.; 82 Ci/mmol) for 2 h at room temperature, with agitation. The samples were centrifuged at 18000 ***g*** for 5 min at 4 °C. The supernatants were incubated with anti-syncollin antibody B (15 μl) for 1 h at 4 °C, with agitation. Protein G–Sepharose

(30 μl of a 1:1 slurry in HBS) was then added, and the incubation was continued for a further 1 h at 4 °C, again with agitation. The samples were centrifuged at 18000 ***g*** for 10 min at 4 °C, and the supernatants were discarded. The pellets were washed four times with 1 ml of HBS, and then resuspended in 100 μl of SDS/PAGE sample buffer. The samples were heated for 10 min at 95 °C, and the beads were pelleted by centrifugation at 18000 ***g*** for 5 min. The radioactivity contained in 90 μl of the supernatant was determined by liquid scintillation counting. The remaining 10 μl was analysed by SDS/PAGE and immunoblotting, using anti-syncollin antibody 87.1.

RESULTS

Previous studies [5,6] have indicated that zymogen granule membranes contain a population of syncollin that resists high-salt (1 M NaCl) washing, but which is removed by treatment of the membranes with sodium carbonate at pH 11.0. The fact that syncollin is not an integral protein of the zymogen granule membrane suggests that there might be a pool of syncollin free in the granule lumen. To investigate this possibility, we lysed a sample of granules, separated the membranes and contents, and determined the relative amounts of syncollin in the two fractions. When equivalent amounts of granule membranes and content were analysed by SDS/PAGE and immunoblotting (Figure 1), it was found that 60 % of the syncollin was membrane-bound and 40 % free in the granule contents. In contrast, the membrane-spanning SNARE protein synaptobrevin 2 was found entirely in the membrane fraction, whereas the content protein amylase was found predominantly (82 %) in the granule contents.

The predicted molecular mass of native syncollin is 13 kDa, although it migrates on SDS/polyacrylamide gels with an apparent molecular mass of 16 kDa [6]. We have shown previously [6] that syncollin in a granule-membrane sample solubilized in Triton X-100 behaves as a monomer, whereas in samples solubilized in deoxycholate it behaves as a protein of approx. 120 kDa. This result suggested that membrane-associated syncollin might form a homo-oligomer. To determine whether this is in fact the case, we prepared syncollin from zymogen granule membranes and investigated its behaviour on SDS/polyacrylamide gels after it had been either left untreated or denatured by heating at 95 °C for 5 min. A Coomassie Brilliant Blue-stained gel of an unheated sample of syncollin showed prominent bands of approx. 55 and 100 kDa that collapsed into a 16 kDa band when the sample was heated (Figure 2A). The corresponding blot, probed with anti-syncollin antibody B, confirmed that the 55 and 100 kDa bands represented higher-order syncollin structures, and also revealed a 34 kDa syncollin band that was resistant to heating. Bands of similar molecular masses were seen when a sample of syncollin was treated with the cross-linking reagent BS^3, although additional bands of approx. molecular masses 20, 64 and 80 kDa were also detected (Figure 2B). The molecular mass of the largest band detected in these experiments (100 kDa) is similar to that seen when taurodeoxycholate-solubilized syncollin is subjected to sucrose-density-gradient centrifugation, and is consistent with a hexameric structure. Furthermore, the family of bands (16, 34, 55, 64, 80 and 100 kDa) are of approximately the correct sizes to represent monomers, dimers, and so on, through to hexamers. The one exception is the 20 kDa band, which we cannot explain at present. The higher-order syncollin structures in unheated samples could only be detected using the polyclonal anti-syncollin antibody B, which is also the only antibody available to us that reliably detects syncollin by immunofluorescence [8]. The monoclonal anti-syncollin antibody 87.1 detected only the syncollin monomer in unheated samples (results not shown), but was able to detect the higher-order structures in heated, cross-linked samples. It appears, then, that the protein undergoes structural rearrangements on oligomerization that render it undetectable by one of the two anti-syncollin antibodies used in these experiments. In contrast, when the syncollin oligomer is held together by cross-linking, but the monomers are denatured, antibody 87.1 is then able to recognize the protein, albeit with relatively low efficiency.

The fact that the zymogen granule contains two distinct populations of syncollin begs the question of how syncollin is recruited to the zymogen granule membrane. Deoxycholate, which extracts syncollin from the membrane as a homo-oligomer, is of course structurally similar to cholesterol, a major component of the inner leaflet of the granule membrane [12]. This raises the possibility that syncollin might bind to the granule membrane through an interaction with cholesterol. To examine this

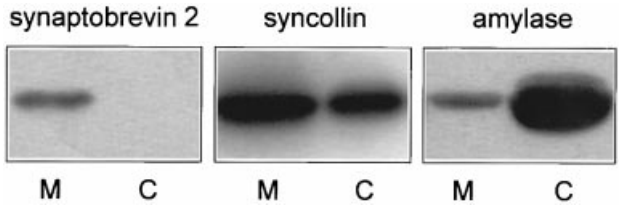

Figure 1 Syncollin is present both free in the granule lumen and attached to the granule membrane

Zymogen granules were lysed, and membranes and contents were separated by centrifugation. Equivalent fractions of membrane (M) and content (C) were analysed by SDS/PAGE and immunoblotting, using antibodies against synaptobrevin 2, syncollin (87.1) and amylase.

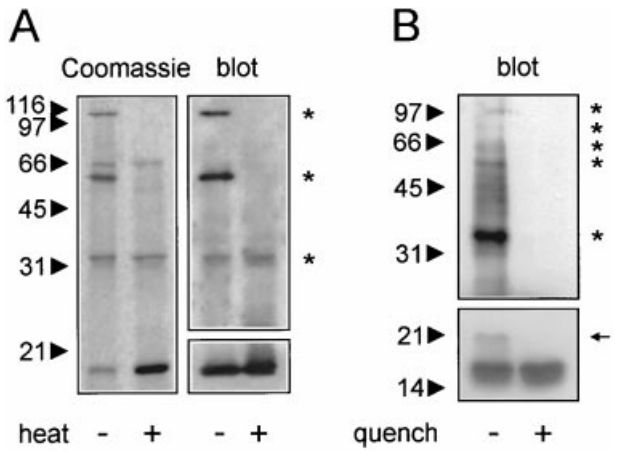

Figure 2 Membrane-associated syncollin exists in homo-oligomeric form

(**A**) Purified syncollin (250 ng of protein) in gel sample buffer was either left unheated or heated at 95 °C for 5 min, and then analysed by SDS/PAGE and either Coomassie Brilliant Blue staining or immunoblotting, using anti-syncollin antibody B. The upper and lower panels of the blot were exposed to film for 5 and 0.5 s, respectively. The positions of molecular-mass markers (in kDa) are shown on the left. Asterisks on the right indicate higher-order syncollin structures. (**B**) Purified syncollin (200 ng of protein) in 0.5 % taurodeoxycholate was incubated with active or pre-quenched BS^3 for 30 min at 4 °C. Active BS^3 was quenched by addition of 100 mM Tris/HCl/100 mM glycine, and the protein was analysed by SDS/PAGE and immunoblotting, using anti-syncollin antibody 87.1. The upper and lower panels of the blots were exposed to film for 5 min and 30 s, respectively. The positions of molecular-mass markers (in kDa) are shown on the left. Asterisks on the right indicate higher-order syncollin structures. The arrow on the right indicates an additional unidentified band.

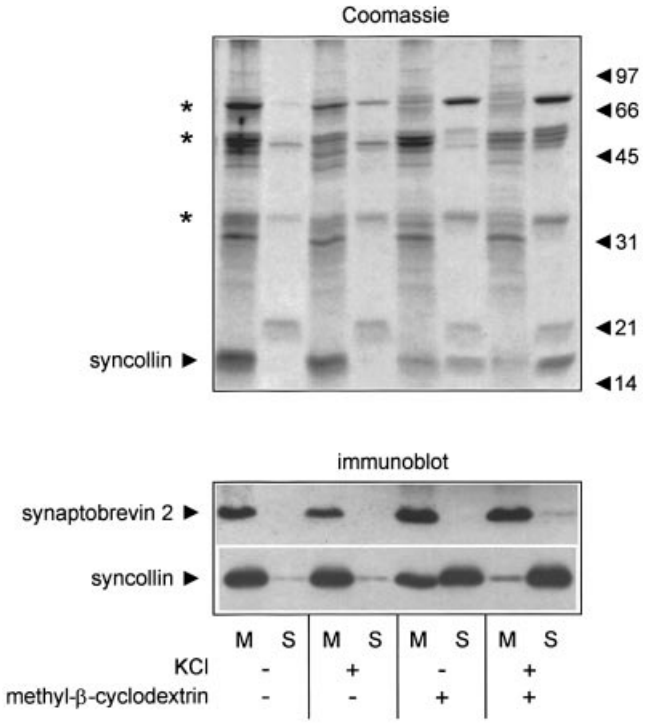

Figure 3 Cholesterol depletion causes the detachment of syncollin from the granule membrane

Zymogen granule membranes were incubated overnight at 4 °C in HBS alone or in HBS containing either 50 mg/ml MβCD or 200 mM KCl, or both. Membranes were collected by centrifugation, and equivalent fractions of membranes (M) and supernatant (S) were analysed by SDS/PAGE and either Coomassie Brilliant Blue staining or immunoblotting, using antibodies against syncollin (87.1) or synaptobrevin 2. The positions of molecular-mass markers (in kDa) are shown on the right of the Coomassie-stained gel. Syncollin is indicated, and the positions of three other proteins that become detached from the membrane after treatment with MβCD are marked with asterisks.

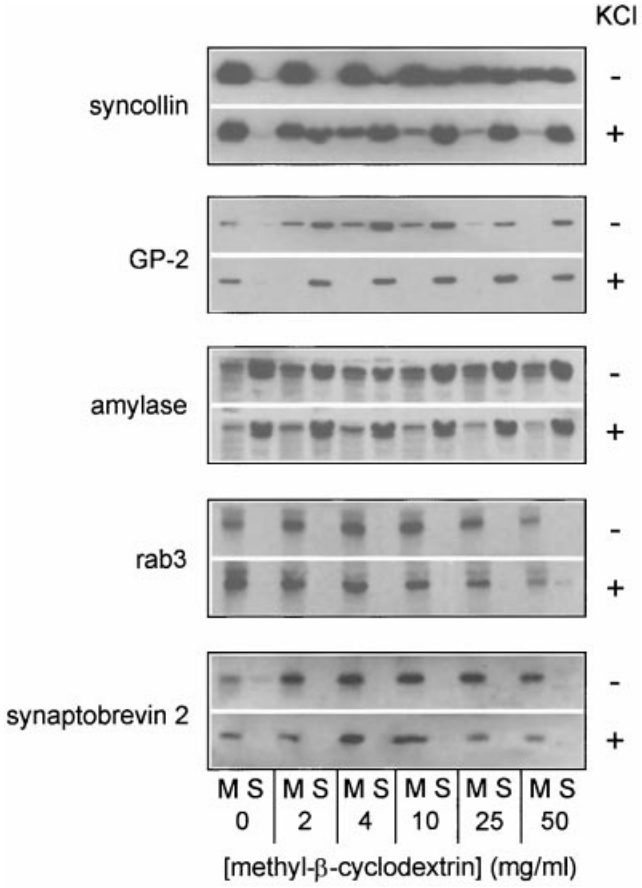

Figure 4 High-salt incubation enhances the ability of MβCD to remove syncollin from the granule membrane

Zymogen granule membranes were incubated overnight at 4 °C with various concentrations of MβCD, in either HBS alone or HBS containing 200 mM KCl. Membranes were collected by centrifugation, and equivalent fractions of membranes (M) and supernatant (S) were analysed by SDS/PAGE and immunoblotting, using antibodies against syncollin (87.1), GP-2, amylase, rab3 and synaptobrevin 2.

possibility we incubated the membranes overnight with the cholesterol-chelating agent MβCD [13,14], either in the presence or absence of 200 mM KCl. We found that MβCD treatment resulted in an 85 % depletion of cholesterol. The membranes that had been incubated under the various conditions were collected by centrifugation, and the proteins in the membrane pellets and the supernatants were analysed. As shown in Figure 3, the distribution of most proteins between the membrane pellet and the supernatant was not affected by treatment of the membranes with MβCD. However, four prominent proteins, of apparent molecular masses 16, 35, 58 and 75 kDa, underwent significant transfer from membranes to supernatant during treatment with MβCD. In all cases, high-salt treatment increased the efficiency of removal of the proteins from the membranes by MβCD. The immunoblot of the same fractions shows that the protein running at 16 kDa is syncollin (Figure 3). In contrast with syncollin, synaptobrevin 2 remained bound to the membranes irrespective of treatment with MβCD or high salt.

As shown in Figure 4, syncollin became progressively detached from the membranes as the concentration of MβCD was raised. Furthermore, this detachment occurred at lower MβCD concentrations when the membranes were incubated in buffer containing 200 mM KCl. The major granule-membrane glycoprotein GP-2, which is attached to the luminal surface of the membrane by a glycosylphosphatidylinositol (GPI) anchor [15], was removed from the membrane at even lower concentrations of MβCD than syncollin, and once again high-salt incubation enhanced the effect of the MβCD. Since the molecular mass of GP-2 is approx. 75 kDa [15], it is likely that the largest Coomassie Brilliant Blue-stained protein that is removed from the membrane in Figure 3 is in fact GP-2. The granule-content protein amylase was predominantly in the supernatant at all concentrations of MβCD, and high-salt incubation removed a further proportion of this protein from the membrane. The protein rab3, which is anchored to the membrane by two geranylgeranyl moieties [16], was found exclusively in the membrane at all concentrations of MβCD, except in the presence of high salt, when the highest concentration of MβCD did cause the removal of a proportion of the protein. Finally, as in Figure 3, synaptobrevin 2 remained exclusively in the membrane fraction throughout.

The results presented so far indicate that syncollin binds to the granule membrane through an interaction with cholesterol. Furthermore, the effect of the high-salt incubation suggests that the binding of syncollin to the membrane might also be stabilized by electrostatic interactions. To test directly the idea that syncollin binds to lipids, we determined whether it was able to migrate with liposomes on sucrose floatation gradients. Initially,

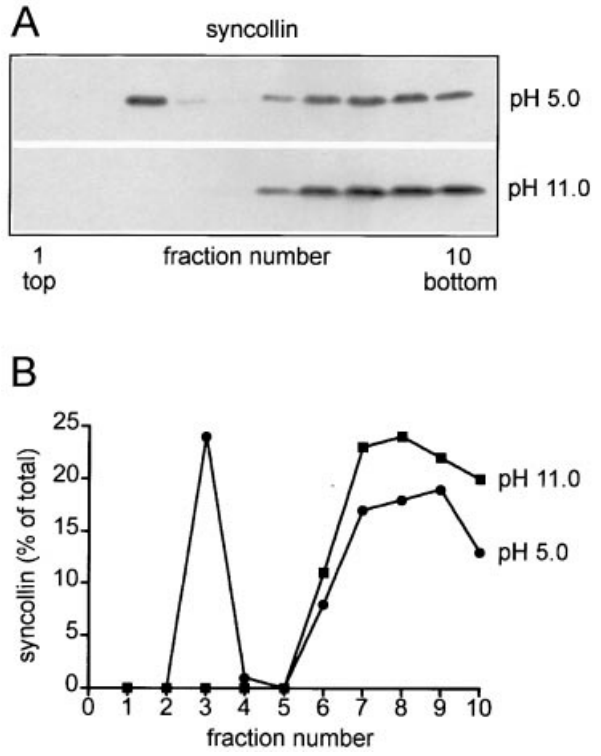

Figure 5 Syncollin binds to brain liposomes

(**A**) Purified syncollin (200 ng) was incubated with 1 mg of brain liposomes for 30 min at 4 °C, at either pH 5.0 or pH 11.0. The liposomes were then floated on a sucrose density gradient (total volume, 5 ml) at the same pH. Fractions (500 μl) were taken from the top of the gradient. Syncollin was detected by SDS/PAGE and immunoblotting, using antibody 87.1. (**B**) Profiles of the distribution of syncollin in the gradients shown in (**A**), obtained by densitometric scanning of the immunoblot. ●, pH 5.0; ■, pH 11.0.

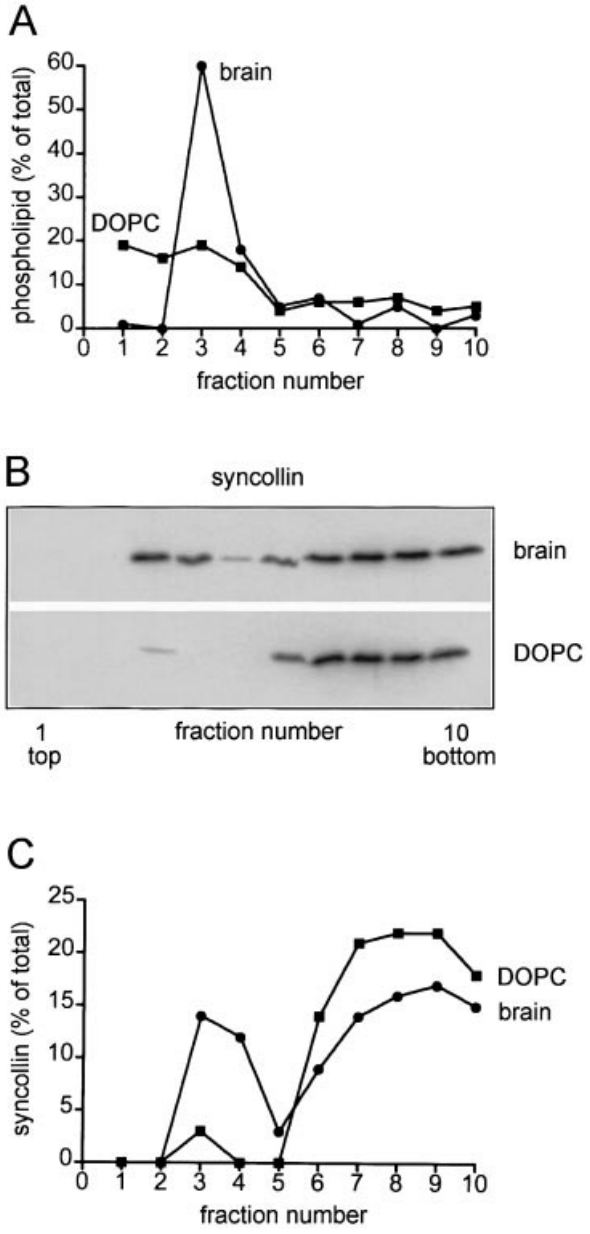

Figure 6 Syncollin binds poorly to DOPC liposomes

(**A**) Liposomes were prepared from either brain lipids (●) or DOPC (■), and in each case 1 mg of lipid was run on a sucrose density gradient (total volume, 5 ml). Fractions (500 μl) were taken from the top of the gradient and assayed for phospholipids. (**B**) Purified syncollin (200 ng) was incubated with 1 mg of either brain or DOPC liposomes at pH 5.0. The liposomes were then floated on a sucrose density gradient (total volume, 5 ml) at the same pH. Fractions (500 μl) were taken from the top of the gradient. Syncollin was detected by SDS/PAGE and immunoblotting, using antibody 87.1. (**C**) Profiles of the distribution of syncollin in the gradients shown in (**B**), obtained by densitometric scanning of the immunoblot. ●, Brain liposomes; ■, DOPC liposomes.

we prepared liposomes from brain lipids. It has been reported previously that synaptosome lipids have a cholesterol/phospholipid ratio of approx. 1:3 (w/w), or 1:1.5 (mol/mol) [17]. We found that cholesterol accounts for 28% (w/w) of the brain lipid extract used in our experiments, giving a ratio of cholesterol to other lipids in close agreement with the reported values. This ratio is also close to the value reported for zymogen granule membranes {approx. 1:4 (w/w) or 1:2 (mol/mol) [18]}. Hence, brain lipids provide a suitable model for zymogen granule lipids, with respect to their cholesterol content. Brain liposomes were incubated with taurodeoxycholate-solubilized syncollin at pH 5.0 (chosen to mimic the acidic conditions in the zymogen granule lumen [19]), and then placed at the bottom of a discontinuous sucrose gradient. At pH 5.0, 25% of the total syncollin in the gradient was found in fractions 3 and 4 after centrifugation, with the remainder staying in the loading zone (fractions 6–10, Figure 5). The majority of the brain lipids in the gradient also migrated to fractions 3 and 4 (see Figure 6A). Hence, syncollin does bind to lipids at the pH it normally experiences in the zymogen granule. In contrast, at pH 11.0, a condition that is known to remove syncollin from granule membranes [6], no syncollin was detected in fractions 3 and 4. There was no difference in the binding of syncollin to liposomes between pH 5.0 and pH 7.6 (results not shown). Further, since it is known that Ca^{2+} modulates some of the properties of syncollin, such as its ability to bind to syntaxin and its sensitivity to trypsin [5], we looked to see whether its binding to liposomes was dependent on Ca^{2+}, but found that it was not (results not shown).

To determine whether the binding of syncollin to liposomes depends on their lipid composition, we again used sucrose-density-gradient centrifugation to compare the ability of syncollin to bind to liposomes prepared from brain lipids with binding to liposomes made from DOPC. The distribution of phospholipids

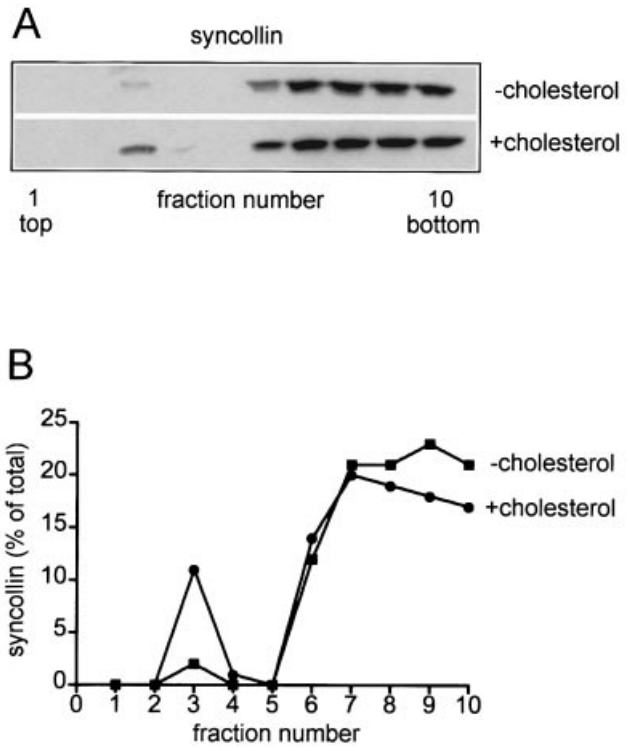

Figure 7 Cholesterol addition enhances syncollin binding to DOPC liposomes

(**A**) Purified syncollin (200 ng) was incubated at pH 5.0 with 1 mg of DOPC liposomes prepared either with or without 0.5 mg of cholesterol. The liposomes were then floated on a sucrose density gradient (total volume, 5 ml) at the same pH. Fractions (500 μl) were taken from the top of the gradient. Syncollin was detected by SDS/PAGE and immunoblotting, using antibody 87.1. (**B**) Profiles of the distribution of syncollin in the gradients shown in (**A**), obtained by densitometric scanning of the immunoblot. ●, With cholesterol; ■, without cholesterol.

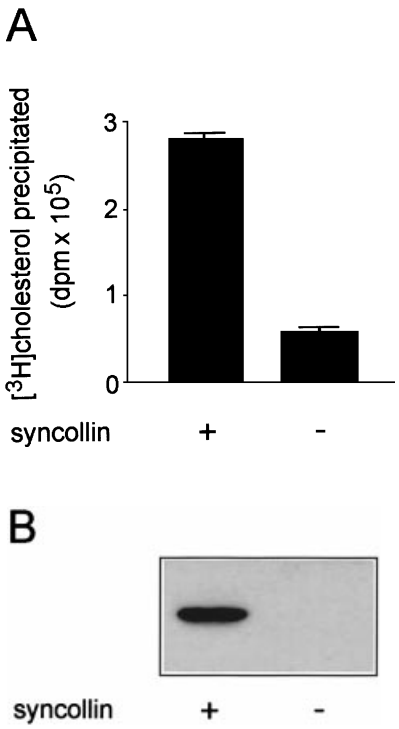

Figure 8 Cholesterol co-immunoprecipitates with syncollin

Syncollin (0.5 μg per sample), or buffer alone, was incubated with [^{3}H]cholesterol. Syncollin was then immunoprecipitated using anti-syncollin antibody B followed by Protein G–Sepharose. The immunoprecipitates were analysed for both [^{3}H]cholesterol content (**A**) and the presence of syncollin (**B**), by SDS/PAGE and immunoblotting, using anti-syncollin antibody 87.1. Data are means ± S.E.M. ($n = 4$).

in the sucrose gradients is shown in Figure 6(A). For brain liposomes, 79 % of the total phospholipid in the gradient was found in fractions 1–4, with the vast majority in fractions 3 and 4 (i.e. at the interface between HBS alone and HBS containing 50 % sucrose). For DOPC liposomes, 69 % of the phospholipid was found in fractions 1–4, although in this case the phospholipid was more evenly distributed between the top four fractions of the gradient. When brain liposomes were used, syncollin in fractions 3 and 4 accounted for 26 % of the total in the gradient (Figures 6B and 6C), in close agreement with the result presented in Figure 5. In contrast, when DOPC liposomes were used, only 3 % of the total syncollin was recovered in fractions 3 and 4. Hence, the composition of the liposomes is a critical determinant of syncollin binding.

In light of the apparent dependence of syncollin binding to granule membranes on cholesterol, we determined whether syncollin binding to DOPC liposomes could be enhanced by provision of cholesterol. It has been reported that the majority of the cholesterol in the membranes of mature zymogen granules is present in the luminal leaflet of the bilayer [12]; hence, the cholesterol/phospholipid ratio in this leaflet will be about 1:2 (w/w), or 1:1 (mol/mol) [18]. Accordingly, liposomes were made from a 1:2 (w/w) mixture of cholesterol/DOPC, and incubated with syncollin as usual. It was found that the addition of cholesterol increased the percentage of syncollin found in fractions 3 and 4 of the gradient from 2 % to 12 % (Figure 7).

In order to obtain evidence for a direct interaction between syncollin and cholesterol, we determined whether cholesterol could be co-immunoprecipitated with syncollin. Syncollin, solubilized in taurodeoxycholate, was incubated with [^{3}H]-cholesterol. The syncollin was then immunoprecipitated with anti-syncollin antibody B, and the amount of [^{3}H]cholesterol present in the precipitate was counted. As shown in Figure 8, [^{3}H]cholesterol did indeed co-precipitate with syncollin. The extent of the co-precipitation was approx. 10 % of the total cholesterol added to the incubation. In the absence of syncollin, the amount of [^{3}H]cholesterol found in the pellet was significantly ($P < 0.001$) reduced. This result is indicative of a direct interaction between syncollin and cholesterol.

DISCUSSION

The N-terminal signal sequence of syncollin directs the nascent protein into the lumen of the endoplasmic reticulum, where it enters the secretory pathway [6,8]. According to the results presented here, by the time the protein has reached the zymogen granule, more than half of it has become tightly attached, via cholesterol, to the luminal surface of the granule membrane. Further, the membrane-bound form of syncollin behaves as a complex of molecular mass approx. 100 kDa. The fact that high-salt treatment enhances the ability of MβCD to remove syncollin from the granule membrane indicates that syncollin is able to interact with either other proteins or phospholipids, and that this interaction might stabilize its association with the membrane.

The identity of its binding partner(s) remains unclear, although one candidate is GP-2, a major glycoprotein of the granule membrane. This protein is also removed from the membrane by MβCD, and high-salt treatment again enhances its removal.

It was shown some time ago [12], using filipin binding combined with freeze–fracture electron microscopy, that the majority of the cholesterol in the membranes of immature pancreatic zymogen granules was in the cytoplasmic leaflet of the bilayer, but that as the granules matured, the cholesterol became relocated to the luminal leaflet. This would of course favour the binding of syncollin to the granule membrane as the granule develops. It is known that, in other membranes, cholesterol, usually in association with sphingolipids, associates laterally to form so-called rafts, into which particular proteins are specifically incorporated [20]. Whether syncollin interacts with rafts in the zymogen granule membrane is certainly worth exploring. Interestingly, the cholesterol-dependent attachment of GP-2 to the granule membrane might point to the involvement of rafts, since these structures are known to recruit GPI-linked proteins [21].

The role of syncollin, and particularly the significance of its cholesterol-dependent association with the granule membrane, remains obscure. Intriguingly, a number of pore-forming bacterial proteins, including perfringolysin-O and pneumolysin, have been proposed to utilize cholesterol as a membrane receptor [22]. In these cases, cholesterol appears to play multiple roles, including promoting oligomerization, triggering membrane insertion and stabilizing the pore. These proteins can also be solubilized in their oligomeric forms using deoxycholate [23]. The protein α-haemolysin is secreted as a 34 kDa monomer by *Staphylococcus aureus*; it then binds to membranes and oligomerizes, forming heptameric trans-membrane pores [24,25]. The oligomeric form of α-haemolysin is able to self-associate in deoxycholate but not in Triton X-100, properties which are reminiscent of syncollin [6]. In another parallel with syncollin, α-haemolysin multimers are extremely resistant to proteases.

Several eukaryotic proteins are also known to oligomerize and to associate reversibly with biological membranes. These proteins include annexin V, which inserts into membranes in the presence of peroxide [26] to form Ca^{2+}-permeable pores. In addition, two proteins found in synaptic vesicles, synaptophysin and the c-subunit of the vesicular proton ATPase (V-ATPase), are both known not only to form homo-oligomers but also to bind cholesterol [13]. Synaptophysin is a 38 kDa protein that is believed to form either homotetramers [27] or homohexamers [28], and has been reported to form pores in membranes [28]. The c-subunit of the V-ATPase is a 17 kDa protein that also penetrates the membrane as a homohexamer [29] and, like syncollin, can be removed from the membrane by alkaline extraction [30]. This protein is of particular interest because it appears to have alternative locations and functions. For instance, it is identical with the mediatophore, a protein from the *Torpedo* electric organ that has been reported to form an acetylcholine-permeable channel in the presynaptic plasma membrane [30,31]. It is also identical with ductin, a putative subunit of intercellular gap junctions [29]. In addition to their oligomeric structures and their interaction with cholesterol, synaptophysin and the V-ATPase c-subunit share another property with syncollin, the ability to bind to SNARE proteins. Whereas syncollin is known to bind to syntaxin *in vitro*, both synaptophysin and the V-ATPase bind to synaptobrevin in synaptic vesicles [9,32].

The parallels between the properties of syncollin and those of the other proteins discussed above suggests that syncollin, too, might be able to form pores in the zymogen granule membrane. This, of course, remains to be established. There has, in fact, been a report, based on a freeze–fracture study of the membranes of zymogen granules, that pores do exist in these membranes [33]. The function of the pores is unclear, although it has been suggested that the granule membrane is permeable even to proteins [34,35]. Finally, if syncollin is indeed able to span the membrane under certain circumstances, then it might after all be able to interact with syntaxin at the cytoplasmic surface.

We thank Reinhard Jahn (Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany) for the gift of antibodies to synaptobrevin 2 and rab3, and Andrew Letcher (Department of Pharmacology, University of Cambridge, Cambridge, U.K.) for help with the phospholipid assays. This work was supported by project grant 053024 from the Wellcome Trust (to J.M.E.). N.J.H. was the recipient of an MRC studentship. J.L. is the recipient of a Cambridge Commonwealth Trust studentship. M.S. is supported by the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg, Germany (SFB 286/B2) and the P. E. Kempkes Foundation (29/99), Marburg, Germany.

REFERENCES

1 Palade, G. E. (1975) Intracellular aspects of protein synthesis. Science **189**, 347–358
2 Kasai, H., Li, Y. X. and Miyashita, Y. (1993) Subcellular distribution of Ca^{2+} release channels underlying Ca^{2+} waves and oscillations in exocrine pancreas. Cell **74**, 669–677
3 Thorn, P., Lawrie, A. M., Smith, P. M., Gallacher, D. V. and Petersen, O. H. (1993) Local and global cytosolic Ca^{2+} oscillations in exocrine cells evoked by agonist and inositol trisphosphate. Cell **74**, 661–668
4 Hansen, N. J., Antonin, W. and Edwardson, J. M. (1999) Identification of SNAREs involved in regulated exocytosis in the exocrine pancreas. J. Biol. Chem. **274**, 22871–22876
5 Edwardson, J. M., An, S. and Jahn, R. (1997) The secretory granule protein syncollin binds to syntaxin in a Ca^{2+}-sensitive manner. Cell **90**, 325–333
6 An, S. J., Hansen, N. J., Hodel, A., Jahn, R. and Edwardson, J. M. (2000) Analysis of the association of syncollin with the membrane of the pancreatic zymogen granule. J. Biol. Chem. **275**, 11306–11311
7 Tan, S. and Hooi, S. C. (2000) Syncollin is differentially expressed in rat proximal small intestine and regulated by feeding behaviour. Am. J. Physiol. **278**, G308–G320
8 Hodel, A. and Edwardson, J. M. (2000) Targeting of the zymogen-granule protein syncollin in AR42J and AtT-20 cells. Biochem. J. **350**, 637–643
9 Edelmann, L., Hanson, P. I., Chapman, E. R. and Jahn, R. (1995) Synaptobrevin binding to synaptophysin: a potential mechansim for controlling the exocytotic fusion machine. EMBO J. **14**, 224–231
10 Matteoli, M., Takei, K., Cameron, P., Hurlbut, P., Johnson, P. A., Südhof, T. C., Jahn, R. and De Camilli, P. (1991) Association of rab3A with synaptic vesicles at late stages of the secretory pathway. J. Cell Biol. **115**, 625–633
11 Nadin, C. Y., Rogers, J., Tomlinson, S. and Edwardson, J. M. (1989) A specific interaction *in vitro* between pancreatic zymogen granules and plasma membranes: stimulation by G-protein activators but not by Ca^{2+}. J. Cell Biol. **109**, 2801–2808
12 Orci, L., Miller, R. G., Montesano, R., Perrelet, A., Amherdt, M. and Vassalli, P. (1980) Opposite polarity of filipin-induced deformations in the membrane of condensing vacuoles and zymogen granules. Science **210**, 1019–1021
13 Thiele, C., Hannah, M. J., Fahrenholz, F. and Huttner, W. B. (2000) Cholesterol binds to synaptophysin and is required for biogenesis of synaptic vesicles. Nat. Cell Biol. **2**, 42–49
14 Nelson, K. L. and Buckley, J. T. (2000) Channel formation by the glycosyl-phosphatidylinositol-anchored protein binding toxin aerolysin is not promoted by lipid rafts. J. Biol. Chem. **275**, 19839–19843
15 Fukuoka, S., Freedman, S. D. and Scheele, G. A. (1991) A single gene encodes membrane-bound and free forms of GP-2, the major glycoprotein in pancreatic secretory (zymogen) granule membranes. Proc. Natl. Acad. Sci. U.S.A. **88**, 2898–2902
16 Novick, P. and Zerial, M. (1997) The diversity of Rab proteins in vesicle transport. Curr. Opin. Cell Biol. **9**, 496–504
17 Hrboticky, N., MacKinnon, M. J., Puterman, M. L. and Innis, S. M. (1989) Effect of linoleic acid-rich infant formula feeding on brain synaptosomal lipid accretion and enzyme thermotropic behavior in the piglet. J. Lipid. Res. **30**, 1173–1184
18 Beaudoin, A. R. and Grondin, G. (1992) Zymogen granules of the pancreas and the parotid gland and their role in cell secretion. Int. Rev. Cytol. **132**, 177–221
19 Titievsky, A. V., Takeo, T., Tepikin, A. V. and Petersen, O. H. (1996) Decrease of acidity inside zymogen granules inhibits acetylcholine- or inositol trisphosphate-evoked cytosolic Ca^{2+} spiking in pancreatic acinar cells. Pflügers Arch. **432**, 938–940
20 Simons, K. and Ikonen, E. (1997) Functional rafts in cell membranes. Nature (London) **387**, 569–572

21 Brown, D. and Rose, J. (1992) Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell **68**, 533–544
22 Rossjohn, J., Feil, S. C., McKinstry, W. J., Tweten, R. K. and Parker, M. W. (1997) Structure of a cholesterol-binding, thiol-activated cytolysin and a model of its membrane form. Cell **89**, 685–692
23 Morgan, P. J., Hyman, S. C., Byron, O., Andrew, P. W., Mitchell, T. J. and Rowe, A. J. (1994) Modelling the bacterial protein toxin, pneumolysin, in its monomeric and oligomeric form. J. Biol. Chem. **269**, 25315–25320
24 Bhakdi, S., Füssle, R. and Tranum-Jensen, J. (1981) Staphylococcal α-toxin: oligomerisation of hydrophilic monomers to form amphiphilic hexamers induced through contact with deoxycholate detergent micelles. Proc. Natl. Acad. Sci. U.S.A. **78**, 5475–5479
25 Song, L., Hobaugh, M. R., Shistak, C., Cheley, S., Bayley, H. and Gouaux, J. E. (1996) Structure of α-hemolysin, a heptameric transmembrane pore. Science **274**, 1859–1866
26 Kubista, H., Hawkins, T. E., Patel, D. R., Haigler, H. T. and Moss, S. E. (1999) Annexin 5 mediates a peroxide-induced Ca^{2+} influx in B cells. Curr. Biol. **9**, 1403–1406
27 Rehm, H., Wiedenmann, B. and Betz, H. (1986) Molecular characterization of synaptophysin, a major calcium-binding protein of the synaptic vesicle membrane. EMBO J. **5**, 535–541
28 Thomas, L., Hartung, K., Langosch, D., Rehm, H., Bamberg, E., Franke, W. W. and Betz, H. (1988) Identification of synaptophysin as a hexameric channel protein of the synaptic vesicle membrane. Science **242**, 1050–1053
29 Finbow, M. E., Harrison, M. and Jones, P. (1995) Ductin – a proton pump component, a gap junction channel and a neurotransmitter release channel. BioEssays **17**, 247–255
30 Israël, M., Morel, N., Lesbats, B., Birman, S. and Manaranche, R. (1986) Purification of a presynaptic membrane protein that mediates a calcium-dependent translocation of acetylcholine. Proc. Natl. Acad. Sci. U.S.A. **83**, 9226–9230
31 Israël, M., Lesbats, B., Morel, N., Manaranche, R., Gulik-Krzywick, T. and Dedieu, J. C. (1984) Reconstitution of a functional synaptosomal membrane possessing the protein constituents involved in acetylcholine translocation. Proc. Natl. Acad. Sci. U.S.A. **81**, 277–281
32 Galli, T., McPherson, P. S. and De Camilli, P. (1996) The V_0 sector of the V-ATPase, synaptobrevin, and synaptophysin are associated on synaptic vesicles in a Triton X-100-resistant, freeze-thawing sensitive, complex. J. Biol. Chem. **271**, 2193–2198
33 Cabana, C., Magny, P., Nadeau, D., Grondin, G. and Beaudoin, A. (1987) Freeze-fracture study of the zymogen granule membrane of pancreas: two novel types of intramembrane particles. Eur. J. Cell Biol. **45**, 246–255
34 Goncz, K. K. and Rothman, S. S. (1992) Protein flux across the membrane of single secretion granules. Biochim. Biophys. Acta **1109**, 7–16
35 Goncz, K. K. and Rothman, S. S. (1995) A trans-membrane pore can account for movement across zymogen granule membranes. Biochim. Biophys. Acta **1238**, 91–93

Received 12 December 2000/23 March 2001; accepted 10 April 2001