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SNAP-23 is located in the basolateral plasma membrane of rat pancreatic acinar cells

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Abstract The SNARE hypothesis proposes that specificity of exocytosis is regulated by the appropriate interactions between the vesicle (v-) SNARE and the target membrane (t-) SNAREs. We show here that pancreatic acinar cells express the SNAP-25 t-SNARE homolog SNAP-23, and find that this t-SNARE is most highly concentrated on the basolateral plasma membrane while being expressed below detectable levels in endocrine islets within the same tissue. This is the first localization of SNAP-23 within a polarized tissue and suggests that this t-SNAREs may interact with syntaxin-4 to mediate basolateral secretion.
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Key words: Exocytosis; Pancreatitis; SNARE; Rat pancreas

1. Introduction

The pancreatic acinar cell has long been used as a model cell type for the study of exocytosis in non-excitable cells [1]. Through intensive study, the kinetics and morphology of complex constitutive and regulated exocytotic pathways have been defined in these highly polarized cells [2]. Regulated exocytosis of zymogen granules (ZG)[1] in response to low, physiologic concentrations of secretagogues, occurs at a limited apical portion of the acinar cell, which constitutes only 5–10% of the total PM surface [1,3]. Enzymatic ZG contents are then delivered to the collecting ducts of the pancreas and subsequently emptied into the intestinal lumen to digest nutrients. In contrast, exposure of acinar cells to supraphysiologic secretagogue concentrations either in vitro or in vivo appears to cause a blockade of apical exocytosis, and aberrant ZG fusion events including fusion with the basolateral plasma membrane [4]. In vivo, this basolateral ZG secretion results in experimental conditions which appear analogous to clinical pancreatitis [4] where serum levels of pancreatic enzymes are elevated. However, the mechanisms controlling normal exocrine secretion, and their possible dysfunction during pancreatitis, remain to be determined.

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Abbreviations: VAMP-2, vesicle associated membrane protein isoform 2; ZG, zymogen granule; SNAP-25 (and 23), synaptosomal associated protein of 25 kDa (and 23 kDa); PM, plasma membrane(s); SNARE, soluble NSF attachment protein (SNAP) receptor; v-SNARE, vesicle SNARE; t-SNARE, target SNARE; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Recent studies by us and others suggest that regulation of ZG fusion may follow the same basic principles as described in the SNARE hypothesis. This general model was derived to explain mechanisms controlling neurotransmitter release, and extrapolated to cover most membrane transport processes in virtually all cells [5]. The SNARE hypothesis predicts that cytosolic NEM-sensitive factors (NSF) and soluble NSF attachment proteins (SNAPs), bind to SNAP receptors (SNAREs), located on the donor vesicle (v-SNARE) and on the target membrane (t-SNAREs), to mediate the docking and fusion of the two membranes [5]. In the nervous system the vesicle associated membrane protein (VAMP) on the synaptic vesicle, along with syntaxin-1 and synaptosomal associated membrane protein of 25 kDa (SNAP-25) on the synaptic plasma membrane, form a stable complex which acts as the receptor (SNARE) for the soluble factors (reviewed in [6]). It is hypothesized that specific v- and t-SNARE combinations may control the accuracy of vesicle-target membrane interactions. The presence of multiple isoforms of VAMP [7] and syntaxin proteins [8], and the specificity of their interactions [9], support this tenet. However, SNAP-25 is expressed predominantly in the nervous system and in neuroendocrine cells [10] leaving non-neural cells without a critical SNARE component. Recently, Ravichandran et al. [11] reported the cDNA cloning of a novel isoform of SNAP-25, called SNAP-23, which is broadly expressed in non-neuronal tissues, including the pancreas. Antibodies specific for SNAP-23 detect a single protein species associated with the plasma membrane which is abundant in most non-neural but not neural cells [12]. This identification provides a complete cassette of SNARE proteins in virtually all cell types.

To determine if exocrine granule secretion is controlled by mechanisms similar to those involved in neurotransmitter release, we began to search for expression of SNARE homologs in pancreatic acinar cells. Initially we demonstrated that VAMP isoform-2 was an integral component of the zymogen granule membrane and cleavage of this protein by tetanus toxin resulted in inhibition of calcium-evoked digestive enzyme secretion [13,14]. More recently we have demonstrated that acinar cells express four isoforms of syntaxin with syntaxin isoform-2 restricted to the apical plasma membrane, syntaxin-3 on the zymogen granule (ZG), and syntaxin-4 on the basolateral plasma membrane of the pancreatic acinar cell [15]. Taken in the context of the SNARE hypothesis, their restricted appearance at these membrane compartments indicates that each of these syntaxins may serve as a distinct exocytotic target for each site [6].

In the present study, we demonstrate that acinar cells also

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express SNAP-23. Using an affinity purified antibody directed to the C-terminus of SNAP-23 [12], we show that SNAP-23, but not SNAP-25, can be detected in the acinar cells while endocrine cells of the pancreas express SNAP-25 predominantly. We also show that SNAP-23, like syntaxin-4, is predominantly localized to the basolateral plasma membrane of the acinar cell. The basolateral location of SNAP-23, along with that of syntaxin-4, suggests that these proteins do not participate in apical secretion, but they may be involved in normal or aberrant secretory events occurring at the basolateral surface.

2. Materials and methods

2.1. Antibody generation

Rabbit polyclonal antibodies to VAMP-2, syntaxins-2 through -4 and SNAP-23 were generated, subjected to affinity purification and their specificity validated as we have previously reported [12–15]. Anti-SNAP-23 specific antibody which recognizes rodent and human forms of the protein was generated against a peptide corresponding to the carboxyl-terminal residues 200–211 of SNAP-23, called αSN23.C12, and affinity purified on columns to which the peptide had been coupled [12]. An antibody raised to the amino-terminus of murine SNAP-23 (also called syndet) [16] was kindly provided by G. Baldini. Antibody raised against SNAP-25, kindly provided by M.K. Bennett, was generated against the bacterially expressed full length fusion protein of SNAP-25 and affinity purified using full length fusion protein immobilized on Affigel beads (Bio-Rad, Richmond, CA). The specificity of these antibodies was determined by immunoblotting against the recombinant full length fusion proteins and against native tissues (see Figs. 1–3) which showed that while the SNAP-23 antibody is specific only for SNAP-23, the SNAP-25 antibody cross-reacts with both SNAP-23 and SNAP-25.

2.2. Subcellular membrane preparations and immunoblotting

Pancreatic acinar plasma membranes were prepared from excised pancreata of Sprague-Dawley rats (250–300 g) made diabetic, as described [13–15], by treatment with streptozotocin which obliterates β-cells. Total membranes were prepared from 3T3-L1 fibroblasts as previously reported [12]. SDS-PAGE and immunoblots were performed as described [13] using primary antibodies diluted to 1:1000. Detection of the antigen on the blot was by enhanced chemiluminescence (ECL, Amersham Corp.). Specificity was determined by preincubating the antibodies with 10 M excess (1 μg/ml) of full length recombinant fusion protein of the appropriate isoform for 1 h at room temperature prior to use as in Fig. 1.

2.3. Immunofluorescence confocal microscopy

These studies were performed as previously described [14,15]. Briefly, pancreata from a diabetic or normal rat that have been perfused in vivo with 4% paraformaldehyde in physiological saline and frozen in liquid nitrogen, were embedded in Tissue Tek OCT. 5 μm cryostat sections were generated, rinsed in PBS and postfixed in 4% paraformaldehyde, rinsed again with PBS, and then blocked with 5% normal goat serum with 0.1% saponin for 30 min. The tissue on the glass slides was then incubated at room temperature for 1 h with the primary antibodies: rabbit anti-SNAP-23 (1:50), rabbit anti-SNAP-25 (1:50), guinea pig anti-insulin (1:100, a gift from R.A. Pedersen, University of British Columbia, Vancouver, Canada). Specificity was determined by preincubation of the primary antibody with 10 M excess (20 μg/ml) of full length recombinant fusion proteins of the appropriate isoforms for 1 h at room temperature prior to use. These were then rinsed with 0.1% saponin, and then treated with appropriate FITC- or rhodamine-labeled secondary antibody (1 h, room temperature): goat anti-rabbit (1:500) and goat anti-guinea pig (1:500). Phalloidin conjugated to FITC (Molecular Probes, Eugene, OR) was added during the secondary antibody step at 1 U/slide. The slides were then mounted with fading retarder; 0.1% p-Phenylenediamine in glycerol, and examined using a laser scanning confocal imaging system (Carl Zeiss, Thornwood, NY).

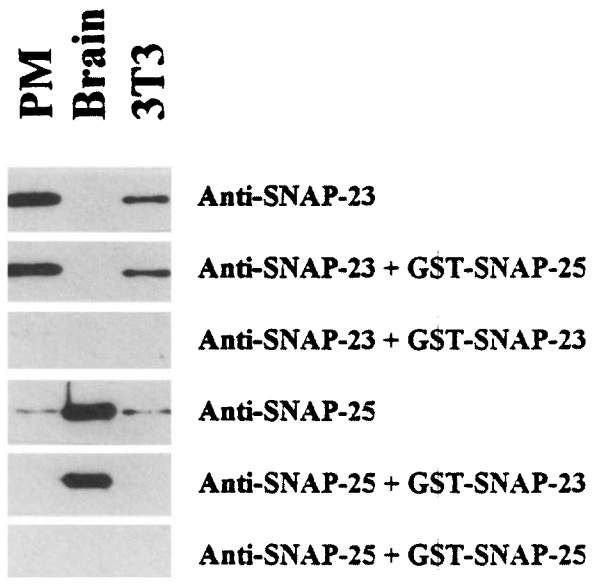

Fig. 1. SNAP-23 is present on pancreatic acinar plasma membranes and zymogen granule membranes. Highly purified pancreatic acinar plasma membranes (PM, 10 μg of protein/lane) from diabetic (streptozotocin-treated) rats, crude brain homogenates (rat brain, 5 μg of protein/lane) and 3T3-L1 fibroblast microsomes (3T3-L1, 10 μg of protein/lane) were prepared, electrophoresed on a 15% SDS-polyacrylamide gel and blotted on Immobilon-P transfer membranes (Millipore) as described in Section 2. These were then immunoblotted with: top row, SNAP-23 antibody; second row, SNAP-23 antibody preincubated with GST-SNAP-25 (1 μg); third row, SNAP-23 antibody preincubated with GST-SNAP-23 (1 μg); fourth row, SNAP-25 antibody; fifth row, SNAP-25 antibody preincubated with GST-SNAP-23; and bottom row, SNAP-25 antibody preincubated with GST-SNAP-25.

3. Results

3.1. SNAP-23 is present in pancreatic acinar cell membranes

To determine the presence and subcellular distribution of SNAP-23 and/or SNAP-25 immunoreactive proteins in the pancreatic acinar cell, we isolated preparations of purified pancreatic acinar PM for immunoblot analyses (Fig. 1). To avoid possible contamination with islet membranes which we [17] and others [18] have reported to be abundant in SNAP-25, membranes were isolated from streptozotocin-treated rats as previously described [13–15]. These were then compared to a homogenate of rat brain, which is abundant in SNAP-25 [19], and 3T3-L1 fibroblast total membranes, which are abundant in SNAP-23 [12]. The top row shows that SNAP-23, which migrated at about 30 kDa, is abundant in both pancreatic PMs and the 3T3-L1 fibroblast membranes, but is virtually absent in the rat brain. The SNAP-23 signal was not affected by preincubation with excess GST-SNAP-25 (1 μg) (second row) but was blocked when preincubated with GST-SNAP-23 (third row), confirming the specificity of the SNAP-23 antibody. The same results were obtained with the anti-syndet/SNAP-23 antibody raised to the amino-terminus of this protein [16] (data not shown). The SNAP-25 antibody gave a strong immunoreactive signal in the rat brain and a weaker signal in the pancreatic PM and 3T3-L1 membranes (fourth row). GST-SNAP-23 had no effect on the SNAP-25 signal in rat brain but significantly inhibited the signal in the pancreatic membranes and 3T3-L1 membranes (row 5), whereas GST-SNAP-25 completely blocked all of the signals

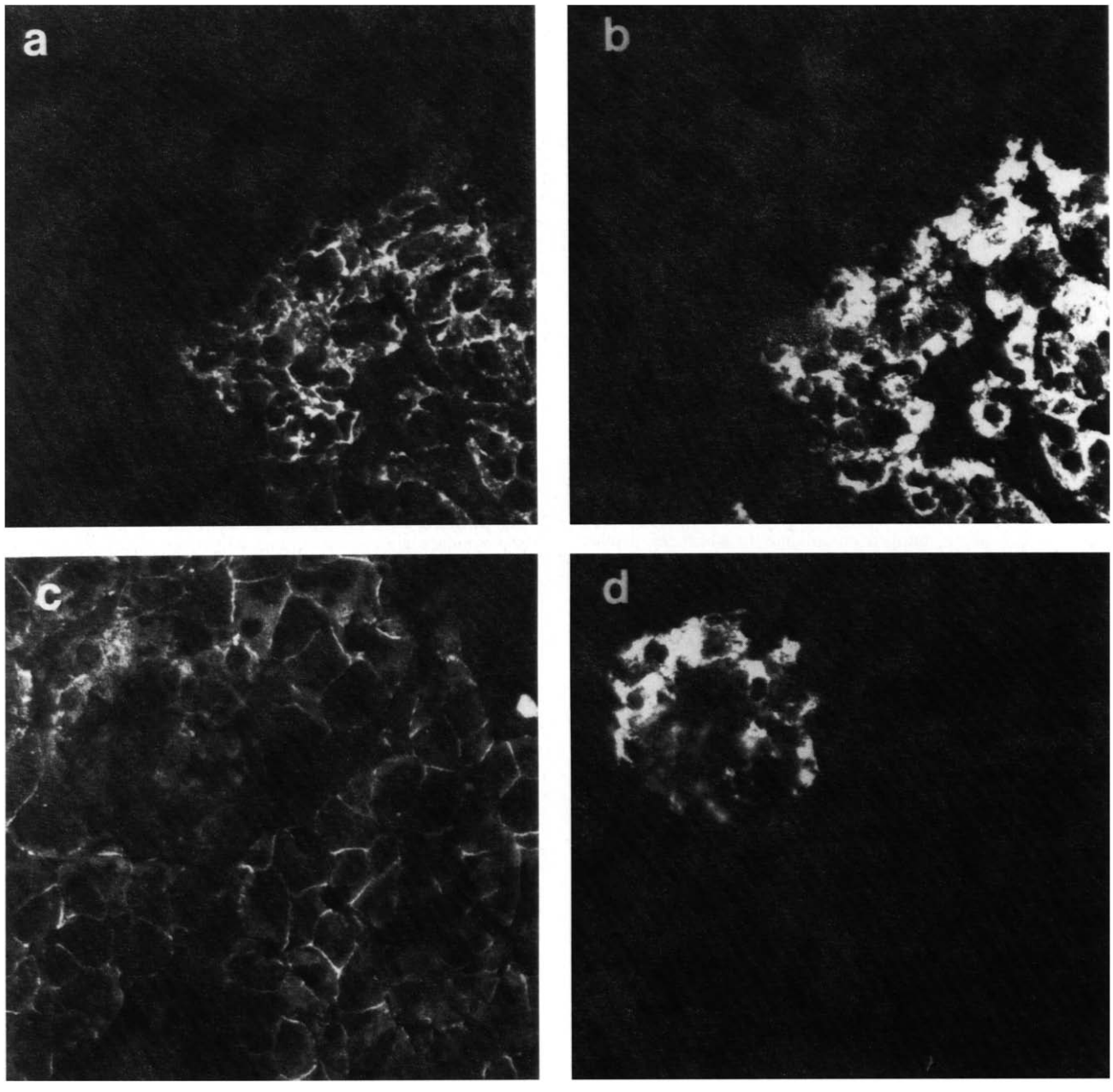

Fig. 2. SNAP-23 is present in pancreatic acinar cell and SNAP-25 is present in pancreatic islets. Laser scanning confocal microscopy was used to distinguish the distribution of SNAP-25 (in a) and SNAP-23 (in c) in normal (non-diabetic) pancreatic tissue from a rat. These tissue sections were double labeled with guinea pig anti-insulin antibody to indicate the location of the islets (in b and d).

from this antibody (row 6). Furthermore, a monoclonal antibody (SMI 81, Sternberger Monoclonal Inc.) which recognizes brain and islet SNAP-25 failed to recognize the acinar isoform. However, this antibody gave a strong cross-reaction to high molecular weight material within the ZG (data not shown) and was not used further. Together, our data indicate that the SNAP-25 immunoreactivity detected in the pancreatic PM and 3T3-L1 membranes, was actually SNAP-23 to which the SNAP-25 antibody cross-reacted.

3.2. SNAP-23 and SNAP-25 are present in pancreatic acinar cells and islets, respectively

The pancreas provides an ideal opportunity to compare the endocrine and exocrine tissues side-by-side for the presence of SNAP-25 and SNAP-23 (Fig. 2). We therefore performed double label immunofluorescence microscopy on pancreatic sections taken from normal (non-diabetic) rats using affinity purified SNAP-23 and SNAP-25 antibodies individually along with antibodies specific to rat insulin. In the islets, insulin (Fig. 2b) was restricted to cells in which SNAP-25 was abundant and localized to the cell membrane (Fig. 2a). In contrast, SNAP-23 antibody strongly stained the plasma membrane of every acinar cell (Fig. 2c), but minimally if at all in the insulin positive islets (Fig. 2d). Therefore, unlike VAMP and syntaxin proteins which are expressed in both the endocrine and exocrine pancreas [14,15,17], SNAP-25 and SNAP-23 appear to be most abundantly expressed in pancreatic endocrine and exocrine cells, respectively.

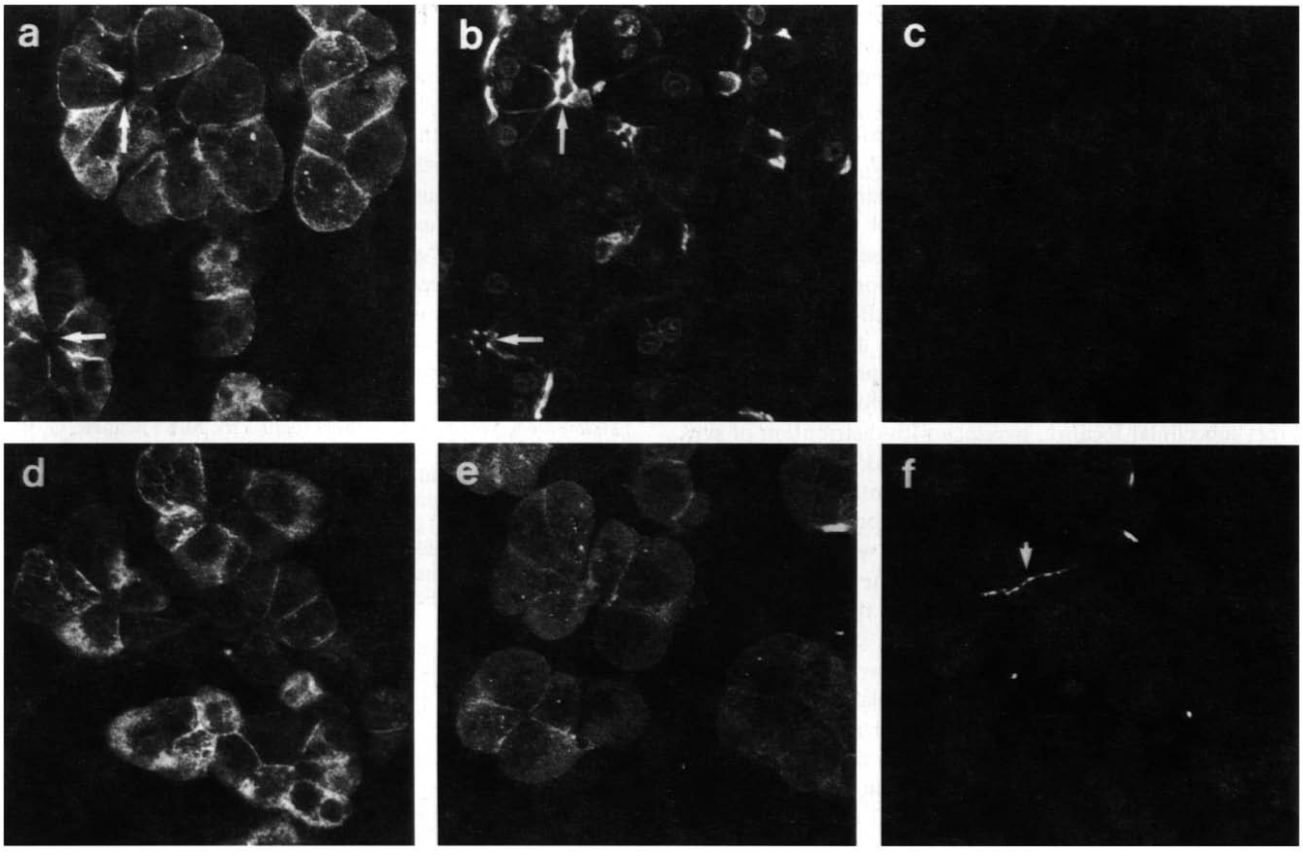

Fig. 3. Subcellular location of SNAP-23 to the basolateral plasma membrane of pancreatic acinar cells by laser confocal microscopy. Pancreatic sections from diabetic rats were labeled with either anti-SNAP-23 (a–d) or anti-SNAP-25 (e–f) antibodies. Preincubation with either GST-SNAP-23 (c, f) or GST-SNAP-25 (d) recombinant proteins were performed prior to immunostaining to demonstrate the specificity of the immunostaining. SNAP-25 in a nerve fiber (arrowhead in f) was not blocked by GST-SNAP-23. Double labeling with FITC-phalloidin (b) was performed to indicate the apical portion of the acinar cells (arrows in a and b).

3.3. SNAP-23 is located on the basolateral plasma membrane of the pancreatic acinar cell

To more precisely map the subcellular location of SNAP-23 in acinar cells we have performed double label immunofluoresence microscopy on sections from the pancreas of diabetic rats with the SNAP-25 and SNAP-23 antibodies along with phalloidin, which labels actin filaments near the apical surface of the acinar cell [15,20]. As shown in Fig. 3a, the SNAP-23 antibody labels the basal and lateral plasma membrane of every acinar cell but was below detectable levels on the apical portions which are labeled by phalloidin (Fig. 3b). The same result was observed with the anti-syndet/SNAP-23 antibody (data not shown). The SNAP-23 signal was completely blocked by GST-SNAP-23 (Fig. 3c) but was not affected by preincubation of the antibody with GST-SNAP-25 (Fig. 3d). A similar pattern was detected with the SNAP-25 antibody (Fig. 3e) when the laser intensity was significantly increased, although this could not be seen at intensities used for SNAP-23 (See Fig. 2a). The SNAP-25 signal on the acinar cell was completely blocked by preincubation of the antibody with GST-SNAP-25 (not shown), as well as with GST-SNAP-23 (Fig. 3f). The GST-SNAP-23, however, did not block the SNAP-25 signal of a nerve fiber straddling the basal surface of an acinus (arrowhead in f). Again, this confirms that the only immunoreactivity detected by the SNAP-25 antibody in exocrine acinar cells was SNAP-23.

4. Discussion

SNAP-23 is a recently described, widely expressed isoform of the neuronal t-SNARE SNAP-25 [11,12,16]. SNAP-23 is 59% identical to SNAP-25 at the amino acid level and its mRNA and protein are expressed in non-neuronal tissues [11,12,16]. To determine which of these two proteins are present in pancreatic acinar cells, we used polyclonal antibodies specific for SNAP-25 [15,17], and SNAP-23 [12,16] in Western blotting and immunocytochemical studies. The antibodies raised against SNAP-25 recognized proteins in the rat brain, pancreatic islet, and exocrine acinar cell. However, the latter signal appears to be the result of cross-reaction since it could be fully blocked by pre-incubation of the antibodies with recombinant SNAP-23 proteins. In contrast, anti-SNAP-23 antibodies gave strong signals for acinar cell membrane preparations and little if any signal for neural membranes, and this signal was not sensitive to blocking by pre-incubation with GST-SNAP-25 but was eliminated by GST-SNAP-23. Together with a previous study which failed to detect SNAP-25 in the acinar cell using an antibody specific to the carboxyl-terminus of SNAP-25 [18], our results show that SNAP-23 is the predominant isoform and that SNAP-25 is not abundantly expressed in these cells.

The pancreas is a tissue which allows simultaneous assessment of protein expression in both endocrine and exocrine

cells. We observed that SNAP-23 was expressed much more abundantly in the exocrine than the endocrine cells, the reciprocal of the pattern seen for SNAP-25. Interestingly, the other SNARE proteins VAMP-2 and syntaxin-1 appear to be expressed in both cell types [13,15,17,18] albeit at much higher levels in endocrine cells. This suggests that the SNAP-23/SNAP-25 component of the secretory machinery may provide specialized features for secretion in each cell type. The nature of this difference will await further characterization of the precise role of SNAP-23 in membrane fusion.

Within the pancreatic acinar cell, SNAP-23 is present in abundance on the basolateral membranes and was virtually absent from the apical surface. We had previously demonstrated that acinar cells express four isoforms of syntaxin and this subcellular location overlaps with distribution of syntaxin-4 in acinar cells [15]. Although SNAP-23 can form binary interactions with all four of the syntaxin isoforms in vitro, it is interesting to see that it co-localizes with only one of them, and that no co-localization was seen with the apically localized syntaxin-2. SNAP-23 is therefore unlikely to be localized solely by its association with the syntaxins, but whether it is targeted by association with other proteins or carries its own targeting signals is not known.

The appearance of SNAP-23 at the basolateral surface represents the first evidence for regional localization of this new t-SNARE isoform within polarized cells and argues that this protein may participate, along with syntaxin-4 and a member of the VAMP family to mediate basolateral membrane fusion events. In vitro studies have shown that VAMP-1 and VAMP-2 can bind to syntaxin-1 or syntaxin-4 with high affinity, but not with syntaxin-2 or -3, suggesting specificity of v-SNARE-t-SNARE interactions [9]. SNAP-25 and SNAP-23, on the other hand, do not appear to contribute to this specificity and are capable of binding to each of the syntaxin and VAMP isoforms [9,11]. Given the presence of VAMP-2 on the ZGM, a complete cassette of interacting v- and t-SNAREs exists for the ZG to fuse with the basolateral PM. Since this rarely occurs under normal circumstances, their interaction must be under additional levels of control. Interestingly, in response to high agonist concentrations, interstitial pancreatitis can result from the inappropriate release of zymogens to the basolateral surfaces [4,21]. 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 [22] is not known.

Finally, the location of SNAP-23 to the basolateral membranes, coupled with the observation that syntaxin isoforms were also distinctly localized within these cells, suggests that apical secretion is quite distinct from basolateral secretion. Since the only identified v-SNARE on the ZGM, VAMP-2, does not form stable complexes with the apical t-SNARE syntaxin-2 in vitro, it is possible that additional isoforms of SNAP-25 and VAMP may exist to provide a complete SNARE cassette for apical secretion. Alternatively, it is also possible that apical secretion may occur by means other than those explained in the SNARE hypothesis. It has been shown in Madin-Darby canine kidney (MDCK) cells that vesicular transport from the trans-Golgi network to the basolateral plasma membrane is dependent on NSF and αSNAP, and is sensitive to tetanus and botulinum neurotoxins which cleave VAMP-2 [23]. In contrast, apical secretion in MDCK cells is independent of NSF, αSNAP and insensitive to tetanus toxin [23]. In our studies, we have found that while Ca^{2+}-evoked amylase secretion from isolated, permeabilized acinar cells can be inhibited by tetanus toxin cleavage of VAMP-2, this inhibition is always partial [13]. We are currently investigating the possibility that the permeabilized preparation may exhibit both apical and basolateral secretion with only the latter being sensitive to tetanus toxin. If so, this may provide a valuable model system to examine the mechanisms controlling normal granule targeting specificity and to develop compounds which could be effective at blocking basolateral secretion events which may be associated with pancreatitis. More work is required to identify the apical and vesicular proteins responsible for regulated apical secretion in acinar cells.

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