Two Rab proteins, vesicle-associated membrane protein 2 (VAMP-2) and secretory carrier membrane proteins (SCAMPs), are present on immunoisolated parietal cell tubulovesicles

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The tubulovesicles of gastric parietal cells sequester H^+/K^+-ATPase molecules within resting parietal cells. Stimulation of parietal cell secretion elicits delivery of intracellular H^+/K^+-ATPase to the apically oriented secretory canaliculus. Previous investigations have suggested that this process requires the regulated fusion of intracellular tubulovesicles with the canalicular target membrane. We have sought to investigate the presence of critical putative regulators of vesicle fusion on immunoisolated gastric parietal cell tubulovesicles. Highly purified tubulovesicles were prepared by gradient fractionation and immunoisolation on magnetic beads coated with monoclonal antibodies against the α subunit of H^+/K^+-ATPase. Western blot analysis revealed the presence of Rab11, Rab25, vesicle-associated membrane protein 2 (VAMP-2) and secretory carrier membrane proteins (SCAMPs) on immunoisolated vesicles. The same cohort of proteins was recovered on vesicles immunoisolated with monoclonal antibodies against SCAMPs and VAMP-2. In contrast, whereas immunoreactivities for syntaxin 1A/1B and synaptosome-associated protein (SNAP-25) were present in gradient-isolated vesicles, none of the immunoreactivity was associated with immunoisolated vesicles. The observation of VAMP-2 and two Rab proteins on immunoisolated H^+/K^+-ATPase-containing tubulovesicles supports the role for tubulovesicles in a regulated vesicle fusion process. In addition, the presence of SCAMPs along with Rab11 and Rab25 implicates the tubulovesicles as a critical apical recycling vesicle population.

INTRODUCTION

The regulation of vesicle transport is critical to the function of polarized epithelial cells. In response to histamine and acetylcholine, the gastric parietal cell secretes large quantities of HCl into the lumen of the gastric gland. The secretion of HCl by the gastric parietal cell is thought to require the regulated fusion of H^+/K^+-ATPase-containing tubulovesicles into an intracellular secretory canaliculus target membrane [1]. The massive vesicle fusion event delivers the H^+/K^+-ATPase to the apical surface, allowing the pumping of HCl into the gastric lumen. After the withdrawal of secretagogues, the H^+/K^+-ATPase-containing membranes are internalized and recycled into tubulovesicles that are competent for another round of fusion [2]. Therefore the modulation of gastric HCl secretion relies on the regulation of the vesicular trafficking of the gastric H^+/K^+-ATPase through a defined recycling vesicle population. Although the parietal cell provides perhaps the most dramatic example of apical vesicle recycling, this process is also implicated in the regulation of a number of other membrane pumps and channels, particularly in the trafficking of the cystic fibrosis transmembrane regulator [3] and water channels [4–6].

Recent discoveries have improved our understanding of the molecular regulation of intracellular vesicle targeting. Studies of classical secretory systems such as the neuron and the budding yeast *Saccharomyces cerevisiae* have converged on an evolutionarily conserved multimeric complex of proteins thought to regulate vesicle docking and fusion [7,8]. In this complex, integral membrane proteins, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), of both the vesicle (v-SNARE) and target membrane (t-SNARE) bind to each other and provide a scaffolding to which soluble factors attach [9]. In the brain, vesicle-associated membrane protein 2 (VAMP-2), a synaptic vesicle protein, was identified as a v-SNARE, and two proteins of the neuronal plasmalemma, syntaxin 1A/1B and synaptosome-associated protein (SNAP-25), were identified as t-SNAREs [9]. The t-SNAREs syntaxin 1 and SNAP-25 are also components of recycling synaptic vesicle membranes [10]. Rothman and colleagues have proposed that the assembly of the SNARE complex might be a molecular regulatory mechanism common to all intracellular vesicle fusion events [11]. Rigorous testing of the SNARE hypothesis is required to determine how generally it should be applied to non-classical secretory systems such as the gastric parietal cell. Isoforms of VAMP and syntaxin [12] are expressed in a variety of non-neural tissues. VAMP-2 resides on synaptic vesicles, pancreatic zymogen granules and Glut-4 vesicles of adipocytes [13–15]. It is currently not clear whether VAMP and syntaxin isoforms assemble into a complex that regulates vesicle docking or fusion in non-neural tissues. Specifically, the presence of the SNARE proteins in the gastric parietal cell has not been investigated.

Rab proteins are small GTP-binding proteins thought to regulate a number of aspects of vesicle trafficking [16–18]. Although small GTP-binding proteins are not found in the SNARE complex, a Rab protein is required for the assembly of the SNARE complex in yeast [19]. We have previously reported that several small GTP-binding proteins are present in enriched preparations of parietal cell tubulovesicles [20]. In particular,

Abbreviations used: AEBSF, 4-(2-aminoethyl)benzenesulphonylfluoride/HCl; CFTR, cystic fibrosis transmembrane regulator; SCAMP, secretory carrier membrane protein; SNAP, soluble *N*-ethylmaleimide sensitive factor attachment protein; SNAP-25, synaptosome-associated protein; SNARE, SNAP receptor; t-SNARE, target membrane SNARE; VAMP, vesicle-associated membrane protein; v-SNARE, vesicle SNARE.

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Rab11 is enriched in the gastric parietal cell and co-distributes with the gastric H^+/K^+-ATPase in resting and stimulated parietal cells [21,22]. The small GTP-binding protein Rab25, originally cloned from parietal cells, is expressed in gastrointestinal mucosa, kidney and lung [23]. Rab25 mRNA is enriched in gastric parietal cells over chief cells, but the subcellular localization of Rab25 in the parietal cell has previously been unknown [23]. Purified zymogen granules from rat pancreatic acinar cells contain at least seven GTP-binding proteins [24], and vesicles immunoisolated from rat hepatocytes with antibodies specific for Rab1A contain at least four small GTP-binding proteins [25]. The number and identity of the small GTP-binding proteins on gastric tubulovesicles are unknown.

The secretory carrier membrane proteins (SCAMPs) are residents of a variety of secretory vesicles including synaptic vesicles, pancreatic zymogen granules and Glut-4 vesicles of adipocytes [26,27]. In addition, SCAMP 37 co-localizes with endocytosed transferrin in transfected NRK fibroblasts [28]. The presence of SCAMPs in regulated secretory vesicles and endocytic vesicles suggests that SCAMPs are involved in a general cell-surface recycling system from which some secretory vesicles might be derived [28,29]. The localization of SCAMPs in the gastric parietal cell has not been examined.

The goals of this study were to investigate the distribution of Rab11 and Rab25 in immunoadsorbed gastric tubulovesicles and to determine whether SNARE proteins and SCAMPs are present in gastric parietal cells. We have hypothesized that Rab proteins enriched in the parietal cell tubulovesicles are involved in the regulation of the membrane trafficking of the gastric H^+/K^+-ATPase. To test this hypothesis, tubulovesicles, prepared by the standard density gradient method [30], were further purified by immunoadsorption. The composition of the immunoadsorbed vesicles was analysed by immunoblotting with monoclonal antibodies specific for SNARE proteins and Rab proteins. Here we report that the gastric H^+/K^+-ATPase,VAMP-2, Rab11, Rab25 and SCAMPs, but not syntaxin 1A/1B or SNAP-25, are present on vesicles immunoisolated with a monoclonal antibody specific for the gastric H^+/K^+-ATPase.

MATERIALS AND METHODS

Materials

New Zealand White rabbits were obtained from Shelton's Bunny Barn. The monoclonal antibody (12.18), specific for the α subunit of the gastric H^+/K^+-ATPase, was a generous gift from Dr. Adam Smolka (Medical University of South Carolina, Charleston, SC, U.S.A.). Anti-syntaxin (HPC-1) and anti-SNAP-25 (SMI 81) monoclonal antibodies were purchased from Sigma (St. Louis, MO, U.S.A.) and Sternberger Monoclonals Incorporated (Baltimore, MD, U.S.A.) respectively. The VAMP-2-specific monoclonal antibody Cl69.1 was a gift from Dr. Reinhard Jahn (Yale University, New Haven, CT, U.S.A.). The anti-SCAMPs antibody (7C12), which recognizes all SCAMPs [26], was a gift from Dr. David Castle (University of Virginia, Charlottesville, VA, U.S.A.). The production of a Rab11-specific monoclonal antibody (8H10) has been described previously [31]. Anti-(mouse IgG)-coated magnetic Dynabeads were obtained from Dynal (Great Neck, NY, U.S.A.). Fc fragment-specific secondary antibodies conjugated with horseradish peroxidase were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.). Enhanced chemiluminescence substrate (SuperSignal) was obtained from Pierce (Rockford, IL, U.S.A.). Non-immune control IgG2b was purchased from Sigma. Peptides were synthesized at either the University of Georgia Peptide Synthesis Core Laboratory or the Medical College of Georgia Core Biochemistry Laboratory, and subsequently reconstituted in distilled water. Immobilon-P PVDF membranes were purchased from Millipore (Bedford, MA, U.S.A.). All other reagents were from standard suppliers and were of the highest purity available.

Tubulovesicle preparation

Gastric tubulovesicles were prepared from resting rabbit gastric mucosa as described by Crothers et al. [30]. Briefly, male New Zealand White rabbits were anaesthetized by intravenous administration of a mixture of ketamine and xylazine; their stomachs were then perfused under high pressure with oxygenated PBS and removed. The gastric mucosa was scraped off the serosa with a glass slide, minced with scissors and homogenized in 5 vol. of homogenization buffer [113 mM mannitol/37 mM sucrose/0.4 mM EDTA/5 mM Mes (pH 6.7)/5 mM benzamidine/0.1 mM 4-(2-aminoethyl)benzenesulphonylfluoride/HCl (AEBSF)] plus a protease inhibitor cocktail (1.75 μg/ml aprotinin/2.5 μg/ml soybean trypsin inhibitor/1 μg/ml chymostatin/1 μg/ml pepstatin A/1 μg/ml leupeptin). The homogenate was centrifuged sequentially at 50 ***g***, 1000 ***g***, 14000 ***g*** and 100000 ***g***. The 100000 ***g*** pellet was resuspended in 10% sucrose buffer [5 mM Hepes/NaOH (pH 7.4)/300 mM sucrose) and fractionated over discontinuous sucrose gradients consisting of layers of 20%, 27% and 33% (w/v) sucrose. The vesicles partitioning at the 10–20% sucrose interface were used for immunoadsorption experiments.

Immunoadsorption of tubulovesicles

For a single immunoadsorption experiment, 750 μg of Dynabeads were washed three times in PBS containing 1% (w/v) BSA, blocked for 30 min at 4 °C in PBS/1% BSA, and washed twice in PBS/0.1% BSA. The blocked beads were incubated overnight at 4 °C with monoclonal antibodies specific for the α subunit of the gastric H^+/K^+-ATPase (12.18), VAMP-2 (Cl69.1), SCAMPs (7C12) and non-immune IgG2b. The beads were washed four times for 30 min at room temperature with PBS/0.1% BSA and incubated with tubulovesicles (20 μg of protein) for 2 h at room temperature in PBS/0.1% BSA plus a protease inhibitor cocktail (5 mM benzamidine/0.1 mM AEBSF/1.75 μg/ml aprotinin/2.5 μg/ml soybean trypsin inhibitor/1 μg/ml chymostatin/1 μg/ml pepstatin A/1 μg/ml leupeptin). After 2 h the unbound material was removed, centrifuged at 20 lb/in^2 (138 kPa) for 5 min at 4 °C in an Airfuge (Beckman Instruments, Stanford, CA, U.S.A.) and resuspended in SDS sample buffer. The bound material was eluted from the beads in SDS sample buffer, and all samples were heated at 65 °C for 5 min. The proteins were separated by SDS/PAGE, electrophoretically transferred to Immobilon-P PVDF membranes and analysed by immunoblotting. For quantification, autoradiographs were digitized with an Alpha Innotech image analyser and integrated densities from three identical experiments were determined. Results were expressed as percentage recovery $\pm$ S.E.M.

Production of monoclonal antibodies against Rab25

His-tagged recombinant rabbit Rab25 was prepared as previously described [23] and purified by nickel-affinity (His-Bind) chromatography (Novagen, Madison, WI, U.S.A.). Monoclonal antibody production was performed at the University of Georgia Monoclonal Antibody Facility as previously described [31]. Initial immunizations were performed with 150 μg of Rab25 with boost injections with 100 μg of protein. Hybridomas were

screened by ELISA simultaneously for immunoreactivity against both recombinant Rab11 and Rab25. Only clones displaying selective immunoreactivity against Rab25 were propagated.

For competition studies, two synthetic peptides were constructed based on the C-terminal variable regions of Rab11 (QKQMSDRRENDMSPSNNVVPIHVPPTTENKPKVQ) and Rab25 (KVSKQIQNSPRSNAIALGSAQAGQEPGPGQKR). In ELISA, the Rab11 peptide inhibited the binding of monoclonal antibody 8H10 to recombinant Rab11 with an IC_{50} of 1 μg/ml (results not shown). Similarly, the Rab25 C-terminal peptide inhibited the binding of 12C3 to recombinant Rab25 in ELISA with an IC_{50} of 1 μg/ml (results not shown). In Western blot competition assays, strips of either recombinant proteins (approx. 25 ng per strip) or gastric mucosal 100000 ***g*** microsomes (approx. 25 μg per strip) were incubated overnight at room temperature with peptides and antibodies added together without preincubation.

RESULTS AND DISCUSSION

Tubulovesicles contain the gastric H^+/K^+-ATPase, Rab11, SNAREs and SCAMPs

An H^+/K^+-ATPase-enriched membrane vesicle fraction was prepared from resting rabbit gastric mucosa by the method of Crothers et al. [30]. The 100000 ***g*** microsomes from rabbit gastric mucosal homogenates were fractionated over a discontinuous sucrose gradient; the vesicles taken from the 10–20 % sucrose interface were recovered as 'enriched tubulovesicles'. Although this preparation is highly enriched for H^+/K^+-ATPase (80–85 % of total protein), the proteins responsible for vesicle trafficking are often minor components. We therefore sought to isolate a tubulovesicle preparation of higher purity to characterize these proteins. The enriched tubulovesicle preparation was the starting material for all the immunoadsorption experiments described below. Tubulovesicles were incubated with antibody-coated magnetic beads, and the vesicles that bound to the beads were collected with a magnet and eluted from the beads in SDS sample buffer. The non-adsorbed material was collected and centrifuged at 110000 ***g***; the sedimented membranes were resuspended in SDS sample buffer. The compositions of the tubulovesicles, the immunoadsorbed pellet and the non-adsorbed supernatant were analysed by immunoblotting. The tubulovesicles were immunoadsorbed with monoclonal antibodies specific for the α subunit of the gastric H^+/K^+-ATPase, VAMP-2, SCAMPs and non-immune IgG2b. Representative results of these experiments are shown in Figure 1. Immunoreactivities for the H^+/K^+-ATPase, Rab11, VAMP-2, SCAMPs and syntaxin 1A/1B were all detected on immunoblots of enriched tubulovesicles. Quantified data for the recovery of immunoreactivities from three experiments are summarized in Table 1.

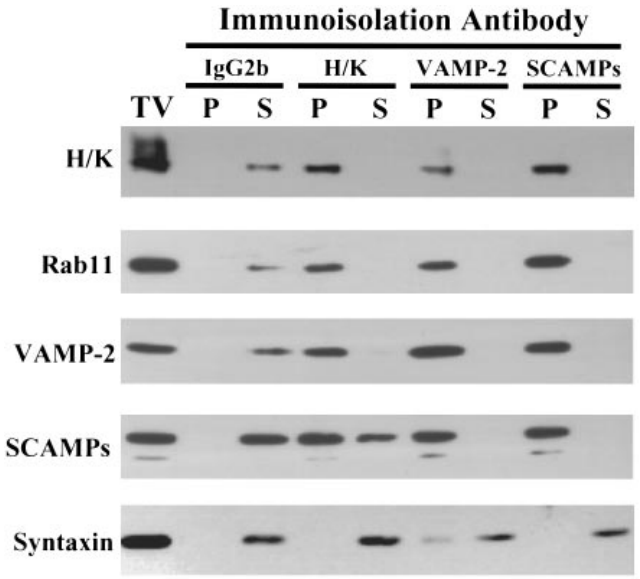

Figure 1 Immunoisolated tubulovesicles contain the gastric H^+/K^+-ATPase, Rab11, VAMP-2 and SCAMPs, but not syntaxin 1A/1B

The distribution of Rab11, Rab25, VAMP-2, SCAMPs and syntaxin 1A/1B in gastric parietal cells was determined by immunoadsorbing gastric tubulovesicles on magnetic beads coated with monoclonal antibodies specific for the α subunit of the gastric H^+/K^+-ATPase (H/K), VAMP-2, SCAMPs and non-immune immunoglobulin (IgG2b), as indicated at the top. Enriched tubulovesicles (TV), the immunoadsorbed tubulovesicles (P, pellet) and the non-adsorbed material (S, supernatant) were analysed by immunoblotting with monoclonal antibodies specific for the gastric H^+/K^+-ATPase, Rab11, VAMP-2, SCAMPs and syntaxin 1A/1B (indicated at the left). H^+/K^+-ATPase, Rab11, VAMP-2 and SCAMPs were all isolated on immunoadsorbed tubulovesicles. Syntaxin 1A/1B immunoreactivity was consistently observed in non-adsorbed vesicles. The results are representative of six separate experiments.

When tubulovesicles were immunoadsorbed with the anti-H^+/K^+-ATPase antibody, the immunoreactivities for the H^+/K^+-ATPase, Rab11, VAMP-2 and SCAMPs were recovered in the immunoadsorbed pellet. Beads coated with non-immune IgG2b did not adsorb significant immunoreactivity. However, the immunoreactivity for syntaxin 1A/1B was not recovered in the immunoadsorbed pellet but was retained in the non-adsorbed supernatant. The recovery of immunoreactivity in the sedimen-

Table 1 Antigen recovery in immunoisolation of gastric tubulovesicles

Autoradiographs from three immunoisolations were quantified by densitometry and expressed as percentages of total recovery (mean ± S.E.M.). The antibodies coupled to magnetic beads for immunoisolation are indicated at the top; the proteins detected by immunoblotting are indicated at the left. Abbreviations: P, the immunoadsorbed material (bead pellet); S, the non-adsorbed fraction (supernatant).

		IgG2b		anti-H/K-ATPase		anti-VAMP-2		anti-SCAMPs	
Western blot	Immunoisolation antibody ...	P	S	P	S	P	S	P	S
H/K-ATPase		10.1 ± 5.1	89.8 ± 5.1	82.3 ± 14.0	17.6 ± 14.0	75.2 ± 10.7	24.8 ± 10.7	78.5 ± 14.2	21.5 ± 14.2
Rab11		4.2 ± 1.9	95.7 ± 1.9	78.5 ± 15.5	21.5 ± 15.5	76.6 ± 17.5	23.3 ± 17.5	84.9 ± 12.7	15.0 ± 12.7
SCAMPs		2.1 ± 0.5	97.8 ± 0.5	68.1 ± 7.5	31.8 ± 7.5	89.2 ± 8.9	10.7 ± 8.9	89.8 ± 8.6	10.1 ± 8.6
VAMP-2		1.0 ± 0.9	98.9 ± 0.9	89.3 ± 5.3	10.7 ± 5.3	88.6 ± 8.1	11.4 ± 8.1	92.2 ± 3.8	7.7 ± 3.8
Syntaxin 1		4.6 ± 0.9	95.4 ± 0.9	7.0 ± 2.6	92.9 ± 2.6	11.9 ± 2.1	88.0 ± 2.1	5.0 ± 2.2	94.9 ± 2.2

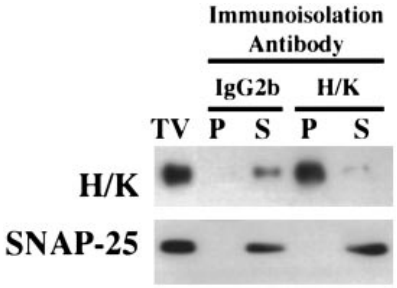

Figure 2 SNAP-25 is not present on immunoisolated tubulovesicles

Enriched gastric tubulovesicles were immunoadsorbed on magnetic beads coated with a monoclonal antibody specific for the α subunit of the gastric H^+/K^+-ATPase (H/K) and non-immune immunoglobulin (IgG2b). Enriched tubulovesicles (TV), the immunoadsorbed tubulovesicles (P, pellet) and the non-adsorbed material (S, supernatant) were analysed by immunoblotting with monoclonal antibodies specific for the gastric H^+/K^+-ATPase and SNAP-25. SNAP-25 immunoreactivity was consistently recovered in the non-adsorbed membranes. The results are representative of three separate experiments.

table non-adsorbed fraction indicated that the immunoadsorption procedure did not disrupt the vesicles. To confirm the specificity of the proteins associated with tubulovesicles, the immunoadsorption experiments were repeated with antibodies specific for VAMP-2 and SCAMPs. In both cases the H^+/K^+-ATPase, Rab11, VAMP-2 and SCAMPs immunoreactivities were recovered in the immunoadsorbed pellet. The syntaxin 1A/1B immunoreactivity was consistently recovered in the supernatant, indicating that the enriched tubulovesicles from the discontinuous sucrose gradient are not a homogeneous population of vesicles containing H^+/K^+-ATPase. These results indicate that H^+/K^+-ATPase-containing tubulovesicles contain Rab11, VAMP-2 and SCAMPs, but not syntaxin 1A/1B. The results highlight the importance of vesicle immunoadsorption in the compositional analysis of secretory vesicles. The detection of syntaxin 1A/1B immunoreactivity in the enriched tubulovesicles, but never in the immunoisolated tubulovesicles, indicates that vesicles other than the H^+/K^+-ATPase-containing tubulovesicles are present in the enriched tubulovesicle preparation.

To explain the marked secretagogue-induced changes in parietal cell morphology, Forte et al. [1] proposed a membrane recycling hypothesis in which the tubulovesicles fuse with the secretory canaliculus of the gastric parietal cell in response to secretagogues. According to the membrane recycling hypothesis, this fusion event inserts the H^+/K^+-ATPase into the cell surface to secrete HCl into the lumen of the gastric gland. After withdrawal of the secretagogue, the H^+/K^+-ATPase-containing membranes are recycled from the secretory canaliculus into tubulovesicles that are competent for another round of fusion. The presence of VAMP-2, a v-SNARE, on immunoisolated tubulovesicles provides evidence for the fusion of the tubulovesicles with the secretory canaliculus. It remains to be determined whether VAMP-2 assembles into a SNARE complex in the gastric parietal cell, and the putative t-SNARE on the secretory canaliculus remains unidentified.

Tubulovesicles do not contain SNAP-25

SNAP-25, originally described as a resident of the presynaptic membrane [32], has been recognized as a t-SNARE in neurons [9,33] and neuroendocrine cells [34,35]. Because SNAP-25 is present on recycling synaptic vesicles [10], we sought to determine whether SNAP-25 resided on the tubulovesicles (Figure 2). As with syntaxin, SNAP-25 immunoreactivity was detected in the gradient-isolated enriched tubulovesicles. However, no significant SNAP-25 immunoreactivity was recovered in vesicles immunoisolated with antibodies against the α subunit of H^+/K^+-ATPase (Figure 2). Densitometric quantification revealed that $95.7 \pm 2.4\%$ of SNAP-25 immunoreactivity was recovered in the non-adsorbed supernatant. In contrast, $85.8 \pm 10.9\%$ of the H^+/K^+-ATPase immunoreactivity partitioned into the bead pellet. These results indicate that neither syntaxin 1A/1B nor SNAP-25 is present on tubulovesicle membranes from resting parietal cells.

Tubulovesicles contain Rab25

Rab25 is a small GTP-binding protein expressed in the gastrointestinal mucosa, kidney and lung [23]. Rab11 and Rab25 are 68% identical in their deduced amino acid sequences, but their C-terminal regions demonstrate little significant sequence similarity [23]. The C-terminus or hypervariable domain of Rab proteins is the region of least similarity among all the Rab proteins and is responsible, at least in part, for targeting Rab proteins to specific intracellular compartments [36]. Immunoadsorption experiments were performed to determine whether these two closely related Rab proteins with divergent C-termini are targeted to the same population of vesicles in the gastric parietal cell.

We have developed a Rab25-specific monoclonal antibody (12C3) that does not cross-react with Rab11. On Western blots 12C3 strongly recognized recombinant Rab25 but no immunoreactivity for recombinant Rab11 was detected (Figure 3A). We have previously demonstrated the specificity of monoclonal antibody 8H10 for Rab11 over Rab25 [31]. Because both proteins have similar molecular masses we have mapped the antigenic sites recognized by the 8H10 and 12C3 monoclonal antibodies by using synthetic peptides constructed against the C-terminal variable regions of the two Rab proteins. Figure 3(B) demonstrates that 8H10 recognized a 25 kDa protein in gastric 100000 ***g*** microsomes. Rab11 immunoreactivity was specifically inhibited by incubation with the Rab11 C-terminal peptide but not by the Rab25 peptide. Similarly, 12C3 recognition of a 25 kDa species in 100000 ***g*** microsomes was inhibited by incubation with the Rab25 C-terminal peptide but not by the Rab11 peptide. Synthetic peptides constructed against the N-termini of both Rab11 and Rab25 had no effect on the binding of either 8H10 or 12C3 to their respective antigens (results not shown).

We have previously demonstrated that Rab11 immunoreactivity partitioned into the tubulovesicle membranes [31], and the above studies have confirmed these findings with immunoisolation. We therefore studied the distribution of Rab25 in membrane fractions obtained during the preparation of enriched tubulovesicle membranes (Figure 3C). The Rab25-specific monoclonal antibody (12C3) detected a 25 kDa polypeptide that co-enriched with immunoreactivity for the gastric H^+/K^+-ATPase in density gradient tubulovesicle preparations (Figure 3C). To confirm the association of Rab25 with tubulovesicles, immunoadsorption experiments were performed with the monoclonal antibody specific for the α subunit of the gastric H^+/K^+-ATPase; the immunoadsorbed tubulovesicles were analysed by immunoblotting with the Rab25-specific monoclonal antibody (12C3). Rab25 was present in the enriched tubulovesicle preparation and recovered in tubulovesicles immunoadsorbed with a monoclonal antibody specific for the α subunit of the gastric H^+/K^+-ATPase (Figure 3D). Similarly to studies described above, $83 \pm 17\%$ of the H^+/K^+-ATPase immunoreactivity was recovered in the bead pellet. Densitometric quantitation of Rab25 immunoreactivity

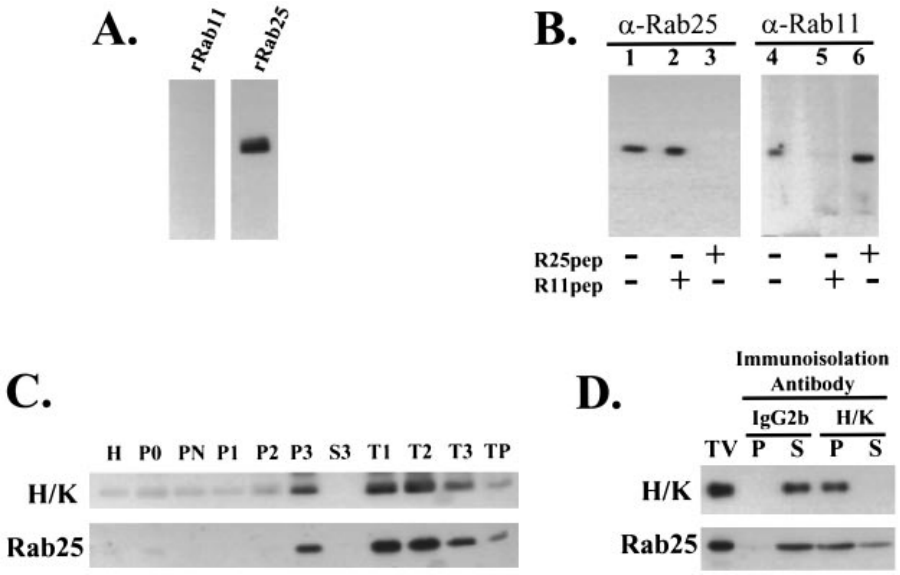

Figure 3 Localization of Rab25 in gastric tubulovesicles

(**A**) The anti-Rab25 monoclonal antibody 12C3 was used to probe Western blots of 50 ng of either recombinant His-tagged Rab11 (rRab11) or recombinant His-tagged Rab25 (rRab25). 12C3 recognized only Rab25. (**B**) Anti-Rab11 (8H10) and anti-Rab25 (12C3) were used to probe Western blots of 100 000 ***g*** microsomes (25 μg of protein) from gastric mucosa in the absence or presence of 10 μg/ml blocking C-terminal-blocking peptides. Both 8H10 (α-Rab11) and 12C3 (α-Rab25) monoclonal antibodies recognized 25 kDa proteins. Immunoreactivity detected with the anti-Rab11 was abolished by the Rab11 C-terminal peptide (R11pep) but was unaffected by the Rab25 C-terminal peptide (R25pep). Conversely, Rab25 immunoreactivity was abolished by R25pep but was unaffected by R11pep. (**C**) Membrane subfractions were prepared from rabbit gastric mucosa as described in the Materials and methods section and analysed (20 μg of protein) by immunoblotting with monoclonal antibodies specific for the α subunit of the gastric H^+/K^+-ATPase (H/K) and Rab25. Abbreviations: H, homogenate; P0, 50 ***g*** pellet; PN, postnuclear supernatant; P1, 1000 ***g*** pellet; P2, 14 000 ***g*** pellet; P3, 100 000 ***g*** pellet; S3, 100 000 ***g*** supernatant; T1, 20% sucrose gradient interface; T2, 27% sucrose gradient interface; T3, 33% sucrose gradient interface; TP, sucrose gradient pellet. Immunoreactivity for Rab25 co-distributed with H^+/K^+-ATPase in the resolved membrane fractions. (**D**) Rab25 immunoreactivity was investigated in immunoisolated tubulovesicles. Enriched tubulovesicles (TV), the tubulovesicles immunoadsorbed (P, pellet) with an anti-(H^+/K^+-ATPase) monoclonal antibody (H/K) or non-immune immunoglobulin (IgG2b), and the non-adsorbed material (S, supernatant) were analysed by immunoblotting with monoclonal antibodies specific for the α subunit of H^+/K^+-ATPase and Rab25. The majority of the Rab25 immunoreactivity was recovered in the immunoisolated tubulovesicles. The results are representative of three separate experiments.

revealed that $68.3 \pm 11.2\%$ of Rab25 immunoreactivity partitioned into the bead pellet. These results indicate that, despite their divergent C-termini, both Rab11 and Rab25 are targeted to the same population of vesicles in gastric parietal cells.

In our original studies of small GTP-binding proteins in parietal cells, we observed a major GTP-binding protein species that translocated into heavy membrane fractions in concert with secretory stimulation [20]. We later identified this protein as Rab2 by using a rabbit polyclonal antiserum raised against whole Rab2 [37]. Although this antibody preparation was thought to be specific for Rab2 at the time, we have recently re-examined these findings with a monoclonal antibody against Rab2 (1C5B; a gift from William Balch, Scripps Research Institute, San Diego, CA, U.S.A.) that has high specificity for Rab2 [38]. These studies have not confirmed our previous findings, and whereas Rab2 was present in parietal cells, Rab2 immunoreactivity did not partition into the enriched tubulovesicles (results not shown). These results were also confirmed in immunoisolated tubulovesicles (results not shown). To identify the GTP-binding species previously labelled as Rab2, we have re-probed Western blots with the 8H10 monoclonal antibody specific for Rab11. These studies have revealed that the protein previously identified as Rab2 is indeed Rab11 (results not shown).

This is the first report of the co-localization of Rab11 and Rab25 in a exocytotic/recycling vesicle membrane. The mechanism or mechanisms for targeting two different Rab proteins to a single vesicle class are not known. Previous investigations have suggested that C-terminal sequences in the Rab protein variable region might determine the vesicle targeting of Rab proteins within a particular cell [36]. However, there are now several reports documenting two or more Rab proteins within individual vesicle populations. Rab3A and Rab3C are present on synaptic vesicles, and both Rabs dissociate from the membrane during neurotransmitter release [39–41]. Rab5A, Rab5B and Rab5C all reside in early endosomes, and all three isoforms of Rab5 stimulate endocytic vesicle fusion [42]. The closely related Rab proteins Rab7 and Rab9 are recruited to late endosomes by kinetically distinct mechanisms, suggesting a role for distinct targeting factors or receptors that recruit highly similar Rab proteins to a single vesicle population [43]. On the basis of the differences in the amino acid sequences of the C-termini of Rab11 and Rab25, it seems likely that these Rab proteins are recruited to gastric tubulovesicles by different targeting factors.

In this paper we demonstrate that immunoadsorbed H^+/K^+-ATPase-containing tubulovesicles contain Rab11, Rab25, VAMP-2 and SCAMPs, but not syntaxin 1A/1B or SNAP-25. These findings indicate that the molecular regulation of the vesicular trafficking of the gastric H^+/K^+-ATPase in parietal cells involves components of the SNARE complex. The presence of VAMP-2, a v-SNARE, and Rab proteins on immunoisolated tubulovesicles supports the membrane recycling model of parietal cell secretion [1]. Moreover the presence of this cadre of vesicle-trafficking proteins on tubulovesicles suggests that the H^+/K^+-ATPase-containing vesicles are an important example of an apical recycling vesicle population. Secretory vesicles such as synaptic vesicles might represent specialized adaptations of such a cell-surface recycling system [29]. In particular the SCAMPs

are thought to be general markers for vesicles involved in this recycling system [28]. Recent studies of synaptic vesicle recycling indicate that synaptic vesicles are regenerated in parallel from the presynaptic membrane and from plasma membrane-derived vesicles internalized by bulk endocytosis [44]. The presence of SCAMPs on immunoisolated tubulovesicles supports the membrane recycling hypothesis and has important implications for the biogenesis of H^+/K^+-ATPase-containing tubulovesicles. The results suggest that tubulovesicles might be derived from a cell-surface recycling system in the gastric parietal cell.

The co-localization of Rab25, Rab11 and SCAMPs on H^+/K^+-ATPase-containing tubulovesicles is of particular importance. We have previously noted the presence of Rab11 in apically oriented vesicles in a number of polarized epithelial cells [31]. Urbe et al. [45] observed Rab11 on both constitutive and regulated vesicles in PC12 cells. In addition, recent studies in transfected CHO cells [46] and K562 cells indicate that Rab11 is present on recycling endosomes in non-polarized cells [47]. These studies suggest that Rab11 might be a critical factor in recycling to the plasma membrane. The results presented here indicate that Rab11 might be a critical regulator and marker of apical membrane recycling systems. Thus Rab11, and perhaps also Rab25, might be specifically associated in polarized cells with vesicles destined for delivery and recycling to the apical membrane. Given the position of Rab11-containing vesicles in the subapical membrane region of a number of polarized epithelial cells, it is likely that similar apical recycling systems occur in many cells. We have recently observed that both Rab11 and Rab25 localize to apical recycling vesicles in transfected MDCK cells (J. R. Goldenring and J. E. Casanova, unpublished work). Such apical vesicle-trafficking systems have been implicated in the modulation of the plasma membrane repertoire of ion transporters and channels such as cystic fibrosis transmembrane regulator [3] and vasopressin-sensitive water channels [4–6] . The presence of Rab11, Rab25, VAMP-2 and SCAMPs on H^+/K^+-ATPase-containing tubulovesicles makes the tubulovesicle an excellent model for the study of the molecular regulation of apical vesicle trafficking. Further investigations will be required to determine whether Rab11 and Rab25 are general components of recycling vesicles destined for fusion with the apical plasmalemmal surface.

We thank Dr. Adam Smolka, Dr. Reinhard Jahn, Dr. David Castle and Dr. William Balch for gifts of monoclonal antibodies; Lorraine Aron of the University of Georgia Monoclonal Antibody Facility for the production of Rab25 monoclonal antibodies; and Jennifer Navarre and Julie Woodrum for excellent technical assistance. This work was supported by grants to J.R.G. from awards from NIH NIDDKD (DK48370 and DK43405), a Veterans Administration Merit Award and a grant from the Medical College of Georgia Research Institute.

REFERENCES

1 Forte, T. M., Machen, T. E. and Forte, J. G. (1977) Gastroenterology **73**, 941–955
2 Forte, J. G., Black, J. A., Forte, T. M., Machen, T. E. and Wolosin, J. M. (1981) Am. J. Physiol. **241**, G349–G358
3 Bradbury, N. A., Cohn, J. A., Venglarik, C. J. and Bridges, R. J. (1994) J. Biol. Chem. **269**, 8296–8302
4 Brown, D. and Orci, L. (1983) Nature (London) **302**, 253–255
5 Brown, D., Weyer, P. and Orci, L. (1988) Eur. J. Cell Biol. **46**, 336–340
6 Strange, K., Willingham, M. C., Handler, J. S. and Harris, Jr., H. W. (1988) J. Membr. Biol. **103**, 17–28
7 Bennett, M. K. and Scheller, R. H. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 2559–2563
8 Ferro-Novick, S. and Jahn, R. (1994) Nature (London) **370**, 191–193
9 Sollner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P. and Rothman, J. E. (1993) Nature (London) **362**, 318–324
10 Walch-Solimena, C., Blasi, J., Edelmann, L., Chapman, E. R., Fischer von Mollard, G. and Jahn, R. (1995) J. Cell Biol. **128**, 637–645
11 Sollner, T., Bennett, M. K., Whiteheart, S. W., Scheller, R. H. and Rothman, J. E. (1993) Cell **75**, 409–418
12 Bennett, M. K., Garcia-Arraras, J. E., Elferink, L. A., Peterson, K., Fleming, A. M., Hazuka, C. D. and Scheller, R. H. (1993) Cell **74**, 863–873
13 Baumert, M., Maycox, P. R., Navone, F., De Camilli, P. and Jahn, R. (1989) EMBO J. **8**, 379–384
14 Gaisano, H. Y., Sheu, L., Foskett, J. K. and Trimble, W. S. (1994) J. Biol. Chem. **269**, 17062–17066
15 Cain, C. C., Trimble, W. S. and Lienhard, G. E. (1992) J. Biol. Chem. **267**, 11681–11684
16 Bourne, H. (1988) Cell **53**, 669–671
17 Zerial, M. and Stenmark, H. (1993) Curr. Opin. Cell Biol. **5**, 613–620
18 Pfeffer, S. R. (1994) Curr. Opin. Cell Biol. **6**, 522–526
19 Sogaard, M., Tani, K., Ye, R. R., Geromanos, S., Tempst, P., Kirchhausen, T., Rothman, J. E. and Sollner, T. (1994) Cell **78**, 937–948
20 Basson, M. D., Goldenring, J. R., Tang, L. H., Lewis, J. J., Padfield, P., Jamieson, J. D. and Modlin, I. M. (1991) Biochem. J. **279**, 43–48
21 Goldenring, J. R., Soroka, C. J., Shen, K. R., Tang, L. H., Rodriguez, W., Vaughan, H. D., Stoch, S. A. and Modlin, I. M. (1994) Am. J. Physiol. **267**, G187–G194
22 Calhoun, B. C. and Goldenring, J. G. (1996) Yale J. Biol. Med. **69**, 1–8
23 Goldenring, J. R., Shen, K. R., Vaughan, H. D. and Modlin, I. M. (1993) J. Biol. Chem. **268**, 18419–18422
24 Padfield, P. J. and Jamieson, J. D. (1991) Biochem. Biophys. Res. Commun. **174**, 600–605
25 Jin, M., Saucan, L., Farquhar, M. G. and Palade, G. E. (1996) J. Biol. Chem. **271**, 30105–30113
26 Brand, S. H., Laurie, S. M., Mixon, M. B. and Castle, J. D. (1991) J. Biol. Chem. **266**, 18949–18957
27 Laurie, S. M., Cain, C. C., Lienhard, G. E. and Castle, J. D. (1993) J. Biol. Chem. **268**, 19110–19117
28 Brand, S. H. and Castle, J. D. (1993) EMBO J. **12**, 3753–3761
29 Cameron, P. L., Südhof, T. C., Jahn, R. and DeCamilli, P. (1991) J. Cell Biol. **115**, 151–164
30 Crothers, J. M., Chow, D. C. and Forte, J. G. (1993) Am. J. Physiol. **265**, G231–G241
31 Goldenring, J. R., Smith, J., Vaughan, H. D., Cameron, P., Hawkins, W. and Navarre, J. (1996) Am. J. Physiol. **270**, G515–G525
32 Oyler, G. A., Higgins, G. A., Hart, R. A., Battenberg, E., Billingsley, M., Bloom, F. E. and Wilson, M. C. (1989) J. Cell Biol. **109**, 3039–3052
33 McMahon, H. T. and Südhof, T. C. (1995) J. Biol. Chem. **270**, 2213–2217
34 Sadoul, K., Lang, J., Montecucco, C., Weller, U., Regazzi, R., Catsicas, S., Wollheim, C. B. and Halban, P. A. (1995) J. Cell Biol. **128**, 1019–1028
35 Roth, D. and Burgoyne, R. D. (1994) FEBS Lett. **351**, 207–210
36 Chavrier, P., Gorval, J.-P., Steltzer, E., Simons, K., Gruenberg, J. and Zerial, M. (1992) Nature (London) **353**, 769–772
37 Tang, L. H., Stoch, S. A., Modlin, I. M. and Goldenring, J. R. (1992) Biochem. J. **285**, 715–719
38 Tisdale, E. J. and Balch, W. E. (1996) J. Biol. Chem. **271**, 29372–29379
39 Fischer von Mollard, G., Mignery, G. A., Baumert, M., Perin, M. S., Hanson, T. J., Burger, P. M., Jahn, R. and Südhof, T. C. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 1988–1992
40 Fischer von Mollard, G., Südhof, T. C. and Jahn, R. (1991) Nature (London) **349**, 79–80
41 Fischer von Mollard, G., Stahl, B., Khokhlatchev, A., Südhof, T. C. and Jahn, R. (1994) J. Biol. Chem. **269**, 10971–10974
42 Bucci, C., Lutcke, A., Steele-Mortimer, O., Olkonnen, V. M., Dupree, P., Chiarello, M., Bruni, C. B., Simons, K. and Zerial, M. (1995) FEBS Lett. **366**, 65–71
43 Soldati, T., Rancano, C., Geissler, H. and Pfeffer, S. R. (1995) J. Biol. Chem. **270**, 25541–25548
44 Takei, K., Mundigl, O., Daniell, L. and De Camilli, P. (1996) J. Cell Biol. **133**, 1237–1250
45 Urbe, S., Huber, L. A., Zerial, M., Tooze, S. A. and Parton, R. G. (1993) FEBS Lett. **334**, 175–182
46 Ullrich, O., Reinsch, S., Urbe, S., Zerial, M. and Parton, R. G. (1996) J. Cell Biol. **135**, 913–924
47 Green, E. G., Ramm, E., Riley, N. M., Spiro, D. J., Goldenring, J. R. and Wessling-Resnick, M. (1996) Mol. Biol. Cell **7**, 591

Received 15 January 1997/19 March 1997; accepted 21 March 1997