Secretagogue-induced translocation of CRHSP-28 within an early apical endosomal compartment in acinar cells

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Thomas, Diana D. H., Ning Weng, and Guy E. Groblewski. Secretagogue-induced translocation of CRHSP-28 within an early apical endosomal compartment in acinar cells. Am J Physiol Gastrointest Liver Physiol 287: G253–G263, 2004. First published February 19, 2004; 10.1152/ajpgi.00033.2004.—Ca2+-regulated heat-stable protein (CRHSP-28) is a member of the TPD52 protein family that has been shown to regulate Ca2+-dependent secretory activity in pancreatic acinar cells. Immunofluorescence microscopy of isolated lobules demonstrated that CRHSP-28 is localized to a supranuclear apical compartment in acini and accumulates immediately below the apical membrane within 2 min of CCK octapeptide (CCK-8) stimulation. Dual-immunofluorescence microscopy demonstrated an endosomal localization of CRHSP-28 that strongly overlapped with early endosomal antigen-1 (EEA-1) on vesicular structures throughout the apical cytoplasm but showed only minimal overlap with the transferrin receptor, which is present in basolaterally derived endosomes. Significant overlapping of CRHSP-28 with the trans-Golgi network marker-38 was also noted in supranuclear regions of acini. Interestingly, treatment of lobules with brefeldin A reversibly disrupted the vesicular localization of CRHSP-28 and EEA-1 within the apical cytoplasm. The CCK-8-induced accumulation of CRHSP-28 in subapical regions of acini was not altered by inhibition of apical endocytosis with the actin filament-disrupting agent latrunculin B. Immunoelectron microscopy confirmed that CRHSP-28 is associated with the limiting membrane of irregularly shaped vesicular structures of low electron density in the apical cytoplasm that are positive for EEA-1 staining. Sparse, but significant, CRHSP-28 immunoreactivity was also observed along the limiting membrane of zymogen granules. Consistent with immunofluorescence data, CRHSP-28 was found to accumulate in clusters on endosomes and positioned between zymogen granules below the cell apex on CCK-8 stimulation. These data indicate that CRHSP-28 is present within endocytic and exocytic compartments of acinar cells and is acutely regulated by secretagogue stimulation.

calcium signaling; membrane trafficking; early endosome; secretion; calcium/calmodulin-dependent protein kinase II

APICAL MEMBRANE TRAFFICKING in pancreatic acinar cells is a highly regulated process whereby membrane proteins and secretory cargo are directed specifically to the apical plasma membrane and away from the basolateral membrane. Transport occurs in specialized zymogen granules (ZGs), which originate in the Golgi apparatus. To reach the cell apex, ZGs must penetrate a prominent subapical actin-cytoskeletal web (40) before undergoing fusion with the apical plasma membrane in a process that is largely mediated by protein components of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex (14, 15). Membrane transport and ZG fusion occur by constitutive and hormone/neurotransmitter-regulated pathways and are generally triggered in response to physiological stimuli, namely, the presence of a meal in the gastrointestinal tract.

After fusion with the apical membrane, ZG phospholipid and the associated regulatory proteins must be retrieved back into the cell by endocytosis. Retrieval of ZGs allows for the efficient recycling of regulatory proteins and phospholipid and also prevents the overexpansion of the apical membrane during high rates of exocytosis (40, 41). Less is known about endocytosis than about studies on the molecular mechanisms that modulate ZG trafficking and exocytosis in acinar cells. A significant proportion of apical membrane endocytosis in acini appears to be mediated by the formation of clathrin-coated pits involving activator protein-2 (AP-2) adapter complexes and the dynamin GTPase (41). Other potential endocytic pathways include the formation of caveolae (28, 41) and the less well-characterized phenomenon of kiss-and-run (37). Similar to what has been reported in cultured Madin-Darby canine kidney cells (16), Valentijn et al. (41) described an essential role for the subapical actin cytoskeleton in mediating membrane retrieval during secretagogue stimulation of acini. Treatment of acini with actin filament-disrupting agents arrested endocytosis, leading to a pronounced dilation of the acinar lumen and accumulation of the endocytic regulatory proteins AP-2, clathrin, dynamin, and caveolin along the overexpanded plasma membrane. Other studies have shown that the alkalization of the acinar lumen by duct cells and the cleavage of glycosyl phosphatidylinositol-anchored proteins in the cell membrane are necessary to activate apical endocytosis in a process that involves src tyrosine kinases and the formation of caveolae (12, 13, 30).

Studies utilizing epithelial cell culture models have shown that newly formed endosomes rapidly undergo homotypic fusion to form an early endosomal compartment (26). Transport vesicles then bud off of early endosomes and may 1) be recycled back to the plasma membrane, 2) fuse with maturing secretory granules to insert/recycle regulatory proteins that are necessary for exocytosis, 3) fuse with the Golgi apparatus, or 4) enter a lysosomal compartment for degradation. Thus the early endosomal compartment acts as a staging area to distribute recycled membrane and its associated regulatory proteins throughout the cell. In the case of pancreatic acinar cells, studies investigating the fluid phase and membrane-associated uptake of luminal markers have shown that, within 2–5 min, fluid phase markers move through an apical endosomal compartment and enter organelles of the secretory pathway, including Golgi cisternae, condensing vacuoles, and ZGs (20, 33).

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Conversely, horseradish peroxidase labeling of apical membrane rapidly (2–5 min) moves through early endosomes to a lysosomal compartment. Although these studies have documented the movement of markers through an apical endosomal compartment, little information is available regarding the molecular composition of these endosomes and, therefore, the signaling processes that regulate phospholipid and protein distribution in the cell after apical membrane retrieval.

Ca\(^{2+}\)-regulated heat-stable protein (CRHSP)-28 is a peripheral membrane protein that is highly expressed in the apical cytoplasm of exocrine epithelial cells throughout the digestive system (17, 18, 29). CRHSP-28 is a member of the TPD52 protein family that has been shown to interact with a number of cytoskeletal regulatory proteins, including MAL2 (42), annexin VI (38), and SNARE proteins (31). With use of a permeabilized acinar cell system, CRHSP-28 was shown to significantly reconstitute Ca\(^{2+}\)-stimulated digestive enzyme secretion after the loss of the endogenous protein from cells (39). Subsequently, CRHSP-28 was shown to undergo a Ca\(^{2+}\)-sensitive interaction with annexin VI, a molecule that has been implicated in endosomal trafficking (38). Investigation of the subcellular distribution of CRHSP-28 in acini indicated that the protein only partially colocalized with ZGs by dual-immunofluorescence microscopy (18) and, furthermore, did not significantly copurify with ZGs after tissue fractionation (38). In the present study, CRHSP-28 is shown to rapidly translocate within an early endosomal pathway in acinar cells after secretagogue stimulation. This endosomal compartment is present exclusively within the apical cytoplasm, shows minimal overlap with endosomal vesicles derived from the basolateral surface of cells, and is reversibly disrupted by the ADP-ribosylation factor inhibitor brefeldin A. Our findings suggest that CRHSP-28 plays an integral role in regulating the movement of phospholipid and proteins through the endocytic pathway of acini after retrieval from the apical membrane.

**METHODS**

**Antibodies.** Anti-early endosomal antigen-1 (EEA-1) N19 was purchased from BD Transduction Laboratories, anti-trans-Golgi network (TGN)-38 clone 2F7.1 from Affinity Bioreagents, anti-transferrin receptor (TR) monoclonal antibody clone OX26 from Cedar Lane Laboratories, and anti-vesicle-associated membrane protein (VAMP)-2 clone c169.1 and anti-VAMP-8 rabbit polyclonal antibodies from Synaptic Systems. The anti-cysteine string protein (CSP) was a gift from Janice Braun. The anti-Ca\(^{2+}\)- and calmodulin (CaM)-dependent protein kinase II (CaMKII) polyclonal antibody G301 (25) was a gift from Angus Nairn. CRHSP-28 polyclonal antibodies were previously characterized (18). All antibodies were characterized before dual-immunofluorescence labeling studies by serial dilutions to determine optimal conditions and negative controls (usually secondary antibody alone) to ensure specificity (data not shown). Various Alexa Fluor-conjugated secondary antibodies and rhodamine-phallolidin were purchased from Molecular Probes. Peroxidase-conjugated donkey anti-goat IgG was obtained from Jackson Immuno Research and peroxidase-conjugated sheep anti-mouse IgG and donkey anti-rabbit IgG from Amersham Biosciences. For immunoelectron microscopy, ultrasmall gold-conjugated goat anti-rabbit and goat anti-mouse IgG and 6-nm gold-labeled protein A were purchased from Electron Microscopy Sciences.

**Other reagents.** Soybean trypsin inhibitor, benzamidine, PMSF, and Triton X-100 were purchased from Sigma, essential amino acid solution from Gibco BRL, and a protease inhibitor cocktail containing leupeptin, and E64 from Calbiochem. Protein determination reagent was obtained from Bio-Rad, brefeldin A from Epicenter Technologies, and latrunculin B from Alexis. All other electron microscopy reagents, including acetylated BSA (BSA-c) and R-gent silver-enhancing kit were purchased from Electron Microscopy Sciences.

**Immunofluorescence microscopy.** The University of Wisconsin Committee on Use and Care of Animals approved all studies involving animals. Pancreatic lobules were prepared by microdissection of a rat pancreas that had been injected with buffer consisting of (in mM) 10 Hepes, 137 NaCl, 4.7 KCl, 0.56 MgCl\(_2\), 1.28 CaCl\(_2\), 0.6 Na\(_2\)HPO\(_4\), 5.5 glucose, and 2 l-glutamine and an essential amino acid solution. The buffer was supplemented with 0.1 mg/ml soybean trypsin inhibitor and 1 mg/ml BSA, gassed with 100% O\(_2\), and adjusted to pH 7.48. Isolated lobules were incubated at 37°C with gentle shaking. After indicated treatments, lobules were gently pelleted and fixed in 4% formaldehyde in PBS. Immunofluorescence microscopy was conducted on 6-μm-thick cryostat sections as described elsewhere (18). Tissue was analyzed using a Bio-Rad model 1024 confocal microscope with a mixed krypton-argon gas laser. For dual-immunofluorescence measurements, fluorophores were individually excited at the appropriate wavelength to ensure no overlapping excitation between channels. Captured images were overlaid using Bio-Rad software and converted to TIFF files for processing using Adobe Photoshop software.

**Immunoelectron microscopy.** In preliminary experiments, CRHSP-28 immunofluorescence was not altered in sections fixed in 2% formaldehyde and 0.25% glutaraldehyde (data not shown); these conditions were therefore used for electron microscopy. Pancreatic lobules were treated as control or with CCK-8 and then fixed for 2 h at room temperature in 4% formaldehyde and 0.25% glutaraldehyde. Tissue was embedded in LR white resin, and 80-nm sections were placed on pioloform-coated nickel grids. Affinity-purified CRHSP-28 antibody was incubated in PBS containing 0.2% BSA, 0.2% Tween 20, and 0.1% goat serum and then with 6-nm gold-labeled protein A. After postfixation in 2% glutaraldehyde and silver enhancement, tissue was stained in Reynolds lead citrate and uranyl acetate. Sections were evaluated with a Philips CM 120 electron microscope. Captured images were converted to TIFF files and edited for publication in Adobe Photoshop.

Because EEA-1 immunoreactivity was not evident with the above technique, dual localization of EEA-1 and CRHSP-28 was conducted using a preembedding double immunogold-silver labeling technique (43). Briefly, lobules were fixed in 4% formaldehyde and embedded in Tissue Tek using liquid nitrogen-cooled isopentane. Cryostat sections (25 μm) were placed on poly-L-lysine-coated coverslips. After aldehyde inactivation in 0.1% sodium borate, tissue was permeabilized with 0.05% Triton X-100 and blocked in PBS containing 5% BSA, 0.1% cold water fish gelatin, and 5% goat serum. Sections were incubated in CRHSP-28 and EEA-1 primary antibodies overnight at 4°C. An ultrasmall gold-conjugated goat anti-mouse antibody was added overnight and then silver enhanced with Aurion R-Gent silver-enhancing kit. Silver enhancement was terminated with 0.3 M sodium thiosulfate. Next, the ultrasmall gold-conjugated goat anti-rabbit secondary antibody was processed using the same method. Sections were postfixed in 2.5% glutaraldehyde and subjected to a second silver enhancement. Tissue was then fixed in 0.5% osmium tetroxide, dehydrated in an ethanol gradient followed by propylene oxide, and then incubated overnight in equal volumes of 1:1 EM bed 812-Spurs low-viscosity resin and propylene oxide. After the tissues were sectioned, they were placed on copper grids and stained with Reynolds lead citrate and uranyl acetate. Because the anti-mouse secondary antibody recognizing the EEA-1 antibody was subjected to silver...
enhancement twice and the anti-rabbit antibody recognizing the CRHSP-28 antibody was subjected to silver enhancement once, there were two groups of silver-enhanced particles on the sections: the larger particles correspond to EEA-1 and the smaller particles to CRHSP-28.

Tissue fractionation. Two rat pancreases were minced in 5 vol of a buffer containing (in mM) 10 MOPS, pH 6.8, 250 sucrose, 0.1 MgCl₂, 0.1 PMSF, and 1 benzamidine. Tissue was homogenized by three strokes of a motor-driven homogenizer (5,000 rpm) using a Teflon pestle with 0.5–1 mm clearance. A postnuclear supernatant was prepared by centrifugation at 600 g for 10 min and then further centrifuged at 1,300 g for 10 min to produce a white pellet enriched in ZGs overlaid by a brown pellet enriched in mitochondria. The remaining supernatant was centrifuged at 100,000 g for 1 h to separate microsomal and cytosolic fractions. Microsomal fractions were further separated on a sucrose gradient composed of 1.3, 0.86, 0.5, and 0.25 M sucrose prepared in homogenization buffer. Crude microsomes were layered between the 0.5 and 0.25 M sucrose layers and then centrifuged at 160,000 g for 1 h. Individual fractions were diluted to 0.5 M sucrose and recovered by centrifugation at 100,000 g for 1 h. Pellets were lysed by sonication in homogenization buffer, and protein content was determined using a Bio-Rad reagent. Equal amounts of protein from each fraction were separated by SDS-PAGE and immunoblotted with indicated antibodies.

RESULTS

CRHSP-28 translocates within an early endosomal compartment. Confocal immunofluorescence localization of CRHSP-28 in pancreatic lobules under basal conditions indicated that the protein is present throughout the apical cytoplasm and most highly concentrated in supranuclear regions of cells (Fig. 1). Secretagogue stimulation of lobules with 1 nM CCK-8 for 2 min induced a rapid and pronounced translocation of CRHSP-28 to subapical regions of cells, where it became concentrated immediately below the acinar lumen. With longer periods of stimulation (15 min), CRHSP-28 staining was abundant on vesicular structures that surrounded the now-dilated lumen of the acinus. Because of the acute nature of this response, 1 nM CCK-8 was chosen in these experiments to promote a rapid and thorough penetration of the agonist into the 1- to 2-mm³ lobules. Lower secretory concentrations of CCK-8 had the same effect on CRHSP-28 localization (see Fig. 8); however, translocation was often less widespread in deeper areas of the lobules.

It was previously reported (18) that, despite its clear apical localization, CRHSP-28 did not significantly colocalize with
pancreatic lipase, a secretory enzyme concentrated within ZGs. Moreover, protein purification studies indicated that CRHSP-28 undergoes a Ca\(^{2+}\)/H\(_{11001}\)-dependent association with annexin VI in acini (38). Consistent with reports that annexin VI is involved in endosomal trafficking (5), subcellular fractionation indicated that annexin VI and CRHSP-28 copurified with endosome-containing light microsomal fractions of pancreas. However, annexin VI also copurified with ZG membranes, whereas CRHSP-28 did not (38). Therefore, dual-immunofluorescence labeling studies were conducted using antibodies to EEA-1, a marker of early endosomes, to investigate a potential association of CRHSP-28 with an endosomal compartment (Fig. 2). Immunodetection of CRHSP-28 and EEA-1 produced an essentially identical pattern of colocalization within the apical cytoplasm under basal and CCK-8-stimulated conditions. Indeed, CRHSP-28 and EEA-1 dual staining was present in a vesicular pattern in acini that markedly accumulated immediately below the apical membrane after secretagogue stimulation of cells.

The intense staining of CRHSP-28 in the supranuclear region of cells under basal conditions would also be consistent with a Golgi pattern of localization. To evaluate a potential association of CRHSP-28 with the Golgi apparatus, experiments were conducted using antibodies to the Golgi marker TGN-38 (Fig. 3). Although overlap of CRHSP-28 and TGN-38 was clearly evident on supranuclear structures, a distinct and independent pattern of localization was also seen for each antibody; CRHSP-28 staining extended toward the cell apex, whereas TGN-38 was concentrated near the nucleus. Identical results were obtained with antibodies to the Golgi resident protein \(\alpha\)-mannosidase II (data not shown). Interestingly, TGN-38 immunoreactivity was also consistently detected in the acinar lumen but was absent from the ZG region. In cultured cells, TGN-38 has been shown to cycle to and from the plasma membrane on an as yet undefined carrier vesicle, suggesting that it may move through a less prominent constitutive pathway to the cell apex (32). Furthermore, insertion of TGN-38 in the apical membrane would be expected to orient the protein toward the acinar lumen and enhance the signal intensity within the luminal space. No luminal staining of \(\alpha\)-mannosidase II was detected (data not shown).

Fig. 2. CRHSP-28 colocalizes with early endosomal antigen-1 (EEA-1). Dual-immunofluorescence microscopy of CRHSP-28 and EEA-1 was performed in pancreatic lobules. Lobules were treated as control (A–C) or with 1 nM CCK-8 (D–F) for 15 min and then fixed in 4% paraformaldehyde. CRHSP-28 was detected with a rabbit polyclonal antibody (1 \(\mu\)g/ml). EEA-1 was detected using a goat polyclonal antibody (N19, 1:100). CRHSP-28 and EEA-1 immunoreactivity were detected using Alexa Fluor 594-conjugated anti-rabbit IgG (1:10,000) and Alexa Fluor 488-conjugated anti-goat IgG (1:1,000), respectively. Note pronounced overlap of CRHSP-28 and EEA-1 localization in control and CCK-8-treated cells. *, Nuclei; arrows, acinar lumen. G and H: close-up DIC and dual-immunofluorescence images of acinus framed in F. Note pronounced accumulation of CRHSP-28/EEA-1 below acinar lumen. Data are from a single representative experiment that was performed on tissues from 3 separate animals with identical results. All immunofluorescence was conducted on duplicate sections.
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Immunoelectron microscopy of CRHSP-28 and EEA-1. High-resolution immunoelectron microscopy was conducted to more fully evaluate localization of CRHSP-28 in the pancreas (Fig. 4). In agreement with the subcellular fractionation of CRHSP-28 to soluble and particulate fractions of acinar lysates, CRHSP-28 immunoreactivity under basal conditions was dispersed throughout the apical cytoplasm and frequently associated with small vesicular structures of low electron density. Less frequently, CRHSP-28 was detected on the limiting membrane of ZGs. Non-specific labeling, as determined by incubation with secondary antibody alone, was generally very low but sometimes detected inside ZGs or in the acinar lumen (Fig. 4, C and D). In regions where Golgi structures, nuclei, endoplasmic reticulum, and mitochondria were clearly identified, no CRHSP-28 was detected (data not shown). CRHSP-28 labeling in control and CCK-8-stimulated cells was often aggregated in clusters of three to four gold particles. Also, CRHSP-28 was consistently localized around large granule structures containing a core of electron-dense material resembling ZG content but lacking a definitive limiting membrane (Fig. 4B). Similar structures were previously identified as condensing vacuoles (20, 33).

Consistent with immunofluorescence microscopy, in multiple experiments, treatment of lobules with CCK-8 for 2 min appeared to increase CRHSP-28 labeling within cytoplasmic regions surrounding the cell apex (Fig. 4D). Again, CRHSP-28 labeling was prominent around small vesicular structures of low electron density located between ZGs and was also clearly detected on the limiting membrane of ZGs. At later times after cell stimulation, CRHSP-28 was concentrated around larger irregularly shaped vesicular structures that were interspersed between ZGs below the acinar lumen (Fig. 4E). To confirm that CRHSP-28 immunoreactivity is localized to an early endosomal compartment, dual labeling was conducted with antibodies to EEA-1 (Fig. 5). Indeed, a striking colocalization of CRHSP-28 and EEA-1 was detected surrounding the limiting membrane of small vesicular structures interspersed between the ZGs in the apical cytoplasm.

CRHSP-28 is not present on basolateral endosomes. The apical and basolateral membranes of epithelial cells are distinct in their lipid and protein composition and, as such, generate distinct early endosomal compartments. To evaluate whether endosomes originating from the basolateral membrane are positive for CRHSP-28 immunoreactivity, dual-immunofluorescence labeling was conducted with antibodies to TfR (Fig. 6). TfR retrieves transferrin from the basolateral plasma membrane to early endosomes, where it may be recycled back to the plasma membrane or enter a late endosomal compartment for degradation (2). In contrast to CRHSP-28, TIR immunoreactivity was clearly evident along the basal aspects of acinar cells and was absent from the apical membrane. TIR immunoreactivity was also evident in perinuclear regions of cells, which potentially represent a late endosomal compartment. Minimal overlap of TIR and CRHSP-28 was detected in these perinuclear regions; however, the majority of each protein clearly displayed a distinct pattern of staining.

CRHSP-28 and EEA-1 localize to a brefeldin A-sensitive endosomal compartment. Brefeldin A is a fungal metabolite that inhibits the exchange of guanine nucleotides onto ADP-ribosylation factors, a group of regulatory proteins that are necessary for the formation of clathrin- and non-clathrin-coated buds on membranes (24). Brefeldin A has been shown to inhibit endoplasmic reticulum-to-Golgi trafficking and induce the tubulation of early endosomes and lysosomes in cells (24). A previous study of acinar cells from the parotid gland demonstrated that a brefeldin A-sensitive apical membrane compartment is crucial in maintaining constitutive and “minor-regulated” secretory pathways in acini (8). Moreover, these pathways were suggested to originate from immature secretory granules and, in the case of the constitutive pathway, to utilize an early recycling endosomal compartment to gain entry to the apical membrane. Consistent with these findings, treatment of lobules with 10 μg/ml brefeldin A induced a complete disruption of the CRHSP-28- and EEA-1-associated vesicles in acinar cells (Fig. 7), creating a diffuse pattern of staining throughout the apical cytoplasm. The effect of brefeldin A was reversible, inasmuch as a washout of the compound from the medium resulted in a complete recovery of the CRHSP-28/EEA-1 colocalization within 30 min. Brefeldin A treatment also fully disrupted Golgi structures, as indicated by TGN-38 and mannosidase II staining (data not shown). Moreover, the effects of brefeldin A were time and concentration dependent, with a 20-min treatment at 5 μg/ml having a more modest, but significant, effect on Golgi and endosomal marker localization (data not shown).

Inhibition of apical endocytosis does not inhibit CRHSP-28 translocation. Valentijn et al. (41) established that disruption of the subapical actin filament network in acinar cells strongly inhibits apical membrane retrieval. Inhibition of membrane retrieval causes a pronounced dilation of the acinar lumen because of the overexpansion of the apical membrane with
accumulating phospholipid as a result of ongoing exocytosis.

We therefore tested whether inhibition of apical endocytosis with actin filament-disrupting agents would alter the localization and/or translocation of CRHSP-28 after secretagogue stimulation (Fig. 8). Treatment of lobules with latrunculin B for 30 min produced an incomplete disruption of actin filaments in acini, as analyzed by rhodamine-phalloidin staining (Fig. 8B). Stimulation of latrunculin B-treated cells with a secretory concentration (100 nM) of CCK-8 for 2 min did not alter CRHSP-28 translocation to the cell apex but did enhance actin filament disruption (Fig. 8G). Latrunculin B inhibits actin filament polymerization by binding to actin monomers. Enhanced actin filament disruption by latrunculin B in the presence of CCK-8 likely reflects a greater filament turnover in response to cell stimulation. After 30 min of CCK-8 stimulation, there was a complete loss of actin filament staining at the cell apex and a pronounced dilation of the acinar lumen (Fig. 8, J and L). Although accumulation of CRHSP-28 along the apical membrane was clearly evident around the dilated lumen (Fig. 8K), the characteristic vesicular pattern of CRHSP-28 staining that was seen at later times of acinar stimulation under normal conditions was not detected (Fig. 1, C and F). Very similar results were obtained using the actin filament-disrupting agent cytochalasin D (data not shown).

CaMKII is present within an apical early endosomal compartment of acinar cells. Using phosphopeptide mapping experiments, it was recently reported (22) that CRHSP-28 is phosphorylated by purified CaMKII in vitro in a pattern that is identical to that seen in cultured mucosal T84 cells. These data confirmed an earlier report that CRHSP-28 isolated from gastric mucosa is phosphorylated by CaMKII in vitro (29). CaMKII was localized to the apex of polarized T84 cells; however, its precise subcellular localization was not determined. To determine whether CaMKII is present in the endo-

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Fig. 4. Immunoelectron microscopy of CRHSP-28 in acinar cells. Pancreatic lobules were treated as control (A–C) or with 1 nM CCK-8 (D and E) and fixed in 4% formaldehyde and 0.25% glutaraldehyde before they were embedded in LR white resin. Microscopy was conducted on 80-nm-thick sections on pioloform-coated grids. Anti-CRHSP-28 antibodies (10 μg/ml) were detected with 6-nm gold-conjugated protein A. A: under control conditions (magnification ×10,500), CRHSP-28 was interspersed throughout cytoplasm, with sparse staining of CRHSP-28 on zymogen granules (ZG). B: CRHSP-28 was often seen in clusters of 3–4 particles and was also present on the outside of large granular structures resembling condensing vacuoles (magnification ×40,000). When clearly identified, Golgi structures, nuclei, endoplasmic reticulum, and mitochondria were negative for CRHSP-28 staining (data not shown). C: section of lobules incubated with gold-conjugated protein alone. D and E: after stimulation with 1 nM CCK-8 for 2 min (D) or 15 min (E), CRHSP-28 immunoreactivity accumulated in regions surrounding the cell apex and was clearly evident on the limiting membrane of irregularly shaped tubular structures of low electron density, consistent with early endosomes [magnification ×25,000 (D) and ×31,000 (E)]. Arrows show distribution of CRHSP-28 labeling. Dashed arrow in D indicates nonspecific labeling that was infrequently seen inside ZGs. Data are a single representative experiment that was performed on tissues from 3–5 separate animals with similar results. All immunolabeling was conducted in duplicate.
somal pathway of acinar cells, a CaMKII antibody was used (Fig. 9). Indeed, CaMKII was found to colocalize with EEA-1 on small vesicular structures in the apical cytoplasm of acinar cells. These data suggest a close association of CRHSP-28 and its putative kinase on an early apical endosomal compartment in acini.

Isolation of CRHSP-28, EEA-1, and CaMKII in light microsomal fractions of the pancreas. Subcellular fractionation revealed that CRHSP-28, EEA-1, and CaMKII are present in membrane and cytosolic fractions of the pancreas. Similarly, all three proteins were absent from purified ZGs but enriched in crude microsomes that were devoid of ZGs and mitochondria. Further separation of crude microsomes by sucrose-gradient centrifugation indicated that the membrane-associated forms of CRHSP-28, EEA-1, and CaMKII partitioned into light microsomal fractions, consistent with their immunolocalization to an early endosomal compartment. In comparison, the ZG membrane proteins VAMP-2, VAMP-8, and CSP are highly expressed in the ZG fraction (Fig. 10). Interestingly, the ZG membrane proteins are also enriched in light microsomal fractions containing CRHSP-28 and EEA-1. In contrast to CRHSP-28, EEA-1, and CaMKII, the VAMPs and CSP are integral membrane proteins and are not present in cytosolic fractions. These data may reflect the retrieval of ZG membrane proteins to early endosomes after endocytosis from the apical plasma membrane.

DISCUSSION

CRHSP-28 (also known as D52) is the archetype of the TPD52 protein family, originally identified as being highly expressed during malignant transformation in breast cancer (6, 7). After the isolation and cloning of the TPD52 family members, considerable attention has been focused on elucidating their molecular function by identifying TPD52 protein interactions. Indeed, CRHSP-28/D52 is a likely candidate for protein interactions: it is highly charged with acidic regions at the NH₂ and COOH ends that flank a basic middle portion, and it has a coiled-coil domain that mediates dimer formation with other members of the TPD52 family (35, 39). A number of diverse TPD52-binding partners have been identified by copurification and yeast two-hybrid screening, and although the precise function of the TPD52 proteins remains elusive, all the interacting proteins have been shown to play a role in membrane trafficking and/or fusion events in cells.

In acinar cells, CRHSP-28 was shown to interact with annexin VI, a phospholipid and actin filament-binding protein that was reported to modulate clathrin-dependent endocytosis in vitro and to be associated with endosomes in cultured cells.
CRHSP-28 was also shown to interact by yeast two-hybrid screening with MAL2, an integral membrane protein on apical carrier vesicles in polarized epithelial cells (42). Selective ablation of MAL2 completely arrested apical trafficking of transcytosed markers in Hep G2 cells (10). Another member of the TPD52 family, D53 was shown to interact with and stabilize the association of synaptobrevin 2 and syntaxin 1 complexes in vitro, implicating D53 in the promotion of SNARE complex formation and the facilitation of membrane fusion in cells (31). Consistent with the present study, D53 was found to localize in an endosomal compartment in PC12 cells (31). More recently, an alternatively spliced isoform of D53 was shown to specify interact with 14-3-3 proteins (4).

The 14-3-3 proteins have been implicated in cellular processes related to membrane trafficking, including cytoskeletal dynamics and exocytosis (34, 36). CRHSP-28 and EEA-1 are peripheral membrane proteins that move between cytosolic and membrane fractions of cells. The translocation of EEA-1 to endosomes is mediated by the COOH-terminal region of the protein, which contains a CaM-binding motif, an Rab5 interaction domain, and an FYVE domain that selectively binds to phosphatidylinositol 3-phosphate (11, 23). EEA-1 has been shown to modulate homotypic fusion of early endosomes, a process that is inhibited by phosphatidylinositol 3-kinase inhibitors and is dependent on elevated cellular Ca\(^{2+}\) and CaM (27). Moreover, EEA-1 has been shown to interact with the endosomal SNARE proteins syntaxin 6 and syntaxin 13, providing a possible mechanism for its membrane fusion properties (27). Similar to the Ca\(^{2+}\)/CaM dependency of EEA-1 function, CRHSP-28 function within the cell appears to be mediated through a Ca\(^{2+}\)-sensitive process, as indicated by its Ca\(^{2+}\)-dependent modulation of digestive enzyme secretion, 2) annexin VI binding, and 3) serine phosphorylation (22, 29, 38, 39). Additionally, the colocalization of the putative CRHSP-28 kinase CaMKII to early endosomes clearly supports a Ca\(^{2+}\)/CaM-dependent role for CRHSP-28 in apical endosomal trafficking. Despite their close association in acinar cells and essentially parallel movement within the cytoplasm on secretagogue stimulation, we have not been able to establish a physical interaction between EEA-1 and CRHSP-28 by coimmunoprecipitation. However, our CRHSP-28 antibodies were raised against full-length protein and, therefore, may displace binding partners in cell lysates.

In vitro homotypic early endosomal fusion assays have clearly established a Ca\(^{2+}\)/CaM dependency for endosome fusion (9). The localization of EEA-1, CRHSP-28, and CaMKII within the apical cytoplasm of acini closely corresponds with the secretagogue-induced pattern of Ca\(^{2+}\) oscillations in these cells (for review see Ref. 3). Secretagogue stimulation of acinar cells induces an immediate rise in cellular Ca\(^{2+}\) that originates at sites in the apical pole of the cell and spreads toward the basal pole. Concomitant with the rise in cellular Ca\(^{2+}\) is the rapid translocation of CaM from perinu-

Fig. 7. CRHSP-28 and EEA-1 are present in a brefeldin A (BFA)-sensitive endosomal compartment. Pancreatic lobules were treated as control (Con) or with 10 \(\mu\)g/ml BFA for 20 min before fixation in 4% formaldehyde. In a separate experiment, lobules were washed with fresh medium after BFA treatment and allowed to recover (Rec) for 30 min at 37°C before fixation. Note dramatic loss in CRHSP-28/EEA-1 staining after BFA treatment and clear reversal of this effect after recovery. *, Nuclei; arrows, acinar lumen. Data are from a single representative experiment that was performed on tissues from 3 separate animals with identical results. All immunofluorescence was conducted on duplicate sections. Essentially 100% of cells in each preparation were similarly affected by BFA treatment.
clear regions to sites within the apical cytoplasm that are suggested to further modulate Ca\(^{2+}\) homeostasis and membrane trafficking (3). Although ZG exocytosis is a Ca\(^{2+}\)-dependent process, the role of CaM in secretion is tenuous. Use of CaM antagonists in isolated acinar cells was shown to have only modest effects on amylase secretion (21). On the basis of research in other cell systems (9, 23), it seems likely that the secretagogue-induced apical elevation in cellular Ca\(^{2+}\) and corresponding CaM translocation is important in modulating endosomal trafficking in acini.

Actin filament disruption by latrunculin B clearly inhibited apical membrane endocytosis, as evidenced by the pronounced dilation of the acinar lumen, but did not inhibit the apical accumulation of CRHSP-28 in response to CCK-8. At later times after stimulation, the enlarged vesicular pattern of CRHSP-28 staining seen under normal conditions was not evident (Fig. 1, C and F). Ultrastructural analysis of normal acini after 15 min of CCK-8 treatment (Fig. 4D) indicated that CRHSP-28 was present on irregularly shaped vesicles that were significantly enlarged compared with control cells or cells stimulated for 2 min. These data suggest that CCK-8 treatment induced an expansion of the early endosomal compartment due to the enhanced retrieval and recycling of apical membrane. Likewise, the finding that brefeldin A rapidly and reversibly disrupted the localization of CRHSP-28/EEA-1 indicates a high rate of phospholipid movement through early endosomes and underscores the dynamic nature of this compartment.

Although some overlap of CRHSP-28 with Golgi markers was detected in acini, especially in control conditions when CRHSP-28 occupied perinuclear regions of cells, ultrastructural analysis did not support the presence of CRHSP-28 on Golgi membranes. Rather, CRHSP-28 was often associated with large granular structures that contained a core of electron-dense material resembling condensing vacuoles (Fig. 4B). Additionally, significant CRHSP-28 staining was seen on the limiting membrane of ZGs but was not present in ZG fractions after tissue fractionation, suggesting a transient association with these structures. Localization of CRHSP-28 on condensing vacuoles supports its partial colocalization with the Golgi apparatus and presents the possibility that CRHSP-28 may move between maturing ZGs and endosomes.

Previous studies indicate that endosomal trafficking plays an integral role in providing important regulatory factors to support the exocytotic pathway in cells. In parotid acinar cells, the insertion of proteins necessary for granule docking and fusion at the apical membrane proceeds through a brefeldin A-sensitive pathway that is distinct from mature secretory granules and may be coordinated through a recycling endosomal compartment (8). Also of significance in acinar cells is the highly coordinated process of compound exocytosis of ZGs. Studies indicate that compound exocytosis is a sequential event where ZG-to-apical membrane fusion precedes ZG-to-ZG fusion, allowing for efficient discharge of granule contents into the acinar lumen (28). Without this sequential process, indiscrimi-
nant ZG-to-ZG fusion would produce large cavities of fused granules in the cytoplasm. The precise mechanism of sequential compound exocytosis is not clear but was suggested to involve the rapid cycling of SNARE proteins from sites of exocytosis on the apical membrane to granules positioned deeper in the cytoplasm (28). An earlier study by Guo et al. (19) demonstrated similar results in mast cells, which undergo massive compound exocytosis and rapidly recycle the SNARE protein SNAP-23 from the plasma membrane to underlying secretory granules. Although the rapid endocytic retrieval of SNARE proteins was not directly addressed in these studies, given that SNARE molecules are integral membrane proteins, endosomal retrieval seems a likely mechanism. More recently, in PC12 cells, which express D53 in an endosomal compartment (31), the rapid recycling of phosphatidylinositol 4,5-bisphosphate and its associated kinase phosphatidylinositol 4-phosphate 5-kinase (PIP5K) through an early endosomal compartment was shown to play an essential role in providing phosphatidylinositol 4,5-bisphosphate to the apical membrane, which is necessary to support dense-core vesicle exocytosis (1). Collectively, these studies underscore the significance of early endosomal trafficking in supporting the process of exocytosis and secretion.

The present study clearly supports mounting evidence that CRHSP-28 and other members of TPD52 family are important regulatory factors in cellular membrane trafficking. Our finding that CRHSP-28 localizes to early endosomes and the limiting membrane of ZGs suggests that CRHSP-28 may play a key role in distributing protein and phospholipid within the secretory pathway of acinar cells. Recent evidence that D53 promotes SNARE complex formation in vitro (31) suggests that the TPD52 proteins may provide specificity to membrane fusion events in cells and, thereby, ensure a high fidelity of phospholipid and regulatory protein distribution in different membrane compartments. The high expression of CRHSP-28 in acinar cells relative to other tissues indicates that further exploration of CRHSP-28 and the TPD52 molecules will provide valuable insight into the process of protein secretion in exocrine epithelia.

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