Analysis of the Association of Syncollin with the Membrane of the Pancreatic Zymogen Granule*

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Syncollin is a pancreatic zymogen granule protein that was isolated through its ability to bind to syntaxin. Here we show that syncollin has a cleavable signal sequence and can be removed from granule membranes by washing with sodium carbonate. When membranes were subjected to Triton X-114 partitioning, syncollin was found predominantly in the aqueous phase, indicating that it is not sufficiently hydrophobic to be embedded in the membrane. Syncollin has intramolecular disulfide bonds and was accessible to water-soluble cross-linking and biotinylating reagents only when granules were lysed by sonication. These results indicate that syncollin is tightly bound to the luminal surface of the granule membrane. In situ, syncollin was resistant to proteases such as trypsin. When granule membranes were solubilized in ionic detergents such as deoxycholate, this trypsin resistance was maintained, and syncollin migrated on sucrose density gradients as a large (150 kDa) protein. In contrast, in non-ionic detergents such as Triton X-100, syncollin became partially sensitive to trypsin and behaved as a monomer. Syncollin in alkaline extracts of granule membranes was also monomeric. However, reduction of the pH regenerated the oligomeric form, which was insoluble. We conclude that syncollin exists as a homo-oligomer and that its ability to self-associate can be reversibly modulated via changes in pH. In light of our findings, we reassess the likely role of syncollin in the pancreatic acinar cell.

The exocrine pancreas is specialized for the synthesis and secretion of digestive enzymes (1). The proteins are synthesized in the rough endoplasmic reticulum, transported via the Golgi apparatus, and ultimately packaged into zymogen granules. Secretagogues such as acetylcholine and cholecystokinin act on receptors at the basolateral pole of the acinar cells to cause a release of Ca2+ from intracellular stores (2, 3). This leads, in turn, to the fusion of the zymogen granules with the apical domain of the plasma membrane and the release of digestive enzymes into the acinar lumen (1). In recent years, several of the steps involved in stimulus-secretion coupling have been studied in great detail. For example, the link between receptor activation and the cytoplasmic Ca2+ signal has been extensively characterized. However, it is still unclear how Ca2+ mediates exocytotic membrane fusion. In particular, the identity of the Ca2+ sensor and the mechanism by which it communicates with the membrane fusion machinery are still unknown.

In recent years, evidence has accumulated that all intracellular fusion events are mediated by a common group of proteins referred to as SNAREs (4–6). These proteins are small and mostly membrane-anchored molecules that spontaneously form tight complexes, which are reversibly disassembled by the chaperone N-ethylmaleimide-sensitive fusion protein in conjunction with cofactors. The neuronal variants are among the best characterized: they include the synaptic vesicle protein synaptobrevin (also referred to as vesicle-associated membrane protein) and the plasma membrane proteins SNAP-25 and syntaxin 1. Based on the identity of a highly conserved amino acid in the center of the complex, SNARE proteins are classified as R-SNAREs (synaptobrevin, vesicle-associated membrane proteins, and relatives) and Q-SNAREs (SNAP-25 and syntaxin families) (5). For fusion, Q- and R-SNAREs need to cooperate to form a tight complex (6). In the pancreatic acinar cell, synaptobrevin 2 is so far the only R-SNARE known to be present on the zymogen granule membrane (7, 8). At least three syntaxin isoforms are also present: syntaxin 2 on the apical plasma membrane, syntaxin 4 on the basolateral membrane, and syntaxin 3 on the granule membrane (9). SNAP-23, the non-neuronal isoform of SNAP-25, is found on the basolateral domain of the plasma membrane (10). By testing the effects of botulinum neurotoxin C1 on zymogen granule-plasma membrane fusion and granule-granule fusion in vitro, we have shown that syntaxin 2 is the isoform that mediates fusion between zymogen granules and the apical plasma membrane, whereas syntaxin 3 mediates homotypic fusion of granules (8). Surprisingly, experiments with tetanus toxin showed that synaptobrevin 2 was responsible for only a minor component of fusion between zymogen granules and plasma membranes, leaving open the identity of the major R-SNARE on the granule membrane (7, 8).

Recently, we identified a novel zymogen granule protein, syncolin, through its high affinity binding to syntaxin 1A (11). Syncollin binds to syntaxin in a Ca2+-sensitive manner and inhibits zymogen granule-plasma membrane fusion, making it a candidate for a Ca2+-dependent regulator protein in pancreatic exocytosis. Syncollin is a small protein (molecular mass of 16 kDa) that is specifically expressed in exocrine secretory cells and highly enriched in zymogen granule membrane fractions. The protein is tightly associated with the granule membrane.

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1 The abbreviations used are: SNAREs, soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors; SNAP, synaptosome-associated protein; PAGE, polyacrylamide gel electrophoresis; biotin-NHS, biotin N-hydroxysuccinimide ester; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.
but the nature of this interaction and the membrane orientation of syncollin have not so far been established.

In this study, we have used a variety of approaches to examine the membrane association and biochemical properties of syncollin. In contrast to our previous conclusions, we now report that the N-terminal domain of syncollin represents a cleavable signal sequence rather than a membrane anchor domain. In agreement with this finding, the protein is associated with the luminal rather than the cytoplasmic side of the granule membrane. Furthermore, the protein in its native state appears to exist as a large homo-oligomer. In light of these data, the role of the protein in the pancreatic acinar cell needs to be reconsidered.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Antibodies used were as follows: FG3, a rabbit polyclonal antibody against an internal peptide sequence from syncollin (11); 87.1, a mouse monoclonal antibody against recombinant syncollin; 69.1, a mouse monoclonal antibody against synaptobrevin 2 (12); 77.1, a mouse monoclonal against α-SNAP (13); and anti-procarboxypeptidase, a rabbit polyclonal antibody against the appropriate band excised from an SDS-polyacrylamide gel of zymogen granule proteins.

**SDS-PAGE and Immunoblotting**—Zymogen granules were isolated and either left undisturbed or sonicated briefly for 3 min. The detergent (lower) and aqueous (upper) phases were recovered separately, and proteins were precipitated with 50% sucrose (6%) cushion containing 0.06% Triton X-114 and centrifuged at 100,000 × g for 3 min. The detergent (lower) and aqueous (upper) phases were then sectioned onto a warmed 30% sucrose cushion containing 0.06% Triton X-114 (Pierce & Warriner) by incubation for 15 min at 4 °C with agitation. Unsolubilized material was pelleted by centrifugation at 100,000 × g for 1 h.

**In Vitro Transcription/Translation**—Coupled transcription/translation reactions were performed with the TNT T7 transcription system and a rabbit reticulocyte lysate according to the manufacturer's instructions (Promega, Southampton, United Kingdom). A typical reaction mixture (25 μl) contained 55% TNT T7 reticulocyte lysate, 0.25 units of T7 RNA polymerase, 1 mM amino acid mixture without t-cysteine, 10 μCi [35S]methionine, 10 units of RNasin, 1 μg/ml DNA substrate in TNT reaction buffer, and dog pancreas microsomes (Promega) where appropriate. Incubations were for 90 min at 30 °C. Proteins were analyzed by SDS-PAGE. Radioactive bands were visualized using horseradish peroxidase-conjugated secondary antibodies (1:1000) and enhanced chemiluminescence (Pierce & Warriner, Chester, United Kingdom). Gated secondary antibodies (1:1000) and enhanced chemiluminescence (Pierce & Warriner, Chester, United Kingdom).

**Syncollin Has a Cleavable Signal Sequence**—For the initial molecular cloning of syncollin (11), the protein was digested with Lys-C, and two internal peptide sequences were obtained. Based on one of these sequences, polymerase chain reaction primers were designed and used to amplify a sequence from a λZAP rat pancreatic cDNA library. A typical reaction mixture (25 μl) contained 55% TNT T7 reticulocyte lysate, 0.25 units of T7 RNA polymerase, 1 mM amino acid mixture without t-cysteine, 10 μCi [35S]methionine, 10 units of RNasin, 1 μg/ml DNA substrate in TNT reaction buffer, and dog pancreas microsomes (Promega) where appropriate. Incubations were for 90 min at 30 °C. Proteins were analyzed by SDS-PAGE. Radioactive bands were visualized using a Molecular Dynamics PhosphorImager with ImageQuant software.

**RESULTS**

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into the type of membrane anchorage, we subjected zymogen granule membranes to three conditions that are commonly used to remove peripherally bound proteins: 1 mM sodium chloride, 0.6 mM potassium iodide, and 0.1 mM sodium carbonate. As shown in Fig. 2A, syncollin remained largely (but not exclusively) associated with the membrane pellet after NaCl and KI washing. However, washing of membranes with carbonate buffer at pH 11.5 removed most of the syncollin into the supernatant. In contrast, the integral membrane protein synaptobrevin 2 remained bound to the membrane under all three conditions.

To examine further the conditions required to remove syncollin from the membrane, we incubated granule membranes in carbonate solutions of varying pH values. We found that syncollin resisted dissociation from the membranes until the pH exceeded 10.0 (Fig. 2B). In contrast, the secretory protein amylase was partially removed from the membranes even at pH 7.6 and was almost completely removed at pH 10.0.

We also tested the partitioning of syncollin during phase separation of Triton X-114 (15). Only proteins with hydrophobic domains large enough to bind detergent micelles such as transmembrane domains or hydrophobic side chains will accumulate in the detergent phase after phase separation. Zymogen granule membranes were solubilized in Triton X-114, and proteins in the aqueous and detergent phases were precipitated with 50% acetone. It was clear from both a Coomassie Blue-stained gel and an immunoblot that syncollin partitioned predominantly into the aqueous phase (Fig. 3), indicating that it does not contain a hydrophobic membrane anchor domain.

Syncollin Has Intramolecular Disulfide Bonds—Syncollin contains seven cysteine residues. If the protein is indeed exposed on the luminal side of the zymogen granule membrane, it is likely that at least some of these cysteines will be disulfide-bonded to each other. To test this possibility, we looked for a change in the mobility of syncollin on SDS-PAGE after treatment with the reducing agent β-mercaptoethanol. Zymogen granule membranes were solubilized in sample buffer with or without β-mercaptoethanol (10%), incubated at 25 °C, and subjected to SDS-PAGE. On a Coomassie Blue-stained gel, some of the proteins in the “minus β-mercaptoethanol” lane migrated as diagonal bands, presumably because of reduction of disulfide bonds by β-mercaptoethanol diffusing out of the “plus” lane (Fig. 4A). The corresponding immunoblot shows that syncollin is one of these diagonally migrating bands and is therefore likely to be disulfide-bonded. To check that disulfide bond formation was not occurring during the preparation of the zymogen granule membranes, a second experiment was carried out in which 100 mM N-ethylmaleimide was included throughout. As shown in Fig. 4B, the presence of N-ethylmaleimide did not affect the mobility shift seen with β-mercaptoethanol. Hence, syncollin does indeed appear to have intramolecular disulfide bonds.

Syncollin Is Accessible to Water-soluble Reagents Only in Lysed Granules—If syncollin is associated with the luminal face of the zymogen granule membrane, syncollin in intact granules should be inaccessible to water-soluble protein-modifying reagents that cannot penetrate membranes. In a preliminary experiment to monitor granule integrity, we determined the sensitivity of three proteins to trypsin. We found that during an incubation for 30 min at 4 °C, the content protein procarboxypeptidase remained resistant to cleavage unless the granules had been sonicated, whereas the cytoplasmically oriented SNARE synaptobrevin 2 was cleaved by trypsin in both unsonicated and sonicated granules (Fig. 5A). However, the unsonicated granules are extremely stable. As expected from previous studies (11), syncollin was resistant to proteolysis under all circumstances. We then tested the accessibility of syncollin to the cross-linker bis(sulfosuccinimidyl) suberate and the biotinylating reagent biotin-NHS. Zymogen granules were isolated and either left undisturbed or sonicated briefly to cause lysis. They were then incubated with either bis(sulfosuccinimidyl) suberate or biotin-NHS for 30 min at 4 °C. As shown in Fig. 5B, syncollin could be cross-linked to form higher order adducts only when the granules had been sonicated. The major adducts seen had molecular masses of ∼40 and 60 kDa, consistent with the formation of syncollin trimers and tetramers (see below). In a similar experiment (Fig. 5C), syncollin was precipitated by immobilized avidin only when biotin-NHS had access to the interior of the granule as a result of prior sonication.

Syncollin Exists as a Multimer—We showed previously that syncollin on intact granule membranes is resistant to trypsin and that even when the membranes are solubilized in Triton...
Zymogen granule membranes were incubated with or without β-mercaptoethanol for 5 min at 25 °C and then subjected to electrophoresis. Total granule membrane proteins were visualized by Coomassie Blue staining, and syncollin was detected by immunoblotting using antibody FG3. The positions of molecular mass markers (in kilodaltons) are indicated on the left. β, zymogen granule membranes were prepared in the presence or absence of N-ethylmaleimide (NEM; 100 mM). Membranes were incubated in sample buffer with or without β-mercaptoethanol for 5 min at 25 °C and then subjected to SDS-PAGE. Syncollin was detected by immunoblotting using antibody FG3. The positions of molecular mass marker proteins. As shown in Fig. 7, syncollin in Triton X-100 ran as a monomer, but syncollin in deoxycholate ran as a much larger species, with a molecular mass of ~150 kDa. The proteins synaptobrevin 2, α-SNAP, and amyrase ran identically in the two detergents (data not shown), indicating that the detergent-dependent change in sedimentation is specific to syncollin.

Analysis of the material washed from zymogen granule membranes by incubation in sodium carbonate at pH 10.5 indicated the presence of a number of proteins, including syncollin (data not shown). When the alkaline supernatant from a granule membrane preparation was dialyzed overnight against Hepes-buffered saline (pH 7.6), a milky precipitate was seen to form. When the suspension was centrifuged and the precipitate was analyzed by SDS-PAGE, it was found to consist of almost pure syncollin. This protocol was refined by the inclusion of a carbonate wash. This protocol was refined by the inclusion of a carbonate wash. This protocol was refined by the inclusion of a carbonate wash. This protocol was refined by the inclusion of a carbonate wash. This protocol was refined by the inclusion of a carbonate wash.
Syncollin and the Zymogen Granule Membrane

We have previously reported that syncollin, a major constituent of the zymogen granule membrane, binds to syntaxin 1A in a Ca$$^{2+}$$-sensitive manner (11). In fact, under appropriate conditions, syncollin binds most strongly to syntaxin 2, the major syntaxin isoform that is involved in exocytosis in the pancreas. Thus, it is likely that the interaction of syncollin with syntaxin 2 is able to prevent this Q-SNARE from forming core complexes with its (still unknown) SNARE partners, a prerequisite for fusion. This effect would explain the ability of syncollin to inhibit the in vitro fusion of zymogen granules with pancreatic plasma membranes (11). Although these properties are entirely consistent with a regulatory role of syncollin in SNARE complex formation, the data reported here question the physiological significance of the interaction of syncollin with syntaxin.

It is now clear that syncollin possesses a signal sequence that directs the protein through the membrane of the endoplasmic reticulum and is then cleaved. Syncollin acquires intramolecular disulfide bonds, which may explain, at least in part, its resistance to proteolytic breakdown. The protein eventually becomes tightly associated with the luminal surface of the zymogen granule membrane, where it is protected from exogenous water-soluble modifying reagents. The conformation of syncollin is clearly affected by detergent treatment in a differential manner. Furthermore, the protein forms higher order oligomers under certain conditions, suggesting that it may assemble into large complexes in the granule membrane.

The balance of the evidence at present suggests that syncollin is more likely to have a role within the zymogen granule (perhaps in the processing, maturation, and concentration of granule content proteins) than in the control of exocytotic membrane fusion. Nevertheless, it must be borne in mind that

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DISCUSSION

cholate (which, like deoxycholate, does not render syncollin sensitive to trypsin) (data not shown). When the solubilized protein was analyzed by sucrose density gradient centrifugation as described above, it again behaved as a large protein, with a molecular mass of ~150 kDa (Fig. 8B). Hence, syncollin appears to exist as a homo-oligomer, perhaps consisting of eight subunits. The cross-linked adducts seen in the experiment illustrated in Fig. 5 might represent the partial stabilization of such an oligomer. In contrast, syncollin in the form that was removed from the granule membrane at a pH of 10.5 behaved as a monomer (Fig. 8B).

Fig. 6. Sensitivity of syncollin to trypsin is detergent-dependent. Zymogen granule membranes were solubilized in various detergents (all at 1%) for 5 min at 37 °C. Samples were then incubated with or without trypsin for an additional 15 min. Reactions were terminated by addition of soybean trypsin inhibitor, and proteins were analyzed by SDS-PAGE, followed by immunoblotting. Syncollin and α-SNAP were detected using antibodies 87.1 and 77.1, respectively. Total granule membrane proteins were visualized by Coomassie Blue staining. The positions of molecular mass markers (in kilodaltons) are indicated on the right.

Fig. 7. Behavior of syncollin on sucrose density gradients is detergent-dependent. Zymogen granule membranes were solubilized in either Triton X-100 or deoxycholate (both at 1%) and layered onto linear 5–15% sucrose gradients, also containing detergent. Gradients were centrifuged overnight at 180,000 × g. Fractions were collected from the top of the gradients, and alternate fractions were analyzed by SDS-PAGE, followed by immunoblotting using antibody 87.1. Molecular mass markers were run in an identical gradient, and their positions were determined by Coomassie Blue staining of the gel. The positions of cytochrome c (12.4 kDa) and alcohol dehydrogenase (150 kDa) are indicated.

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S. J. An and J. M. Edwardson, unpublished observations.
Syncollin is a highly unusual protein. The dramatic changes in the structure and properties of the protein in response to changes in pH are of particular interest. Consequently, we cannot completely exclude the possibility that under particular circumstances, syncollin is able to embed itself into the membrane to the extent that it can still interact with syntaxin 2, for instance at the point of exocytosis. There are precedents for such behavior. Annexin V, for example, inserts into membranes at low pH (19) or at neutral pH in the presence of peroxide (20) to form a Ca^{2+} channel. Further work is clearly needed to clarify the role of syncollin in the pancreatic acinar cell.

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