Distinct Cellular Locations of the Syntaxin Family of Proteins in Rat Pancreatic Acinar Cells

Herbert Y. Gaisano,^{*,†} Menisha Ghai,[‡] Per N. Malkus,[§] Laura Sheu,^{*} Antony Bouquillon,[‡] Mark K. Bennett,[§] and William S. Trimble[‡]

^{*}Department of Medicine, University of Toronto and The Toronto Hospital, Toronto, Ontario M5S 1A8, Canada; [‡]Departments of Physiology and Biochemistry and Center for Research in Neurodegenerative Diseases, University of Toronto, Toronto, Ontario M5S 1A8, Canada; and [§]Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, California 94720

Submitted June 27, 1996; Accepted September 4, 1996
Monitoring Editor: Ari Helenius

Syntaxins are cytoplasmically oriented integral membrane soluble NEM-sensitive factor receptors (SNAREs; soluble NEM-sensitive factor attachment protein receptors) thought to serve as targets for the assembly of protein complexes important in regulating membrane fusion. The SNARE hypothesis predicts that the fidelity of vesicle traffic is controlled in part by the correct recognition of vesicle SNAREs with their cognate target SNARE partner. Here, we show that in the exocrine acinar cell of the pancreas, multiple syntaxin isoforms are expressed and that they appear to reside in distinct membrane compartments. Syntaxin 2 is restricted to the apical plasma membrane whereas syntaxin 4 is found most abundantly on the basolateral membranes. Surprisingly, syntaxin 3 was found to be localized to a vesicular compartment, the zymogen granule membrane. In addition, we show that these proteins are capable of specific interaction with vesicle SNARE proteins. Their nonoverlapping locations support the general principle of the SNARE hypothesis and provide new insights into the mechanisms of polarized secretion in epithelial cells.

INTRODUCTION

The SNARE hypothesis is a general model proposed by Rothman and Warren (1994) to explain the basic principles of membrane fusion responsible for intracellular membrane movements and secretion (Bennett and Scheller, 1994). This hypothesis predicts that a stepwise progression of interactions between soluble cytoplasmic proteins and membrane receptors ultimately leads to the fusion of the two membranes. The soluble factors, including the ATPase NEM-sensitive factor (NSF) and soluble NSF attachment proteins (SNAPs), are thought to bind to SNAP receptors (SNAREs), located on transport vesicles (v-SNAREs) and on target membranes (t-SNAREs), to mediate the fusion of the two membranes. A stable complex be-

tween t-SNARE and v-SNARE proteins is thought to serve as the target site for the binding and action of the soluble factors. The central tenet for this hypothesis is that specificity of membrane fusion events is controlled in part by the correct interaction of cognate v- and t-SNAREs. For such an hypothesis to be true, each membrane compartment within the cell must contain specific t-SNARE components for interaction with their v-SNARE partners.

The v-SNARE proteins are all members of the VAMP protein family and in mammals include the proteins VAMP-1 and -2 (also known as synaptobrevins; Trimble *et al.*, 1988; Baumert *et al.*, 1989; Elferink *et al.*, 1989), and cellubrevin (McMahon *et al.*, 1993). Although differentially expressed in a wide range of tissues, these proteins reside predominantly in vesicular fractions within cells. Recently, two additional members of this family, rsec22 and rbet1, have been shown to share significant sequence similarity to the

[†] Corresponding author: Division of Cell Biology, Hospital for Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8, Canada.

yeast v-SNARE proteins sec22 and bet1 and are localized to the endoplasmic reticulum and Golgi apparatus, respectively (Hay *et al.*, 1996).

t-SNARE proteins are classified into two distinct families: SNAP-25 and the syntaxins (syns). SNAP-25 is a 25-kDa peripheral membrane protein anchored to the intracellular membrane via palmitic acid modification of cysteine residues near the middle of the sequence (Oyler *et al.* 1989). Expression of SNAP-25 is most abundant in the nervous system and to date no other isoforms have been detected which are expressed in non-neuronal cells. In contrast, many members of the syn family of t-SNARE proteins have been shown to be expressed outside the nervous system. This family includes six isoforms (Bennett *et al.*, 1992, 1993): syns 1A and 1B are expressed abundantly in the nervous system; syn 2 through syn 5 mRNAs are present in a wide variety of non-neuronal tissues (Bennett *et al.*, 1993). When expressed in COS cells, syn 1, syn 2, and syn 4 were shown to localize to the plasma membrane (PM) and syn 5 was shown to accumulate in intracellular membranes resembling the Golgi complex (Bennett *et al.*, 1993). The subcellular location of syn 3 has not been determined.

Consistent with the SNARE hypothesis, *in vitro* studies have demonstrated that only certain combinations of VAMP, SNAP-25, and syn isoforms are capable of forming stable ternary complexes (Calakos *et al.*, 1994; Pevsner *et al.*, 1994a). SNAP-25 associates equally with each syn isoform *in vitro*, yet VAMP-2 binds well with recombinant syn 1A or syn 4, but poorly if at all to syn 2 or syn 3. The stability and strength of these interactions is greatly enhanced by SNAP-25 in ternary complexes. To date, no specific v-SNARE partner has been identified for syn 2 or syn 3.

To confirm the widespread validity of the SNARE hypothesis and to develop other model systems in which to test basic principles of it, we have searched for the presence of SNARE components in non-neuronal cell types. In particular, we have examined the role of these proteins in regulating secretion from the exocrine acinar cells of the pancreas. These polarized epithelial cells have long been used as a model system to study secretory events in nonexcitable cells, and their study has contributed greatly to our current understanding of the secretory pathway. We have previously demonstrated that the VAMP isoform 2 (VAMP-2) v-SNARE was expressed in these cells and was present on the acinar secretory (zymogen) granule membrane (Gaisano *et al.*, 1994). Furthermore, we found that cleavage of this protein by tetanus toxin resulted in inhibition of Ca^{2+} -evoked enzyme secretion (Gaisano *et al.*, 1994), indicating an important role for this protein.

In the present study, to determine whether any of the known t-SNARE components were also expressed

within these cells, we have used antisera generated against syn 1 through syn 4. Surprisingly, we have found that all four of these syn isoforms were expressed within these cells. By subcellular fractionation and immunofluorescence microscopy, we show that syn 2 and syn 4 are differentially distributed on the acinar PM. Surprisingly, syn 3 was concentrated intracellularly on zymogen granule membranes, in contrast to its predicted role as a t-SNARE. Finally, we demonstrate that specific interactions can be detected between v- and t-SNAREs expressed within these cells. Together, these results support the generality of the SNARE hypothesis and suggest that syn isoforms 2–4 may play important roles in regulated exocrine secretion.

MATERIALS AND METHODS

Materials

Monoclonal mouse anti-synaptophysin (Boehringer Mannheim, Indianapolis, IN), mouse anti-human amylase (Calbiochem, La Jolla, CA), and polyclonal rabbit anti-amylase (Sigma, St. Louis, MO) antisera were from commercial sources. The following antisera were gifts, including monoclonal mouse anti-syntaxin 1 (HPC-1; from Dr. C. J. Barnstable), monoclonal mouse antisynaptotagmin-1 (from Dr. M. Takayashi), and rabbit polyclonal antisera directed against GP-2 (from Dr. A. Beaudoin) and nSEC1 (Drs. J. Pevsner and R. Scheller).

Pancreatic Acinar PM and Zymogen Granule Membrane Preparations

Pancreatic PMs were prepared according to previously described methods (Gaisano *et al.*, 1989) from pancreata of streptozotocin-induced diabetic (blood glucose > 18 mM for 1 wk) male Sprague Dawley rats (250–300 g) to eliminate contaminating islet membranes from the membrane preparations. Hematoxylin and eosin staining, as well as insulin staining, of the diabetic pancreata was used to confirm the absence or destruction of the islets. Dispersion of rat pancreatic acini was performed as we have reported previously (Gaisano *et al.*, 1994). Zymogen granule membranes (ZGM) were prepared from nondiabetic male Sprague Dawley rats (250–300 g) as described previously (Gaisano *et al.*, 1994). We have previously demonstrated that synaptophysin, abundantly present in islet secretory granules (Wheeler *et al.*, 1996), was uniformly absent in our ZGM preparations of either diabetic or nondiabetic rats (Gaisano *et al.*, 1994), indicating the purity of our ZGM preparations. To inhibit nonspecific proteolysis of these protease-sensitive synaptic proteins (Gaisano, unpublished observations), the following protease inhibitors were present in all of the buffers used for the membrane preparations, including 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 $\mu\text{g}/\text{ml}$ pepstatin A, 1 $\mu\text{g}/\text{ml}$ leupeptin, and 0.2 mg/ml soybean trypsin inhibitor.

Antibody Generation and Western Blot Analysis

Rabbit polyclonal antisera to bacterially expressed SNAP-25 and syns 2 through 4 (prepared by Berkeley Antibody, Richmond, CA) were generated against full-length fusion proteins of SNAP-25 and syn isoforms 2–4. These antisera were then subjected to affinity purification utilizing full-length fusion proteins of the respective antigen immobilized on Affigel beads (Bio-Rad, Richmond, CA) according to standard procedures (Cold Spring Harbor Antibody Manual). Selectivity of each antisera was determined by its ability to identify its respective syn fusion protein and the limited cross-

reactivity with other syn fusion proteins (Figure 1). A rabbit anti-VAMP-2-specific antibody was generated and validated as we described previously (Gaisano *et al.*, 1994). SDS-PAGE and immunoblotting were performed as described previously (Gaisano *et al.*, 1994). Briefly, blots were incubated for 2 h at room temperature in 1% bovine serum albumin/0.05% Tween 20 (in phosphate-buffered saline (PBS)) with the following primary antisera: 1:700 dilution of anti-VAMP-2 and 1:1000 dilution with anti-syn 1 through syn 4, anti-SNAP-25, anti-synaptotagmin, and antisynaptophysin. Antigen detection was with goat anti-mouse (1:5000) and goat anti-rabbit (1:2000) immunoglobulin coupled to horseradish peroxidase. Visualization was by chemiluminescence (ECL, Amersham, Arlington Heights, IL) and exposure of the membranes to Kodak BMR film for 0.5 to 5 min.

Immunofluorescence Confocal Microscopy

The pancreas was excised from a diabetic rat that had been perfused with 4% paraformaldehyde in physiological saline and immediately frozen in liquid nitrogen. This was then embedded in Tissue-Tek OCT and 5- μ m cryostat sections were obtained, rinsed in PBS for 5 min, postfixed in 4% paraformaldehyde, rinsed in PBS, and then blocked with 5% normal goat serum with 0.1% saponin (in PBS) for 0.5 h at room temperature. The tissue was then incubated at room temperature for 1 h with the following 1:100 dilutions of primary antibodies: control (no antibody), anti-syn 1 through syn 4, or anti-amylase. To eliminate potential cross-reactivity to other isoforms, the antisera were preincubated with a 10 M excess of full-length recombinant fusion proteins of the inappropriate isoforms for 1 h at room temperature prior to use. In double-labeling experiments, primary antibodies were added concurrently. After rinsing with 0.1% saponin, these were incubated with an appropriate fluorochrome-conjugated secondary antisera (fluorescein isothiocyanate (FITC) or Texas Red) for 1 h at room temperature: sheep anti-mouse (1:500) and goat anti-rabbit (1:250; Serotec, United Kingdom). Benzamide (1 mM) and leupeptin (1 μ g/ml) were added to the buffers as protease inhibitors because we noticed that some of the antigens were extremely sensitive to proteolysis. Phalloidin conjugated to Texas Red or FITC (Molecular Probes, Eugene, OR) was added during the secondary antibody step at 1 U/coverslip. The coverslips were then mounted on slides in a fading retarder, 0.1% *p*-phenylenediamine in glycerol, and examined using a laser scanning confocal imaging system (Carl Zeiss, Thornwood, NY).

Immunoprecipitation

syn-containing complexes were immunoprecipitated from a solubilized PM preparation utilizing syn 2-specific through syn 4-specific antibodies immobilized on protein A-Sepharose beads. PMs from diabetic rats were suspended at a protein concentration of 2 mg/ml in HK buffer (10 mM HEPES, pH 7.4, 140 mM potassium acetate, 2 mM EDTA, 0.1 mM EGTA) supplemented with 2% Triton X-100 and a cocktail of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml pepstatin, 1 μ g/ml leupeptin, 0.2 mg/ml soybean trypsin inhibitor). After mixing for 1 h at 4°C, insoluble material was removed by centrifugation at 25,000 $\times$ g for 15 min. Two hundred fifty micrograms of the detergent extract were incubated with each of the immobilized syn antibody bead preparations (2.5–5 μ g antibody) for 2 h at 4°C followed by extensive washing (four times, 500 μ l) with HK buffer supplemented with 0.1% Triton X-100 and 0.1% gelatin. Immunoprecipitated complexes were eluted with SDS-PAGE sample buffer and analyzed with Western blotting.

RESULTS

Analysis of the Selectivity of the Antisyntaxin Antibodies

Western blots were performed using 10 ng of recombinant glutathione-S-transferase-syn fusion proteins.

As shown in Figure 1, monoclonal anti-syn 1 (hybridoma HPC-1) and anti-syn 4 appeared to be specific, whereas anti-syn 3 cross-reacted slightly with syn 1. Anti-syn 2 antibody cross-reacted the most, detecting syn 1 moderately and syn 3 slightly. This cross-reactivity could be completely eliminated by preincubation of the antibodies with a 10 M excess of the non-specific isoforms (our unpublished observations). In all subsequent experiments, the antisera were routinely preincubated in this way before use.

syn Isoforms 1 through 4 Are Present in Pancreatic Acinar Cells

To determine which of the syn t-SNARE isoforms are expressed in the acinar cell, we performed immunoblot analyses using purified pancreatic PMs. To compare the relative contribution of acinar cells with any possible contamination from islet cells during membrane preparation, PMs were isolated from control untreated or streptozotocin-treated rats. Streptozotocin renders rats diabetic through ablation of β cells in the pancreas. Rats were maintained in the diabetic state for at least 7 days before harvest of membrane fractions to ensure complete elimination of β cells. As shown in Figure 2, streptozotocin treatment had no effect on the relative levels of any of the SNARE proteins tested, with the exception of SNAP-25. We and others have documented the abundance of SNAP-25 in the neuroendocrine cells of the islet (Sadoul *et al.*, 1995; Wheeler *et al.*, 1996), and we see a significant reduction of levels within our acinar cell population after ablation of these cells. The levels of GP2, an abundant PM protein in acinar cells, remained unchanged following streptozotocin treatment (our unpublished observations). Hence, the lack of effect on VAMP-2 or syn 1 through syn 4 indicates that these proteins are also abundantly expressed within the acinar cells of the pancreas. Occasionally, syn 4 bands

Figure 1. Analysis of syn antibody selectivity to individual syn isoforms. Ten nanograms of each purified GST-syn fusion protein were electrophoresed in lanes indicated at top, and probed on individual blots with the antibodies shown at the left. The strips of the blots shown were the only immunoreactive bands detected.

Streptozotocin

Figure 2. Presence of SNARE proteins in pancreatic acinar cells. Pancreatic PM fractions were prepared from rats treated with streptozotocin and maintained in the diabetic state or from untreated control rats (see MATERIALS AND METHODS). Ten micrograms of membrane protein from each preparation was loaded in adjacent lanes and blots were probed with antisera specific for the SNARE proteins as indicated at the right.

appeared as a doublet with a faster migrating species in variable quantities (see Figures 3 and 6). This observation was inconsistent and most likely reflects a partial degradation of syn 4 during protein preparation from this protease-rich tissue. The relative abundance of the syns was estimated by comparison of the Western blot signals obtained from PM preparations with those from known amounts of syn fusion proteins. We found that syn 4 was the most abundant, at concentrations greater than 10 ng/10 μ g of PM protein. syn 2 and syn 3 were both present at about 1 ng/10 μ g of PM protein (although, as shown below, syn 3 is much more abundant on zymogen granule membranes), and syn 1 was the least abundant at about 0.2 ng/10 μ g (our unpublished observations).

Figure 3. Differential distribution of syns 1–4 in acinar PM and ZGM fractions. Ten micrograms of protein from a PM fraction, zymogen granule membrane fractions before or after washing with 0.25 M NaBr, and from dispersed acini were electrophoresed, blotted, and probed with the antibodies indicated at the right.

syns 1 through 4 Are Localized to Distinct Membrane Fractions

To examine the subcellular distribution of syn isoforms within the acinar cell, we isolated preparations of purified pancreatic acinar ZGM and PM for Western blot analysis (Figure 3). To confirm the absence of islet and neuronal contamination in our membrane preparations, we have probed the ZGM and PM fractions for synaptophysin and nSEC-1/Munc-18 using specific antisera. Synaptophysin is an abundant neuronal synaptic vesicle protein (Walch-Somilena *et al.*, 1995), whereas nSEC-1/Munc-18 is a soluble protein also found on the PM where it is associated with syn (Pevsner *et al.*, 1994b). Both have been previously reported to be present in neurons and islets. We found neither synaptophysin (Gaisano *et al.*, 1994) nor nSEC-1/Munc-18 to be present in these membrane fractions. However, from crude tissue homogenates we cannot rule out that the small amount of syn 1, which is predominantly expressed in neural tissues, is not due to neural contamination of these fractions since the sensitivities of the individual antibodies may vary. We therefore prepared PM from dispersed acinar cells which had been allowed to attach to culture dishes before membrane harvest. Through this dissociation and culture step, neural contamination, which should not attach to the dishes, would be eliminated. However, we found that the immunoreactivity to syn 1 from this PM fraction was not diminished, indicating that the syn 1 is a component of the exocrine cell (our unpublished results).

Some peripheral proteins remain associated with the zymogen granule membranes, despite the many steps associated with their purification. Further purification of the membranes was achieved by treatment with NaBr, which removes remaining peripheral proteins. As shown in Figure 2, the residual amylase, which is one of the most abundant digestive proteins within the intact granules, is effectively removed by this treatment.

syns 1, 2, and 4 were highly enriched in the PM fraction. In all cases, with long exposures, lower levels can also be detected in ZGM fractions, but this is likely to reflect some PM contamination in this ZGM preparation. In contrast, syn 3 was highly enriched in the ZGM fraction relative to the PM. Furthermore, a NaBr wash of the ZGM fraction did not decrease the signal of syn 3, indicating that these are integral components of the ZGM and not simply adhering to it. The enrichment of syn 3 in the ZGM is unexpected given its predicted role as a t-SNARE. Preincubation of the antisera with full-length recombinant fusion proteins resulted in blockade of the signal if the analogous isoform was used, but had no effect if inappropriate isoforms were used, confirming the specificity of the antisera (our unpublished observations).

Localization of syns 2–4 within Pancreatic Acinar Cells by Confocal Immunofluorescence Microscopy

The exocrine pancreas is composed of highly polarized acinar cells in which the kinetics and morphology of complex constitutive and regulated exocytotic pathways are well characterized (Arvan and Castle, 1987; Beaudoin and Grondin, 1992). We have performed double-label immunofluorescence microscopy on pancreatic sections taken from diabetic rats. Affinity-purified antibodies specific for syns 1 through 4 were used along with phalloidin. Phalloidin labels actin filaments, the majority of which are typically found concentrated beneath the apical surface of these epithelial cells (Braun *et al.*, 1994), thereby allowing us to determine the cell polarity. The apical poles are located adjacent to the tubular ducts into which the zymogen granule contents are secreted. Within a single section, the apical surfaces of only a subset of the cells can be detected and the ducts can be cut longitudinally or transverse. From our studies, we found that the majority of syn 2 immunoreactivity (Figure 4) colocalized with the patches of phalloidin staining at the apical poles of the cells and appeared to be concentrated in the membrane there. However, significant staining was observed in a granular intracellular pattern. These structures did not correspond to zymogen granules, and their nature is not known. In contrast, syn 3 was predominantly present intracellularly within the apical half of the cell in a granular compartment. Double-labeling experiments with anti-syn 3 and antibodies against a granule constituent, amylase, confirmed that syn 3 is present on the zymogen granules (Figure 5). syn 4, like syn 2, appeared to be present on the PM, but interestingly, did not overlap with the region stained positive with phalloidin. This indicates that syn 4 is restricted to the basal and lateral membranes of these cells. Again, intracellular staining was also observed for syn 4, but its nature is not known. In all cases, the immunoreactivity could be blocked if the antibody was preincubated with recombinant fusion proteins of the appropriate syn isoform (Figure 4, C, F, and I). Although some cross-reactivities were detected with these antibodies in the absence of preincubation with nonspecific isoforms in Figure 1, the differences in electrophoretic mobility of the different isoforms, their varied abundance, and their nonoverlapping subcellular distributions preclude any of the observations presented herein being the result of cross-reactivity. We were unable to detect a syn 1 signal in the acinar cell using immunocytochemical procedures.

Anti-syn 4 but not 2 or 3 Immunoprecipitates VAMP-2 from Pancreatic Membranes

Immunoprecipitations were performed to determine whether the v- and t-SNARE proteins present in the

acinar cell membrane preparations were capable of interaction. Triton X-100-solubilized membrane preparations were immunoprecipitated with anti-syn 2, 3, or 4 antisera and the precipitates were immunoblotted to detect the presence of SNARE proteins. As shown in Figure 6, each of the antibodies was able to immunoprecipitate its appropriate syn isoform, but we did not detect immunoprecipitation of the inappropriate isoform (our unpublished observations). VAMP-2 coprecipitation only occurred when anti-syn 4 was used for the immunoprecipitation, as predicted from previous observations (Calakos *et al.*, 1994). This therefore confirms the potential for specific VAMP-2-syn 4 interactions and the lack of VAMP-2 interactions with either syn 2 or 3 in pancreatic tissues.

DISCUSSION

In this study, we have examined the possibility that isoforms of the syn family of t-SNAREs are expressed in the pancreatic acinar cell where they may have a role in polarized exocytotic events. Indeed, our results indicate that within this single cell type, syns 1 through 4 are simultaneously expressed. The presence of multiple isoforms within a single cell type indicates that the individual isoforms are not each specialized for individual tissues but rather supports the hypothesis that these distinct forms may have unique and nonredundant functions. The pancreatic acinar cell may therefore provide an ideal model system to examine the individual functions of these proteins in a context in which they are all simultaneously present.

The SNARE hypothesis predicts that the fidelity of transport is ensured by appropriate interactions of cognate v- and t-SNARE pairs, with each being restricted to distinct membrane compartments (Bennett and Scheller, 1994; Rothman and Warren, 1994). The results presented here support this possibility by demonstrating that individual syn isoforms have nonoverlapping distribution patterns. Previous studies had shown that upon transfection into COS cells, syns 1, 2, and 4 were localized to the PM, whereas syn 5 was restricted to a perinuclear location corresponding to the Golgi apparatus (Bennett *et al.*, 1993; Dascher *et al.*, 1994). Here, we show that although endogenously expressed syn 4 is present over the basal and lateral PMs, syn 2 is specifically restricted to the apical portion of the PM. In contrast, syn 3 is enriched on intracellular membrane structures which appear to be coincident with zymogen granules. The observation that within a single cell type each isoform has a unique distribution pattern implies that each contains different targeting signals responsible for their accumulation. Although the identity of such signals remain to be determined, they must reside in regions of sequence divergence between the isoforms. The coincident location of syns 1, 2, and 4 to the PM

Figure 4. Confocal microscopy of pancreatic tissue sections probed with antibodies specific to syns. Laser scanning confocal microscopy was used to distinguish the subcellular location of syn 2 (A), syn 3 (D), and syn 4 (G) within acinar cells. Sections were double labeled with a rabbit anti-syn antibody and FITC-phalloidin (B, E, and H). Control sections (C, F, and I) were incubated with anti-syn 2 (C), anti-syn 3 (F), and anti-syn 4 (I) antibodies that had been preincubated with their respective recombinant syn protein prior to immunostaining. Bar, 10 μ m.

of COS cells in the previous study indicates that these cells lack the sorting machinery and polarity necessary for differential placement of the proteins.

The restricted appearance of syn 4 on the basolateral PM and syn 2 on the apical membrane suggests that each isoform plays a role in secretory events restricted to these surfaces. The contents of the zymogen granules are secreted almost exclusively by fusion of the granule with the apical PM, and we and others have previously found VAMP-2 to be a functional component of the granule membrane (Braun *et al.*, 1994;

Gaisano *et al.*, 1994). However, as in previous studies (Calakos *et al.*, 1994, Pevsner *et al.*, 1994a), we find here that VAMP-2 associates with syn 4, but not syn 2. Therefore, the v-SNARE/t-SNARE interactions responsible for polarized secretion from the acinar cell remain unclear. It is possible that additional t-SNARE species are present on the apical surface capable of interaction with VAMP-2, of which our antibodies do not recognize. If so, then additional control mechanisms must exist to ensure that VAMP-2 does not interact with its known cognate t-SNARE syn 4 on the

Figure 5. Double-immunofluorescent labeling of syn 3 and amylase. Sections were double labeled with rabbit anti-syn 3 and monoclonal mouse anti-amylase and detected with FITC-conjugated goat anti-rabbit and Texas Red sheep anti-mouse secondary antibodies. Colocalization of syn 3 (A and C) with amylase (B and D) on granular cytoplasmic structures is most clearly resolved at higher magnifications (C and D), as indicated by arrows. Bars, 10 μ m.

basolateral surfaces. Interestingly, in response to high agonist concentrations, interstitial pancreatitis can result from the inappropriate release of zymogens to the basolateral surfaces (Lampel and Kern, 1977). Whether such events result from a failure of such control mechanisms or entry of the vesicles into minor constitutive exocytosis pathways operating through the basolateral surfaces (Arvan and Castle, 1987) remains to be determined.

An alternative explanation could be that additional unrecognized VAMP isoforms may be present in the zymogen granule membranes within these cells (Braun *et al.*, 1994) and may interact specifically with syn 2. To date, no cognate v-SNARE partner has been identified for syn 2. Interestingly, in our previous studies we found that in permeabilized preparations, tetanus toxin could inhibit exocytosis significantly, but only by approximately 30% compared with controls (Gaisano *et al.*, 1994). It remains possible, therefore, that the VAMP-2 protein proteolytically attacked by tetanus toxin does not play the principal role in regulating zymogen granule fusion in the exocrine pancreas.

The observation that syn 3 is predominantly associated with the zymogen granule membrane was

unexpected, given its putative role as a t-SNARE, but not unprecedented. Previous studies have shown that syn 1 is a component of the synaptic vesicle (Walch-Solimena *et al.*, 1995), and it has been suggested that t-SNARE contamination of that compartment may occur as a result of rapid membrane retrieval events. However, more recent studies have also demonstrated the presence of syn 1 on the chromaffin granule membrane (Tagaya *et al.*, 1995). Both chromaffin granules and zymogen granules are thought to arise from budding events from the trans-Golgi network and, unlike the synaptic vesicle, are unlikely to be associated with endocytic cycles. The presence of putative t-SNAREs in these compartments is inconsistent with the simplest predictions derived from the SNARE hypothesis. However, it is possible that this isoform is not involved in the terminal stages of the secretory pathway and that the ZGMs themselves may in some cases represent target membranes for subsequent fusion events. In fact, in the acinar cell, examples of intergranule fusions between mature zymogen granules (Edwardson *et al.*, 1993; Anderson and McNiven, 1995) as well as fusion of Golgi-derived progranules to form immature zymogen granules (Lew *et al.*,

Figure 6. Immunoprecipitation of SNARE proteins with anti-syn antibodies. Anti-syns 1 through 4 were used to immunoprecipitate SNARE complexes from detergent-solubilized extracts of pancreatic PMs. For each antibody, 250 μ g of membrane protein were incubated with 2.5–5 μ g antibody, bound to protein A-Sepharose, and precipitated as described in MATERIALS AND METHODS. The precipitates labeled IP were then electrophoresed beside 25 μ g of total membrane protein (labeled T) and blotted. The blots were cut into strips and probed with the antibodies listed at the right. For each precipitate, the blot was probed with the precipitating anti-syn (top row) and anti-VAMP-2 (bottom row).

1994) have been demonstrated. The presence of syn 3 in the zymogen granules may provide insights into the mechanisms by which such homotypic fusion events occur in acinar cells.

Another feasible explanation for the abundance of syn 2 on the apical PM and syn 3 on the zymogen granule membranes is that these proteins could collaborate in regulating apical membrane fusion events by mechanisms not explained by current versions of the SNARE hypothesis. It is possible that the v-SNARE/t-SNARE designations are too restrictive and that syn 3 could function as a v-SNARE, possibly through interactions with syn 2. Alternatively, it has recently been shown for constitutive transport processes in epithelial cells that, in contrast to basolateral transport events, some apical transport may occur in an NSF-independent manner (Ikonen *et al.*, 1995; Wilson, 1995). The presence of syns 2 and 3 on the zymogen granule and apical PMs, and their failure to associate with VAMP-2, may mean that these proteins use novel mechanisms to mediate membrane fusion events. Additional work will be required to identify proteins with which syns 2 and 3 interact to mediate membrane fusions.

The pancreatic acinar cell has long been studied as a model for vesicular transport and exocytotic pathways in nonexcitable cells. Our surprising observations regarding the multiplicity and restricted subcellular distributions of the syn isoforms within this cell type indicate that it will continue to be a valuable model system in which to examine these processes in health and disease.

ACKNOWLEDGMENTS

We thank Dr. Gabrielle Boulianne for critical comments on the manuscript. This work was supported by grants from the Medical Research Council of Canada to H.Y.G. and W.S.T., the Fraser Elliott Foundation to H.Y.G., and the National Institutes of Health grant GM-51313 to M.K.B. W.S.T. is supported by the Alzheimer's Associations of Ontario, and H.Y.G. is a recipient of an American Gastroenterology Association/Industry (Pharmacia, Uppsala, Sweden and Upjohn, Kalamazoo, MI) Research Scholar Award.

REFERENCES

- Anderson, K.L., and McNiven, M.A. (1995). Vesicle dynamics during regulated secretion in a novel pancreatic acinar cell in vitro model. *Eur. J. Cell Biol.* 66, 25–38.
- Arvan, P., and Castle, J.D. (1987). Phasic release of newly synthesized secretory proteins in the unstimulated rat exocrine pancreas. *J. Cell Biol.* 104, 243–252.
- Baumert, M., Maycox, P.R., Navone, F., De Camilli, P., and Jahn, R. (1989). Synaptobrevin: an integral membrane protein of 18 000 daltons in small synaptic vesicles of rat brain. *EMBO J.* 8, 379–384.
- 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, 177–122.
- Bennett, M.K., Calakos, N., and Scheller, R.H. (1992). Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. *Science* 257, 255–259.
- Bennett, M.K., Garcia-Ararras, J.E., Elferink, L.A., Peterson, K., Fleming, A.M., Hazuka, C.D., and Scheller, R.H. (1993). The syntaxin family of vesicular transport receptors. *Cell* 74, 863–873.
- Bennett, M.K., and Scheller, R.H. (1994). A molecular description of synaptic vesicle membrane trafficking. *Annu. Rev. Biochem.* 63, 42–100.
- Braun, J.E.A., Fritz, B.A., Wong, S.M.E., and Lowe, A.W. (1994). Identification of a vesicle-associated membrane protein (VAMP)-like membrane protein in zymogen granules of the rat exocrine pancreas. *J. Biol. Chem.* 269, 5328–5335.
- Calakos, N., Bennett, M.K., Peterson, K.E., and Scheller, R.H. (1994). Protein-protein interactions contributing to the specificity of intracellular vesicular trafficking. *Science* 263, 1146–1149.
- Dascher, C., Matteson, J., and Balch, W.E. (1994). Syntaxin 5 regulates endoplasmic reticulum to Golgi transport. *J. Biol. Chem.* 269, 29363–29366.
- Edwardson, J.M., MacLean, C.M., and Law, G.J. (1993). Synthetic peptides of the rab3 effector domain stimulate a membrane fusion event involved in regulated exocytosis. *FEBS Lett.* 320, 52–56.
- Elferink, L.A., Trimble, W.S., and Scheller, R.H. (1989). Two vesicle-associated membrane protein genes are differentially expressed in the rat central nervous system. *J. Biol. Chem.* 264, 11061–11064.
- Gaisano, H.Y., Klueppelberg, U., Pinon, D.I., Pfenning, M., Powers, S.P., and Miller, L.J. (1989). Novel tool for the study of cholecystokinin-stimulated pancreatic enzyme secretion. *J. Clin. Invest.* 83, 321–325.
- Gaisano, H.Y., Sheu, L., Foskett, J.K., and Trimble, W.S. (1994). Tetanus toxin light chain cleaves a vesicle-associated membrane protein (VAMP) isoform 2 in rat pancreatic zymogen granules and inhibits enzyme secretion. *J. Biol. Chem.* 269, 17062–17077.
- Hay, J.C., Hirling, H., and Scheller, R.H. (1996). Mammalian vesicle trafficking proteins of the endoplasmic reticulum and Golgi apparatus. *J. Biol. Chem.* 271, 5671–5679.

- Ikonen, E., Tagaya, M., Ullrich, O., Montecucco, C., and Simons, K. (1995). Differential requirements for NSF, SNAP, and Rab proteins in apical and basolateral transport in MDCK cells. *Cell* 81, 571–580.
- Lampel, M., and Kern, H.F. (1977). Acute interstitial pancreatitis in the rat induced by excessive doses of a pancreatic secretagogue. *Virchows Arch. A Pathol. Histol.* 373, 97–117.
- Lew, S., Hammel, I., and Galli, S.J. (1994). Cytoplasmic granule formation in mouse pancreatic acinar cells. Evidence for formation of immature granules (condensing granules) by aggregation and fusion of progranules of unit size, and for reductions in membrane surface area and immature granule volume during granule maturation. *Cell Tissue Res.* 178, 327–336.
- McMahon, M.T., Ushkaryov, Y.A., Edelman, L., Link, E., Binz, T., Niemann, H., Jahn, R., and Sudhof, T.C. (1993). Cellubrevin is a ubiquitous tetanus-toxin substrate homologous to a putative synaptic vesicle fusion protein. *Nature* 364, 346–349.
- Oyler, G.A., Higgins, G.A., Hart, R.A., Battenberg, E., Billingsley, M., Bloom, F.E., and Wilson, M.C. (1989). The identification of a novel synaptosomal-associated protein SNAP-25, differentially expressed by neuronal subpopulations. *J. Cell Biol.* 109, 3039–3052.
- Pevsner, J., Hsu, S.-C., Braun, J.E.A., Calakos, N., Ting, A.E., Bennett, M.K., and Scheller, R.H. (1994a). Specificity and regulation of a synaptic vesicle docking complex. *Neuron* 13, 353–361.
- Pevsner, J., Hsu, S.-C., and Scheller, R.H. (1994b). n-SEC1: a neural-specific syntaxin binding protein. *Proc. Natl. Acad. Sci. USA* 91, 1445–1449.
- Rothman, J.E., and Warren, G. (1994). Implications of the SNARE hypothesis for intracellular membrane topology and dynamics. *Curr. Biol.* 4, 220–233.
- Sadoul, K., Lange, J., Montecucco, C., Weller, U., Regazzi, R., Catsicas, S., Wollheim, C.B., and Halban, P.A. (1995). SNAP-25 is expressed in islets of Langerhans and is involved in insulin release. *J. Cell Biol.* 128, 1019–1028.
- Tagaya, M., Toyonaga, S., Takahashi, M., Yamamoto, A., Fujiwara, T., Akagawa, K., Moriyama, Y., and Mizushima, S. (1995). Syntaxin-1 (HPC-1) is associated with chromaffin granules. *J. Biol. Chem.* 270, 15930–15933.
- Trimble, W.S., Cowan, D.M., and Scheller, R.H. (1988). VAMP-1: a synaptic vesicle associated integral membrane protein. *Proc. Natl. Acad. Sci. USA* 85, 4538–4542.
- Walch-Solimena, C., Blasi, J., Edelman, J., Chapman, E.R., Fischer von Mollard, G., and Jahn, R. (1995). The t-SNARE syntaxin-1 and SNAP-25 are present on organelles that participate in synaptic vesicle recycling. *J. Cell Biol.* 128, 637–645.
- Wheeler, M.B., Sheu, L., Ghai, M., Bouquillon, A., Grondin, C., Weller, U., Beaudoin, A.R., Bennett, M.K., Trimble, W.S., and Gaisano, H.Y. (1996). Characterization of SNARE protein expression in β cell lines and pancreatic islets. *Endocrinology* 137, 1340–1348.
- Wilson, K.L. (1995). NSF-independent fusion mechanisms. *Cell* 81, 475–477.