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Association of Syntaxin 3 and Vesicle-associated Membrane Protein (VAMP) with H^+/K^+-ATPase-containing Tubulovesicles in Gastric Parietal Cells

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H^+/K^+-ATPase is the proton pump in the gastric parietal cell that is responsible for gastric acid secretion. Stimulation of acid secretion is associated with a reorganization of the parietal cells resulting in the incorporation of H^+/K^+-ATPase from a cytoplasmic membrane pool, the tubulovesicle compartment, into the apical canalicular membrane. To better characterize the role of membrane trafficking events in the morphological and physiological changes associated with acid secretion from parietal cells, we have characterized the expression and localization of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) in these cells. Each of the six different SNARE proteins examined [syntaxins 1 through 4 of 25-kDa synaptosome-associated protein, and vesicle-associated membrane protein] were found to be expressed in parietal cells. Furthermore, two of these SNAREs, vesicle-associated membrane protein and syntaxin 3, were associated with H^+/K^+-ATPase-containing tubulovesicles while the remainder were excluded from this compartment. The expression of syntaxin 1 and synaptosome-associated protein of 25 kDa in parietal cells, two SNAREs previously thought to be restricted to neuroendocrine tissues, suggests that parietal cells may utilize membrane trafficking machinery that is similar to that utilized for regulated exocytosis in neurons. Furthermore, the localization of syntaxin 3, a putative target membrane SNARE, to the tubulovesicle compartment indicates that syntaxin 3 may have an alternative function. These observations support a role for intracellular membrane trafficking events in the regulated recruitment of H^+/K^+-ATPase to the plasma membrane after parietal cell stimulation.

INTRODUCTION

Parietal cells (also called oxyntic cells) line the lumen of tubular glands in the gastric mucosa and are responsible for the secretion of gastric HCl in response to a variety of stimuli, including histamine, gastrin, and acetylcholine. The secretion of acid by parietal cells is mediated by the transport of H^+ into the gastric lumen by an apical plasma membrane-localized H^+/K^+-ATPase (Forte and Soll, 1989). The transition between the resting nonsecreting state and the activated acid-secreting state is associated with dramatic changes in the morphology of the cells. In its resting state, the parietal cell has a relatively limited apical membrane area that includes an extensive network of canals, known as the secretory canaliculi, that extend into the cytoplasm from the apical cell surface. These canaliculi are surrounded by tubular and vesicular membrane structures, known as the tubulovesicle compartment, that occupy about 50% of the cytoplasmic volume. In resting parietal cells, the H^+/K^+-ATPase is predominantly retained within intracellular tubulovesicles where it is inactive due to the low K^+

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permeability of the tubulovesicle membrane (Forte *et al.*, 1981; Smolka *et al.*, 1983). Upon stimulation, a 5- to 10-fold increase in the apical surface area and a corresponding decrease in the abundance of intracellular tubulovesicles is observed (Helander and Hirschowitz, 1972; Forte *et al.*, 1981). As a result of this membrane reorganization, the intracellular H^+/K^+-ATPase is translocated to the membranes of the secretory canaliculi. The coupling of the H^+/K^+-ATPase with K^+ and Cl^- channels in the canalicular membrane facilitates the H^+–K^+ exchange that drives HCl secretion across the apical membrane into the gastric lumen (Wolosin and Forte, 1984; Forte and Soll, 1989).

Membrane trafficking events have been proposed to play a central role in the dramatic changes in parietal cell structure and function associated with the transition to the active acid secreting state (Forte *et al.*, 1977; Forte and Yao, 1996). Although little is known about the intracellular machinery mediating membrane trafficking in parietal cells, biochemical and genetic studies of membrane trafficking in animal cells and yeast have begun to reveal the molecular mechanism underlying transport vesicle targeting and fusion (Bennett and Scheller, 1994; Ferro-Novick and Jahn, 1994; Südhof, 1995). According to a current model known as the soluble *N*-ethylmaleimide-sensitive factor (NSF)[1] attachment protein receptor (SNARE) hypothesis (Söllner *et al.*, 1993a,b; Rothman and Warren, 1994), vesicle targeting is mediated by the unique pairing between membrane proteins on the transport vesicle (known as v-SNAREs) and proteins on the target membrane (known as t-SNAREs). The SNARE complexes then provide a binding site for the general membrane trafficking factors known as soluble NSF attachment protein α and NSF. Three neuronal membrane proteins have been identified as components of the SNARE complex at the synapse (Söllner *et al.*, 1993b): the synaptic vesicle protein vesicle-associated membrane protein (VAMP, also known as synaptobrevin; Trimble *et al.*, 1988; Baumert *et al.*, 1989) and the presynaptic plasma membrane proteins syntaxin 1 (Bennett *et al.*, 1992) and synaptosome-associated protein of 25 kDa (SNAP-25; Oyler *et al.*, 1989). Proteins sharing sequence homology with these three synaptic proteins are required for efficient secretion from yeast. Furthermore, in both yeast and animal cells, syntaxin and VAMP are members of small protein families that have been implicated in a variety of vesicle docking and fusion events including both constitutive and regulated exocytosis (Bennett and Scheller, 1994; Ferro-Novick and Jahn, 1994). These observations highlight the general importance of SNARE proteins in membrane trafficking.

To better characterize the role of membrane trafficking events in the morphological changes associated with the secretion of gastric acid, we have characterized the expression and localization of SNARE proteins in gastric parietal cells.

[1] Abbreviations used: BSA, bovine serum albumin; NSF, *N*-ethylmaleimide-sensitive factor; SNAP-25, synaptosome-associated protein of 25 kDa; SNARE, soluble NSF attachment protein receptor; t-SNARE, target membrane SNARE; VAMP, vesicle-associated membrane protein; v-SNARE, vesicle membrane SNARE.

MATERIALS AND METHODS

Tissue Preparation and Membrane Fractionation

Subcellular membrane fractions were obtained from the gastric mucosa of fasted Sprague Dawley rats (about 300 g) as described by Reenstra and Forte (1990) with slight modification. Briefly, gastric oxyntic mucosa was minced and resuspended in 25 volumes of ice-cold homogenization buffer [5 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)/Tris, pH 6.7, 125 mM mannitol, 40 mM sucrose, 1 mM EDTA, 30 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin], and homogenized with 20 strokes in a Potter-Elvehjem homogenizer at a speed of 200 rpm. The homogenate was centrifuged at 80 × *g* for 10 min to remove whole cells and the connective tissue, and the resulting supernatant (S_0) was saved as starting material. Three membrane fractions were then generated by sequential centrifugation steps: P_1 (4300 × *g* for 10 min), P_2 (10,000 × *g* for 10 min), and P_3 (100,000 × *g* for 1 h). The P_1 fraction is enriched for the apical plasma membrane and the P_3 microsomal fraction is enriched for tubulovesicle membranes. The final high-speed supernatant (S_3) was saved as the cytosol.

Isolation of Rabbit Parietal Cells and Cell Lysate Preparation

Gastric parietal cells were purified to >85% homogeneity from rabbit gastric mucosa by Nycodenz sedimentation as described (Chew *et al.*, 1989). The cells from each layer of the gradient were examined by phase-contrast microscopy and counted as parietal cell or nonparietal cell on the basis of morphology. The top layer of cells was assayed as 85–90% parietal cells whereas the pellet contained negligible parietal cells, consisting mainly of chief cells and mucous cells. Parietal and nonparietal cell preparations (each containing 1–2 × 10^6 cells) were pelleted to remove the Nycodenz gradient medium and resuspended in 500 μl of phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM NaCl, pH 7.4) containing 4% Triton X-100 and protease inhibitors (30 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin). After mixing end-over-end at 4°C for 1 h, insoluble material was removed by centrifugation at 18,500 × *g* for 15 min, the resulting supernatant was collected, and its protein concentration was determined (Bio-Rad Dc protein assay, Bio-Rad, Hercules, CA). Rabbit brain homogenate (5 μg of protein) as well as parietal and nonparietal cell lysates (each containing 100 μg) were separated by SDS-PAGE, transferred to a nitrocellulose filter, and probed for the presence of the SNARE proteins by immunoblotting.

Sucrose Gradient Purification of H^+/K^+-ATPase-containing Vesicles

A gastric microsomal membrane fraction (P_3; 10–20 mg protein) from rats was resuspended in isotonic suspension medium (300 mM sucrose, 5 mM Tris-HCl, pH 7.4, 1 mM EDTA), homogenized with 10 strokes in a Potter-Elvehjem homogenizer at a speed of 200 rpm, and brought to a final sucrose concentration of 50%. The sample was then transferred to an SW28 centrifuge tube (Beckman Instruments, Palo Alto, CA) and overlaid with a 10–40% continuous sucrose

gradient (in 5 mM Tris-HCl, pH 7.4, 1 mM EDTA). After centrifugation at 135,000 × *g* for 16 h at 4°C, 1-ml fractions were collected and analyzed by SDS-PAGE. The presence of H^+/K^+-ATPase and SNARE proteins in the fractions was monitored by Coomassie blue staining and immunoblotting.

Topology of Microsomal Membrane Proteins

Rat gastric microsomal membranes enriched for H^+/K^+-ATPase by sucrose gradient flotation (50 μg of protein) were incubated with 1 μg of trypsin either in the presence or the absence of 0.5% Triton X-100 for 30 min at 37°C. The trypsin digestion was terminated by addition of an equal volume of 20% trichloroacetic acid, and precipitated protein was recovered by centrifugation at 18,500 × *g* for 15 min. The samples were then analyzed by SDS-PAGE and immunoblotting.

Immunoisolation of H^+/K^+-ATPase-containing Membranes

H^+/K^+-ATPase-enriched tubulovesicles were isolated from rat gastric microsomal membranes (P_3; 10–20 mg of protein) by continuous sucrose gradient flotation. Fractions containing H^+/K^+-ATPase (identified by SDS-PAGE and Coomassie blue staining) were pooled, and the amount of H^+/K^+-ATPase α-subunit was estimated by comparison to known amounts of bovine serum albumin (BSA) on a Coomassie blue-stained gel. Aliquots of the pooled fractions containing 10 μg of H^+/K^+-ATPase were used as the starting material for immunoisolation. The magnetic beads used for immunoisolation (Dynabeads M-280, 6–7 × 10^8 beads/ml from Dynal, Lake Success, NY) were cross-linked to secondary antibody (either rabbit anti-mouse IgG or goat anti-rabbit IgG, depending on the primary antibody used) and blocked by incubation for 30 min at 4°C in PBSE (PBS supplemented with 2 mM EDTA) containing 5% BSA. Immunoisolation was carried out according to one of the following two procedures. In the first procedure, the membranes were incubated with 2 μg of 2G11 (anti-H^+/K^+-ATPase β-subunit) for 2 h at 4°C followed by the addition of increasing amounts of magnetic beads (in 400 μl of PBSE containing 5% BSA) and incubation overnight at 4°C with gentle agitation. In the second procedure, the membranes were first incubated with primary antibody [monoclonal antibodies directed against H^+/K^+-ATPase (2G11; 10 μg), syntaxin 1 (HPC-1; 5 μl of ascites), or SNAP-25 (SMI-81 from Sternberger Monoclonals, Baltimore, MD; 5 μl of ascites) or polyclonal antibodies directed against syntaxin 3 (3 μg of affinity purified) or VAMP (5 μl of serum)] for 2 h at 4°C. The samples were then brought to 60% sucrose, and the membranes were isolated by flotation through 40% sucrose prior to incubation with secondary antibody-coupled magnetic beads (40 μl in PBSE containing 5% BSA) overnight at 4°C with gentle agitation. In each procedure, the beads were recovered with a magnet and washed three times with PBSE containing 5% BSA and once with PBSE containing 0.1% BSA. Where indicated, the membranes remaining in the supernatant after isolation of the magnetic beads were recovered by centrifugation at 200,000 × *g* for 1 h. Proteins in the different fractions were solubilized in SDS-PAGE sample buffer containing no β-mercaptoethanol and analyzed by SDS-PAGE and immunoblotting. In control reactions, magnetic beads cross-linked with secondary antibody were either incubated with membrane alone or with primary antibody alone.

SDS-PAGE and Immunoblotting

Subcellular fractions of gastric tissue (10–40 μg of protein) were solubilized in SDS-PAGE sample buffer (1% SDS, 500 mM urea, 1% β-mercaptoethanol, 0.125 mM EDTA, 10% glycerol, 0.025% bromphenol blue, and 15 mM Tris-HCl, pH 6.8), separated by SDS-PAGE, and protein composition was detected by Coomassie blue staining. For immunoblotting, SDS-PAGE was followed by transfer to nitrocellulose and probing for the presence of H^+/K^+-ATPase and SNARE proteins. The following primary antibodies were used for Western blotting: monoclonal antibodies against H^+/K^+-ATPase α-subunit (1:10,000 dilution), H^+/K^+-ATPase β-subunit (2G11, 2.5 μg/ml), or syntaxin 1 (HPC-1, 1:2000 dilution) or affinity-purified rabbit polyclonal antibody against syntaxins 2 through 4 (1:1000 dilutions), SNAP-25 (1:2000 dilution), or VAMP-2 (1:5000 dilution). The immunoreactivity was detected either by ^{125}I-labeled goat anti-mouse or anti-rabbit IgG (ICN Biochemical, Irvine, CA) and quantitated by phosphorimaging (Molecular Dynamics, Sunnyvale, CA), or by peroxidase-labeled goat anti-mouse or anti-rabbit secondary antibody and chemiluminescence detection (Amersham, Arlington Heights, IL).

RESULTS

Identification of Syntaxins 1 through 4, SNAP-25, and VAMP in Subcellular Fractions of Rat Gastric Parietal Cells

Subcellular fractions from rat gastric mucosa were obtained by differential centrifugation and subjected to SDS-PAGE. The presence of H^+/K^+-ATPase was monitored by Coomassie blue staining (Figure 1A) and immunoblotting (Figure 1B). H^+/K^+-ATPase was enriched (3.6-fold) in the microsomal P_3 fractions with lower levels detected in other membrane fractions, confirming that P_3 is the tubulovesicle-rich fraction. All subcellular fractions were then examined by immunoblotting for the presence and quantity of a variety of SNARE proteins, including syntaxins 1 through 4, SNAP-25, and VAMP (Figure 2A; Table 1). To differentiate between the different syntaxin isoforms, affinity-purified antibodies raised against bacterially ex-

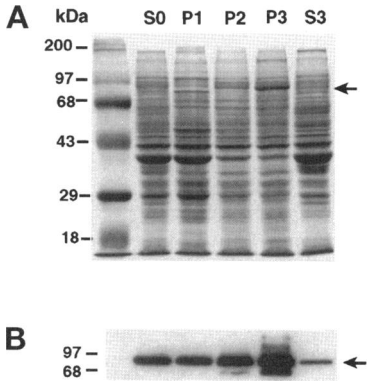

Figure 1. Protein profile of the subcellular fractions of rat gastric mucosa. Tissue preparation and membrane fractionation were performed as described in MATERIALS AND METHODS. Equivalent amounts (40 μg) of total protein from S_0, P_1, P_2, P_3, and S_3 fractions were separated on a 10% SDS-polyacrylamide gel and stained with Coomassie brilliant blue (A). Aliquots of the same samples (10 μg) were resolved on a parallel gel, and the proteins were transferred to nitrocellulose and immunoblotted with an antibody against the α-subunit of H^+/K^+-ATPase by using chemiluminescent detection (B). Enrichment of the 95-kDa α-subunit of the H^+/K^+-ATPase (arrows) was seen in the microsomal P_3 fraction on the Coomassie blue-stained gel and confirmed on the immunoblot.

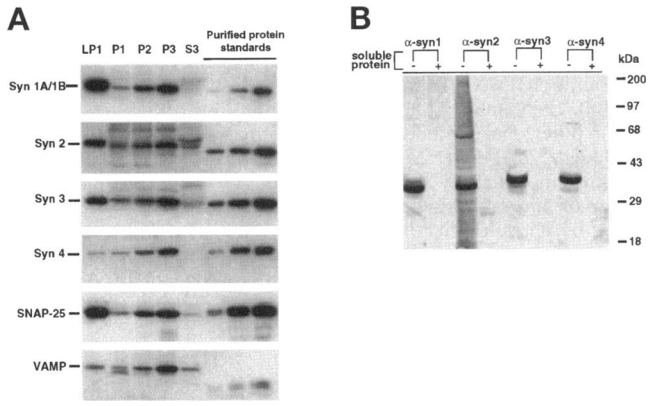

Figure 2. Immunoblot analysis of syntaxins, SNAP-25, and VAMP in gastric subcellular fractions. Tissue preparation and membrane fractionation were performed as described in MATERIALS AND METHODS. (A) A rat brain membrane fraction (LP1; 1 μg) and gastric P_1, P_2, P_3, and S_3 fractions (each containing 40 μg) were separated on a 12% SDS-polyacrylamide gels. Known amounts of bacterially expressed SNARE proteins were analyzed simultaneously. After transfer to nitrocellulose, immunoblot analysis was performed with the indicated antibodies and ^{125}I-labeled secondary antibodies for detection. The following amounts of purified protein standards were used: 1 ng, 5 ng, and 10 ng, syntaxin 1A, syntaxin 4, and SNAP-25; 1 ng, 2.5 ng, and 5 ng, syntaxin 2 and syntaxin 3; 5 ng, 10 ng, and 25 ng, VAMP-2. Because the bacterially expressed syntaxins and VAMP-2 lack their carboxyl-terminal membrane anchors, they have greater electrophoretic mobility than the native proteins. (B) Nitrocellulose blots (each containing 40 μg of gastric mucosal P_3 fraction) were probed with the indicated anti-syntaxin isoform antibodies either in the absence (−) or presence (+) of competing syntaxin isoform (10 μg/ml), and the immunoreactivity was detected by chemiluminescence. The position of molecular weight markers are indicated. For clarity in subsequent figures, only the region of the immunoblot corresponding to the molecular weight range of the appropriate SNARE protein is shown.

pressed syntaxins 2 through 4 were used along with a monoclonal antibody (HPC-1; Barnstable *et al.*, 1985) that specifically recognizes syntaxin 1A/1B. Previous studies have demonstrated that the syntaxin isoform antibodies selectively recognize the appropriate bacterially expressed syntaxin (Gaisano *et al.*, 1996). In the gastric mucosal microsome fraction, each syntaxin isoform antibody recognizes a single prominent band of 34–35 kDa, and this immunoreactivity is eliminated when the corresponding purified bacterially expressed syntaxin isoform is added to the antibody during the immunoblotting (Figure 2B). In the case of syntaxin 2, a second immunoreactive species with lower abundance and electrophoretic mobility was often observed on immunoblots of gastric membrane fractions (Figure 2A). This heterogeneity may be the result of alternative splicing (Bennett *et al.*, 1993). In addition, the mobility of the syntaxin 1A/1B immunoreactive species was consistently faster in gastric mucosal membranes than in rat brain membrane extracts. The basis for this difference in mobility remains to be established. Polypeptides of 18 kDa and 28 kDa corresponding to VAMP and SNAP-25, respectively, were also detected in the gastric parietal cell membranes. Antibodies that distinguish between VAMP-1 and VAMP-2 (kindly provided by Dr. William Trimble, University of Toronto, Toronto, Canada) demonstrated that VAMP-2 corresponds to the prominent 18-kDa polypeptide (our published results). It remains to be determined whether the faster migrating VAMP-immunoreactive species represents a third isoform of VAMP known as cellubrevin (McMahon *et al.*, 1993). The SNAP-25 immunoreactive species was also detected with the monoclonal antibody SMI-81 (our unpublished data), suggesting that the signal is not due to cross-reaction with SNAP-23, a recently identified SNAP-25 isoform (Ravichandran *et al.*, 1996).

The amount of the different SNARE proteins present in the gastric membrane fractions was estimated by directly comparing the immunoblot signals (quantitat-

Table 1. Quantitation of SNARE proteins in subcellular fractions of the rat gastric mucosa

Fraction	Total protein (% of S_0)	H^+/K^+-ATPase fold enrichment	Syntaxin 1A/B ng/mg	Syntaxin 1A/B Fold enrichment	Syntaxin 2 ng/mg	Syntaxin 2 Fold enrichment	Syntaxin 3 ng/mg	Syntaxin 3 Fold enrichment	Syntaxin 4 ng/mg	Syntaxin 4 Fold enrichment	SNAP-25 ng/mg	SNAP-25 Fold enrichment	VAMP ng/mg	VAMP Fold enrichment
S_0	100	1	110	1	32	1	17	1	22	1	32.5	1	65	1
P_1	17	0.9	50	0.4	32.5	0.9	12.5	0.7	10	0.4	20	0.6	25	0.4
P_2	6.6	1.5	200	1.8	50	1.5	27.5	1.5	31.3	1.4	55	1.6	40	0.6
P_3	19.8	3.6	375	3.4	62.5	1.9	52.5	3.1	87.5	4.3	100	3.0	200	3.0
S_3	56.2	0.1	25	0.21	20	0.63	5.0	0.3	2.5	0.1	10	0.3	35	0.5

Subcellular fractions of the rat gastric mucosa were prepared as described in MATERIALS AND METHODS. Equivalent amounts of total protein (40 μg) from S_0, P_1, P_2, P_3, and S_3 fractions were separated on a 12% SDS-polyacrylamide gel and immunoblotted with antibodies against different SNARE proteins, and the immunoreactivity was detected with ^{125}I-labeled secondary antibodies. The amount of different SNARE proteins present in the samples (quantitated by phosphorimaging) was compared to the signals generated with known amount of bacterially expressed SNARE proteins. The fold enrichment of H^+/K^+-ATPase and SNARE proteins in each fraction was calculated by comparing the signals of S_0 with signals of P_1, P_2, P_3, and S_3 fractions and values from S_0 were set as 1.

ed by phosphorimaging) to the signals generated with known amounts of bacterially expressed SNARE proteins. Among the syntaxins, syntaxin 1A/1B was found to be the most abundant in gastric mucosal membranes (110 ng/mg protein) followed by syntaxins 2 (32 ng/mg), 4 (22 ng/mg), and 3 (17 ng/mg). VAMP was the most abundant SNARE detected (200 ng/mg) and SNAP-25 was present at a level comparable to the syntaxins (32 ng/mg). Surprisingly, all of the markers displayed an enrichment in the microsomal P_3 fraction. However, the level of enrichment varied. For example, syntaxin 2 exhibited less than twofold enrichment relative to the starting S_0 fraction whereas syntaxins 1A/1B, 3, and 4, SNAP-25, and VAMP were all enriched three- to fourfold.

Parietal Cells Are the Primary Source of the Gastric SNARE Proteins

Previous studies have demonstrated that syntaxin 1A/1B and SNAP-25 expression is restricted to neuronal and endocrine cells (Oyler *et al.*, 1989; Bennett *et al.*, 1993; Bark *et al.*, 1995). Therefore, it was surprising to detect high levels of these proteins expressed in gastric mucosa. Although parietal cells make up 50% of the cell mass in gastric mucosa, several other cell types are also present, including pepsinogen-secreting chief cells, mucous neck cells, endothelial cells, endocrine cells, and cells associated with the connective tissue (Forte and Soll, 1989). To better define the source of the SNARE proteins in the gastric mucosal membrane fractions, parietal cells from rabbit gastric mucosa were separated from other cell types on a Nycodenz gradient yielding a fraction consisting of >85% parietal cells (Chew *et al.*, 1989). Both parietal cells and the nonparietal cells were collected and the levels of the SNARE proteins in the respective cell lysates were compared by immunoblotting. As shown in Figure 3, all of the SNAREs were detected in the parietal cell lysate, including syntaxin 1A/1B and SNAP-25. Only syntaxins 2 and 3 and VAMP were detected in the nonparietal cell lysate. Because many of the antibodies used in this study were generated in rabbits against rat proteins and because of a high background on immunoblots with anti-rabbit secondary antibodies with samples prepared from rabbit gastric mucosa, the remaining experiments utilized membranes derived from rat gastric mucosa.

Cofractionation of Syntaxin 3 and VAMP with H^+/K^+-ATPase-containing Tubulovesicles

As noted above (Figure 2A), each of the syntaxin isoforms as well as SNAP-25 and VAMP were found to be enriched in the microsomal fraction P_3, the fraction that includes the H^+/K^+-ATPase-containing tubulovesicles. To better characterize the localization of SNARE proteins in parietal cells, the microsomal P_3 fraction was further fractionated by equilibrium flotation through a continuous 10–40% sucrose gradient. The gradient fractions were analyzed by immunoblotting for the distribution of different syntaxin isoforms, SNAP-25, VAMP, and the α-subunit of the H^+/K^+-ATPase. Among the different syntaxin isoforms, only syntaxin 3 cofractionated with the H^+/K^+-ATPase (Figure 4A). Syntaxins 1A/B, 2, and 4 and a small amount of syntaxin 3 were found to be distributed in gradient fractions with lower density (<1.090 g/ml) than that of H^+/K^+-ATPase-containing membranes (>1.108 g/ml). VAMP immunoreactivity was also found to cofractionate with the H^+/K^+-ATPase-containing membranes while SNAP-25 was recovered in lighter membranes that overlap with the distribution of syntaxins 1A/B, 2, and 4 (Figure 4B).

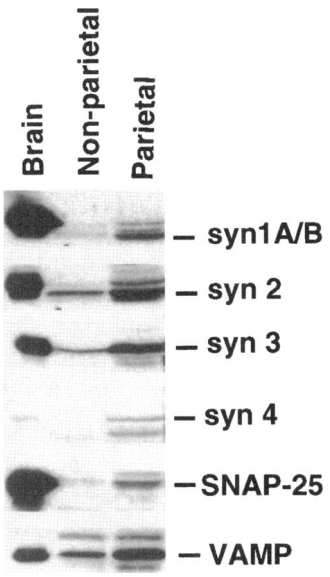

Figure 3. Parietal cells are the primary source of syntaxins, SNAP-25, and VAMP in gastric mucosa tissue. Parietal and nonparietal cell lysates were prepared as described in MATERIALS AND METHODS. Samples of rabbit brain homogenate (5 μg) and lysates of rabbit parietal cells and nonparietal cells (each containing 100 μg) were first separated on SDS-polyacrylamide gels and then transferred to nitrocellulose membranes for immunoblotting with the indicated antibodies. The immunoreactivity was detected by chemiluminescence.

SNARE Protein Topology

The SNARE proteins present in the gastric microsomal fraction could be derived from intracellular organelles (endosomes, Golgi complex, tubulovesicles, and transport vesicles) or from plasma membrane vesicles generated during tissue homogenization. In the former case, the SNAREs should be exposed on the surface of the membrane, and in the latter case, they should be at least partially sealed within the lumen of the vesicles due to the formation of outside-out plasma membrane vesicles (Hirst and Forte, 1985). To distinguish between these possibilities, a membrane preparation enriched for the H^+/K^+-ATPase was isolated on a sucrose gradient and the topology of each protein was examined by trypsin digestion (in the presence or absence of detergent) followed by immunoblotting (Figure 5). In the presence of Triton X-100, each of the SNARE proteins was digested, demonstrating that

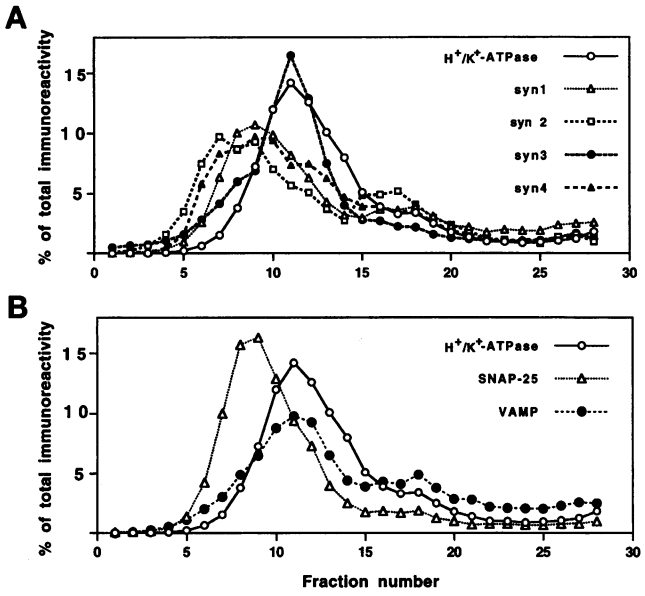

Figure 4. Distribution of H^+/K^+-ATPase, syntaxins, SNAP-25, and VAMP after sucrose gradient fractionation of P_3. The microsomal P_3 fraction (20 mg) was resuspended in a solution containing 45% sucrose, 5 mM Tris-HCl, and 1.0 mM EDTA; overlaid with a continuous 10–40% sucrose gradient; and subjected to centrifugation at 135,000 × *g* for 16 h. Fractions were collected and numbered from the top. The levels of H^+/K^+-ATPase, syntaxin isoforms, SNAP-25, and VAMP in the fractions were measured by immunoblotting with ^{125}I-labeled secondary antibody and quantitated with a PhosphorImager. The immunoreactivity of the protein in each fraction is expressed as percentage of the sum of all of the fractions assayed.

none of them are intrinsically protease resistant. However, in the absence of detergent, only syntaxin 3, VAMP, and H^+/K^+-ATPase were highly susceptible to proteolysis (>75% digested), indicating that they are derived from intracellular organelles. In contrast, syntaxins 1, 2, and 4 along with SNAP-25 were largely protected from proteolysis (20–40% digested), suggesting that these SNARE proteins may be derived, in part, from vesiculated plasma membrane. The portion of these markers that are sensitive to protease treatment may be present on intracellular membranes or inside-out plasma membrane vesicles.

Immunoisolation of H^+/K^+-ATPase-containing Vesicles

The results of the gradient fractionation analysis indicated that syntaxin 3 and VAMP (but not syntaxins 1, 2, or 4 or SNAP-25) may be associated with the tubulovesicle fraction containing the H^+/K^+-ATPase. To determine whether the syntaxin 3 and VAMP proteins actually reside in the same membrane as the H^+/K^+-ATPase, vesicles from sucrose gradient fractions were immunoisolated with a monoclonal antibody that recognizes the cytoplasmic domain of the H^+/K^+-ATPase β-subunit (Chow and Forte, 1993). The H^+/K^+-ATPase-containing vesicles were recovered on magnetic beads and analyzed for the presence of H^+/K^+-ATPase as well as the SNARE proteins by immunoblotting (Figure 6). As a control, antibody-conjugated beads that were not incubated with membranes were also analyzed to monitor the immunoblot signal generated by the antibody eluted from the beads. Incubation of gradient fractions with magnetic beads in the absence of primary antibody resulted in the isolation of low or undetectable levels of the various markers. However, in the presence of the primary antibody, approximately 25% of the H^+/K^+-ATPase in the starting fraction was recovered on the magnetic beads. Of the markers tested, only syntaxin 3 was recovered with comparable efficiency. The recovery of the other syntaxin isoforms (1, 2, and 4) and SNAP-25 was not consistently above control levels. In contrast, VAMP was coimmunoisolated with H^+/K^+-ATPase-containing vesicles, although with an efficiency that was

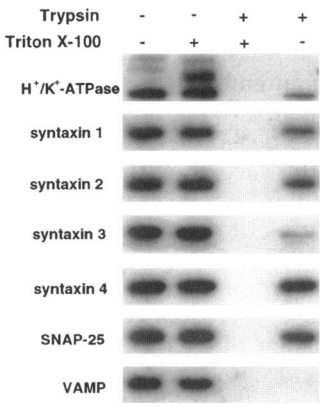

Figure 5. Susceptibility of H^+/K^+-ATPase, syntaxins, SNAP-25, and VAMP to trypsin digestion. H^+/K^+-ATPase-enriched membranes (50 μg) isolated from a sucrose density gradient as in Figure 4 were mixed with or without Triton X-100 in the presence or absence of trypsin (1 μg) at 37°C for 30 min. The incubation was terminated by addition of trichloroacetic acid to a final concentration of 10%. The precipitated proteins were recovered by centrifugation, separated on SDS-polyacrylamide gels, transferred to a nitrocellulose membranes, and probed with the indicated antibodies and ^{125}I-labeled secondary antibody for detection.

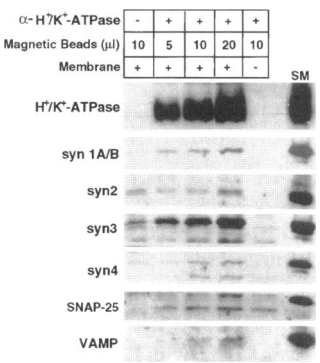

Figure 6. Coimmunoisolation of syntaxin 3 and VAMP with H^+/K^+-ATPase-containing membranes. H^+/K^+-ATPase-containing vesicles were immunoisolated with increasing amounts of magnetic beads and 2G11 (anti-H^+/K^+-ATPase β-subunit) as the primary antibody as described in MATERIALS AND METHODS. The membranes recovered on the magnetic beads were analyzed by SDS-PAGE and immunoblotted for the presence of H^+/K^+-ATPase, syntaxin isoforms, SNAP-25, and VAMP. For comparison, an aliquot of the starting membranes (SM) equivalent to 25% of the input fraction was included in the immunoblot analysis.

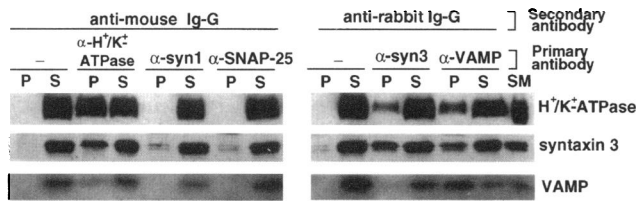

Figure 7. Syntaxin 3 and VAMP are resident proteins of a subpopulation of H^+/K^+-ATPase-containing vesicles. Vesicles first incubated with the indicated primary antibodies were isolated by flotation through a sucrose gradient and immunoisolated as described in MATERIALS AND METHODS. The membranes recovered on the secondary antibody-conjugated magnetic beads (P) and 50% of the membranes remaining in the supernatant fraction (S, recovered by sedimentation at 200,000 × *g*) were analyzed by SDS-PAGE and immunoblotted for the presence of H^+/K^+-ATPase, syntaxin 3, and VAMP. The equivalent of 10% of the starting membrane (SM) was also analyzed. The results presented are representative of three independent experiments.

lower than that of syntaxin 3. Similar results were obtained when anti-syntaxin 3 and anti-VAMP were used for the immunoisolation (Figure 7). The VAMP antibody was able to coimmunoisolate both syntaxin 3 and H^+/K^+-ATPase, and similarly, the syntaxin 3 antibody was able to coimmunoisolate both VAMP and H^+/K^+-ATPase. In contrast, neither anti-syntaxin 1 or anti-SNAP-25 were able to immunoisolate H^+/K^+-ATPase, syntaxin 3, or VAMP. These results clearly demonstrate the specificity of the immunoisolation procedure and indicate that syntaxin 3 and VAMP are associated with at least a portion of the H^+/K^+-ATPase-containing tubulovesicles. The varying efficiency of syntaxin 3, VAMP and H^+/K^+-ATPase coisolation may reflect heterogeneity in the distribution, abundance, or accessibility of these markers among the membranes in the starting fraction. Since none of the antibodies was able to quantitatively immunoisolate vesicles containing the targeted antigen, it is difficult to determine the extent to which the markers exist in mutually exclusive membrane populations. Based on experiments in other cell types, it is possible that VAMP is also present in endosomes or other endosome-derived compartments (Chilcote *et al.*, 1995). Furthermore, since VAMP and syntaxin 3 expression is not restricted to parietal cells (Figure 3), it is likely that at least a portion of these markers present in the total gastric mucosal membrane fractions are derived from nonparietal cells.

DISCUSSION

The recruitment of H^+/K^+-ATPase-containing tubulovesicles from a cytoplasmic pool to the apical canalicular membrane is central to the regulation of acid secretion by gastric parietal cells (Forte and Soll, 1989). The prospect that membrane trafficking processes are responsible for the regulated recruitment of H^+/K^+-ATPase (Forte and Yao, 1996) is strongly supported by our finding that a variety of SNARE proteins are present in parietal cells. A role for SNARE proteins in parietal cell activation is also supported by the observation that members of two protein families that regulate the assembly of SNARE protein complexes, the rab family of low molecular weight GTP-binding proteins (Pfeffer, 1994) and the sec1 family (Pevsner, 1996), are expressed by parietal cells (Tang *et al.*, 1992; Goldenring *et al.*, 1994; Peng, unpublished observation).

Two SNARE proteins, VAMP and syntaxin 3, are localized to the H^+/K^+-ATPase-containing tubulovesicle membrane. Members of the VAMP family have been found to be associated with a variety of exocytic vesicles including synaptic vesicles in neurons (Trimble *et al.*, 1988; Baumert *et al.*, 1989), chromaffin granules in adrenal chromaffin cells (Höhne-Zell *et al.*, 1994), zymogen granules in pancreatic acinar cells (Braun *et al.*, 1994; Gaisano *et al.*, 1994), and GLUT4 transporter-containing vesicles in adipocytes and muscle cells (Cain *et al.*, 1992; Volchuk *et al.*, 1995). In several systems, the cleavage of VAMP by tetanus toxin or botulinum toxins is associated with an inhibition of exocytosis (Gaisano *et al.*, 1994; Höhne-Zell *et al.*, 1994; Regazzi *et al.*, 1995). Thus, it is likely that the VAMP associated with the tubulovesicle compartment in parietal cells functions as a v-SNARE in the delivery of H^+/K^+-ATPase to the plasma membrane. The localization of syntaxin 3 to the H^+/K^+-ATPase-containing tubulovesicles was unexpected because syntaxin 3 has been proposed to function as a t-SNARE (Bennett *et al.*, 1993). Several possibilities could account for this finding: 1) syntaxin 3 could function as a v-SNARE, rather than a t-SNARE, during the recruitment of tubulovesicles to the plasma membrane; 2) syntaxin 3 could act as a t-SNARE during the homotypic fusion of tubulovesicles (homotypic fusion events are likely to contribute to the morphological changes associated with parietal cell activation); and 3) syntaxin 3 could function as a t-SNARE for endocytic vesicles during H^+/K^+-ATPase retrieval from the plasma membrane. The recent observation that syntaxin 3 is associated with zymogen granules in pancreatic acinar cells demonstrates that localization of syntaxin 3 to exocytic vesicles is not restricted to gastric parietal cells (Gaisano *et al.*, 1996). The identification and characterization of the physiologically relevant binding partners for syntaxin 3 will be required to elucidate its function in parietal cells and other cell types.

The remaining SNARE proteins expressed in parietal cells (SNAP-25 and syntaxins 1, 2, and 4) are excluded from the tubulovesicle compartment. In other cell types, each of these SNARE proteins is localized, at least partially, to the plasma membrane

(Bennett *et al.*, 1993; Söllner *et al.*, 1993a; Garcia *et al.*, 1995). The fact that SNAP-25 and syntaxins 1, 2, and 4 in the gastric microsomal fraction are partially protected from proteolysis is consistent with localization to plasma membrane-derived vesicles (Hirst and Forte, 1985). Since the plasma membrane of epithelial cells, including parietal cells, is composed of distinct apical and basolateral domains (Roush *et al.*, 1994), the localization of plasma membrane SNARE proteins should be reflective of distinct polarized functions. Of particular relevance in parietal cells is the fact that the H^+/K^+-ATPase is delivered exclusively to the apical plasma membrane domain (Chow and Forte, 1995). Therefore, any target membrane SNARE proteins involved in this process should be apically localized. The prospect that syntaxins are differentially localized to the apical and basolateral membranes in polarized epithelial cells is supported by recent observations in pancreatic acinar cells and transfected MDCK cells (Gaisano *et al.*, 1996; Low *et al.*, 1996). Further subcellular fractionation and immunolocalization studies will be required to better define the localization of SNAP-25 and syntaxins 1, 2, and 4 in gastric parietal cells.

The detection of syntaxin 1 and SNAP-25 in parietal cells was unexpected since the expression of these two SNARE proteins had previously been reported to be restricted to neuronal and endocrine cells (Oyler *et al.*, 1989; Bennett *et al.*, 1993; Bark *et al.*, 1995). The fact that both of these SNAREs are recovered in a cell preparation that consists of >85% parietal cells indicates that endocrine cells present in the gastric mucosa are not the source of syntaxin 1 and SNAP-25. Furthermore, since the gastric mucosa receives minimal direct innervation and the neuronal membrane marker synaptophysin (Jahn *et al.*, 1985) is not detected in gastric mucosal membranes (Peng, unpublished observation), the prospect of neuronal contamination is unlikely. In neurons, syntaxin 1 and SNAP-25 are colocalized on the plasma membrane (Garcia *et al.*, 1995), where they are thought to form a high-affinity binding site for VAMP (Pevsner *et al.*, 1994). SNAP-25 can be coimmunoisolated from a P_3 microsome fraction with an anti-syntaxin 1 antibody (Peng, unpublished observation), suggesting that these markers are also colocalized in parietal cells. Because of the similarity between axonal protein targeting in neurons and apical protein targeting in epithelial cells (Dotti and Simons, 1990), it is tempting to speculate that syntaxin 1 and SNAP-25 may be localized to the apical plasma membrane in parietal cells. If this were the case, syntaxin and SNAP-25 would provide an optimal target for the VAMP-mediated recruitment of H^+/K^+-ATPase-containing tubulovesicles to the apical canalicular membrane.

The fact that multiple SNARE proteins are expressed in gastric parietal cells and that at least some of these proteins are differentially localized is consistent with the prospect that SNARE proteins contribute to the specificity of transport vesicle targeting. The variety and abundance of SNARE proteins expressed by parietal cells indicates that these cells will provide an experimental system in which the biochemical and functional properties of the different SNARE proteins can be systematically investigated.

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