Identification of Annexin VI as a Ca\textsuperscript{2+}-sensitive CRHSP-28-binding Protein in Pancreatic Acinar Cells*

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CRHSP-28 is a member of the tumor protein D52 protein family that was recently shown to regulate Ca\textsuperscript{2+}-stimulated secretory activity in streptolysin-O-permeabilized acinar cells (Thomas, D. H., Taft, W. B., Kaspar, K. M., and Groblewski, G. E. (2001) J. Biol. Chem. 276, 28866–28872). In the present study, the Ca\textsuperscript{2+}-sensitive phospholipid-binding protein annexin VI was purified from rat pancreas as a CRHSP-28-binding protein. The interaction between CRHSP-28 and annexin VI was demonstrated by coimmunoprecipitation and gel-overlay assays and was shown to require low micromolar levels of free Ca\textsuperscript{2+}, indicating that these molecules likely interact under physiological conditions. Immunofluorescence microscopy confirmed a dual localization of CRHSP-28 and annexin VI, which appeared in a punctate pattern in the supranuclear and apical cytoplasm of acini. Stimulation of cells for 5 min with the secretagogue cholecystokinin enhanced the colocalization of CRHSP-28 and annexin VI within regions of acini immediately below the apical plasma membrane. Tissue fractionation revealed that CRHSP-28 is a peripheral membrane protein that is highly enriched in smooth microsomal fractions of pancreas. Further, the content of CRHSP-28 in micromes was significantly reduced in pancreatic tissue obtained from rats that had been infused with a secretory dose of cholecystokinin for 40 min, demonstrating that secretagogue stimulation transiently alters the association of CRHSP-28 with membranes in cells. Collectively, the Ca\textsuperscript{2+}-dependent binding of CRHSP-28 and annexin VI, together with their colocalization in the apical cytoplasm, is consistent with a role for these molecules in acinar cell membrane trafficking events that are essential for digestive enzyme secretion.

Secretion of digestive enzymes from the exocrine pancreas is a nutrient-driven process whereby the ingestion of a meal stimulates neural and hormonal pathways that directly mediate the activation of pancreatic acinar cells (reviewed in Refs. 1 and 2). Cell stimulation involves the acute elevation of intracellular Ca\textsuperscript{2+}, a pivotal signaling event for the exocytosis of zymogen-containing secretory granules at the apical plasma membrane (1–3). In screening for signaling proteins that regulate acinar cell function, CRHSP-28\textsuperscript{1} was identified based on its Ca\textsuperscript{2+}-sensitive phosphorylation in response to secretagogue stimulation (4). We recently established an important regulatory role for CRHSP-28 in the acinar cell secretory pathway by demonstrating that introduction of CRHSP-28 protein into streptolysin-O-permeabilized acini significantly enhanced Ca\textsuperscript{2+}-stimulated digestive enzyme secretion following the loss of cytosolic proteins from the intracellular compartment (5).

CRHSP-28, also known as CSPP28 in gastric mucosa (6), is a member of the tumor protein D52 (TPD52) family (7, 8) that is highly expressed in exocrine epithelial cells throughout the digestive system (9). The TPD52 proteins share in common a conserved coiled-coil motif that supports homo- and hetero-meric interactions among family members (10). In acinar cells, chemical cross-linking studies indicate CRHSP-28 is part of a large insoluble protein complex (5) that localizes in a punctate pattern on vesicular structures in the supranuclear and apical cytoplasm (9). Supporting these findings, CRHSP-28/D52 has been localized to vesicular structures in the perinuclear cytoplasm of cultured cells (11) and was recently reported to interact with MAL2 by yeast two-hybrid screening (12). MAL2 is a member of a family of lipid-associated proteins that regulate apical targeting of intracellular vesicles in epithelial cells (13, 14).

In screening for proteins that interact with CRHSP-28 in pancreas, we recently identified 35- and 70-kDa binding proteins that co-immunoprecipitated with CRHSP-28 from acinar lysates and bound with recombinant CRHSP-28 in a gel-overlay assay (5). The 70-kDa protein co-purified with zymogen granule membranes, consistent with a secretory role for CRHSP-28 in acinar cells. Further, subcellular fractionation of the binding proteins was markedly altered when lysates were prepared in the presence of Ca\textsuperscript{2+}, resulting in a redistribution of both molecules from a cytosolic to a Triton X-100 insoluble fraction. The present study describes the purification of the 70-kDa CRHSP-28-binding protein as annexin VI and further demonstrates a colocalization of these molecules in the apical cytoplasm of acinar cells. Based on recent studies establishing a role for annexin VI in endocytic trafficking (15–17), it is proposed that CRHSP-28 may function at multiple steps in acinar cell membrane trafficking involving digestive enzyme secretion and zymogen granule membrane retrieval from the apical plasma membrane.

* This work was supported by Grants WISO4221 and WISO4444 from the United States Department of Agriculture Cooperative State Research Education and Extension Service Program, by American Cancer Society Institutional Research Grant IRG-58-011-42-2, and by National Science Foundation Award MCB-0094154 (to G. E. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: CRHSP-28, calcium-regulated heat-stable protein of 28 kDa; TPD52, tumor protein D52 family; HPLC, high performance liquid chromatography; CCK, cholecystokinin; MOPS, 4-morpholinepropanesulfonic acid.

This paper is available on line at http://www.jbc.org

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**EXPERIMENTAL PROCEDURES**

**Materials**—Soybean trypsin inhibitor, benzamidine, phenylmethylsulfonyl fluoride, and Triton X-100 were purchased from Sigma, essential amino acid solution from Invitrogen, and a protease inhibitor mixture from Calbiochem. Bovine serum albumin and peroxidase-conjugated anti-rabbit secondary antibody were from Amersham Biosciences, protein A beads from Pierce, and protein determination reagent from Bio-Rad. Purified bovine brain annexin VI and anti-rabbit annexin VI antibody (K80102R) were purchased from Bio Design International. Goat anti-human annexin VI (sc930) and corresponding antigen, rabbit anti-human Rab4 (sc312), fluorescein isothiocyanate-conjugated anti-goat IgG, and peroxidase-conjugated anti-goat IgG secondary antibodies were purchased from Santa Cruz Biotechnology. The buffer was supplemented with 0.1 mg/ml soybean trypsin inhibitor and 1 mg/ml bovine serum albumin, gassed with 100% O₂, and adjusted to pH 7.4.

**Immunofluorescence Microscopy**—Pancreatic lobules were prepared by microdissection of a rat pancreas that had been injected with phosphate-buffered saline containing (in mM) 137 NaCl, 2.7 KCl, 4.3 Na₂HPO₄, 1.4 KH₂PO₄, and 0.1 mg/ml soybean trypsin inhibitor. Isolated acinar lobules were incubated in the acinar buffer at 4 °C with gentle shaking. Following indicated treatments, lobules were gently pelleted and fixed in 4% formaldehyde prepared from paraformaldehyde. Immunofluorescence microscopy was conducted on 6-μm-thick cryostat sections as described (9). Annexin VI immunoreactivity with antibody sc1390 was characterized using a fluorescein isothiocyanate-conjugated anti-goat IgG (1:200). Antigen competition studies were conducted by preincubating annexin VI antibody with a 10-fold molar excess of peptide antigen for 2 h at room temperature, prior to an overnight incubation with the tissue. For dual immunofluorescence localization of CRHSP-28 and annexin VI, CRHSP-28 immunoreactivity was detected using Alexa fluor 549-conjugated anti-rabbit IgG (1:10,000). 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 occurred between channels. Captured images were overlaid using Bio-Rad software and then converted to TIFF files for processing using Photoshop software.

**Annexin VI Purification**—Acini prepared from two pancreases were sonicated at 4 °C in a buffer containing (in mM) 50 Tris base (pH 7.4), 2 MgCl₂, 1 CaCl₂, 0.2% Triton X-100, 0.1 phenylmethylsulfonyl fluoride, 2 benzamidine, and protease inhibitor mixture. Following a 30-min incubation at 4 °C, a detergent-insoluble fraction (P1) was obtained by centrifugation at 100,000 × g for 30 min. Detergent-insoluble proteins were sonicated again in the same buffer without CaCl₂ and containing 5 mM EGTA. Proteins released by EGTA treatment were pelleted and fixed in 4% formaldehyde prepared from paraformaldehyde. Immunofluorescence microscopy was conducted on 6-μm-thick cryostat sections as described (9). Annexin VI immunoreactivity with antibody sc1390 was characterized using a fluorescein isothiocyanate-conjugated anti-goat IgG (1:200). Antigen competition studies were conducted by preincubating annexin VI antibody with a 10-fold molar excess of peptide antigen for 2 h at room temperature, prior to an overnight incubation with the tissue. For dual immunofluorescence localization of CRHSP-28 and annexin VI, CRHSP-28 immunoreactivity was detected using Alexa fluor 549-conjugated anti-rabbit IgG (1:10,000). 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 occurred between channels. Captured images were overlaid using Bio-Rad software and then converted to TIFF files for processing using Photoshop software.

**RESULTS**

**Annexin VI Is a CRHSP-28-binding Protein**—Using a gel overlay technique to screen for proteins that interact with CRHSP-28, we recently identified two binding proteins of 35 and 70 kDa (5). Both proteins partitioned into a Triton X-100-insoluble fraction of lysates prepared in the presence of Ca²⁺. The Ca²⁺ dependence of this redistribution was exploited to purify the 70-kDa binding protein from pancreatic acinar cells (Fig. 1A). Lysates were prepared in the presence of 0.2% Triton X-100 and 1 mM CaCl₂, and a detergent-insoluble fraction (P1) highly enriched in binding proteins was obtained by centrifugation. The 70-kDa protein was subsequently released from the insoluble material by chelating Ca²⁺ using EGTA. Proteins in the EGTA released fraction (S2) were combined from multiple preparations and then separated by two-dimensional electrophoresis as a final purification step (Fig. 1B). Of the two CRHSP-28-binding proteins, only the 70-kDa molecule was readily detected by Coomassie staining, and was present as a closely spaced doublet with a pI of ~5.5–6.0. Tryptic fragments of the 70-kDa binding protein were separated by HPLC (Fig. 1C). Microsequence analysis of fraction 21 from the HPLC trace yielded high quality sequence of a 14-amino acid peptide with 100% homology to amino acids 472–485 of rat annexin VI, a Ca²⁺-sensitive phospholipid and cytoskeletal binding protein (24).

As the molecular mass, pI and Ca²⁺-mediated redistribution of the 70-kDa binding protein in subcellular fractions were essentially identical to that reported for annexin VI in other cell types, specific antibodies were used to verify its identity in acinar cells. A polyclonal antibody raised against the N-termi- nal 16 amino acids of rabbit annexin VI specifically reacted with the purified 70-kDa binding protein in two-dimensional gels (Fig. 1B). The annexin VI immunoreactive protein migrated as a closely spaced doublet following SDS-PAGE and was identical in size to signals obtained from an annexin VI-enriched membrane fraction from lung, as well as purified bovine annexin VI protein (Fig. 2A). Similar results were obtained using a separate polyclonal antibody raised against the N-terminal 19 amino acids of human annexin VI (Fig. 2C). The annexin VI immunoreactive protein underwent a pronounced redistribution from cytosolic to detergent-insoluble fractions of lysates prepared in the presence of 1 mM Ca²⁺. These results are identical to our recent report showing a Ca²⁺-sensitive translocation of the 70-kDa CRHSP-28-binding protein from...
A 14-amino acid peptide was sequenced with 100% homology to amino acids 472 of the 70-kDa binding protein. The peptide was digested with trypsin and peptides were separated by HPLC. Fraction 21 was submitted for microsequence analysis.

Ca²⁺-dependent CRHSP-28/Annexin VI Binding in Vitro—The interaction between CRHSP-28 and annexin VI was further demonstrated by coimmunoprecipitating the proteins from an acinar cell lysate (Fig. 3). Interestingly, no CRHSP-28/annexin VI binding was detected when lysates were prepared in the absence of Ca²⁺. However, inclusion of Ca²⁺ in the lysis buffer promoted a strong association between the two proteins, which readily coimmunoprecipitated together. Conducting immunoprecipitations in the presence of Ca²⁺ resulted in ~50% of the annexin VI protein translocating to the detergent-insoluble fraction, which was then removed by centrifugation prior to immunoprecipitation (see Fig. 2C). This sedimentation of annexin VI made it difficult to quantify the Ca²⁺ sensitivity of CRHSP-28/annexin VI binding by coimmunoprecipitation, as variable amounts of annexin VI moved to the detergent-insoluble fraction in the presence of Ca²⁺. As an alternative, the Ca²⁺ sensitivity of CRHSP-28/annexin VI binding was measured in vitro using the gel-overlay assay (Fig. 4). In initial experiments, gel overlays were conducted in the presence of milk protein, which contains ample amounts of Ca²⁺. Removal of Ca²⁺ from the buffer using EGTA resulted in a complete loss of CRHSP-28 binding (data not shown). As a substitute, bovine serum albumin was included in the buffer and CRHSP-28 binding was measured while clamping the free Ca²⁺ concentration at various levels. Consistent with the Ca²⁺-sensitive coimmunoprecipitation, CRHSP-28 binding to annexin VI occurred over a micromolar range of free ionized Ca²⁺. CRHSP-28 binding reached a plateau at 10–100 μM Ca²⁺ with an EC₅₀ of ~2.5 μM Ca²⁺. Binding increased slightly (<20%) at 1 mM Ca²⁺; however, no further interaction was detected at Ca²⁺ concentrations as high as 10 mM (data not shown).

Tissue Fractionation of CRHSP-28 and Annexin VI in Pancreas—CRHSP-28 is a hydrophilic protein that partitions into both soluble and membrane fractions following cell lysis (9, 5, 11). To characterize the association of CRHSP-28 with membranes, a crude microsomal fraction of a pancreatic homogenate was treated under alkaline conditions with Na₂CO₃, then re-isolated by centrifugation to separate peripheral and integral membrane proteins (Fig. 5). As a positive control, the small G-protein Rab4, which anchors to phospholipids via a geranylglyceran moiety (25), was analyzed by immunoblotting and found to remain largely associated with the membrane fraction following alkaline treatment. In contrast, CRHSP-28 was completely recovered in the soluble fraction following alkaline treatment, indicating that it is not an integral membrane protein but instead is peripherally associated with these structures.

Subcellular fractionation of rat pancreas demonstrated that CRHSP-28 was largely localized to a microsomal fraction (Fig. 6). As previously reported (9), no CRHSP-28 signal was detected in purified zymogen granules. Further, little or no CRHSP-28 was present in fractions enriched in mitochondria or lysosomes. In contrast, annexin VI was present at similar levels in all subcellular fractions tested including zymogen granules, mitochondria, and lysosomes. Interestingly, treatment of animals with a secretory dose of CCK for 40 min prior to tissue fractionation significantly increased the amount of CRHSP-28 recovered in the cytosolic fraction and, correspondingly, decreased the amount of the protein recovered in the microsomal fraction. Hormone treatment had no effect on annexin VI fractionation. Further fractionation of crude microsomes by sucrose gradient centrifugation demonstrated that both CRHSP-28 and annexin VI were predominantly associated with the smooth microsomal fraction composed mainly of Golgi, plasma membrane, and endosomes.

Immunofluorescence Localization of Annexin VI in Acinar Cells—Immunofluorescence localization of annexin VI was conducted on 0.5-μm-thick optical sections of pancreatic lobules and demonstrated that the protein was present in a punctate pattern throughout the basal and apical cytoplasm (Fig. 7). Annexin VI staining was evident in the juxtanuclear regions of cells but was largely absent from nuclei. This same pattern of annexin VI immunofluorescence was detected using both anti-human and anti-rabbit annexin VI polyclonal antibodies. No signal was observed in sections when primary antibody was omitted from the incubations (data not shown). Further, pre-
absorption of the annexin VI antibody with a 10-fold molar excess of antigen completely abolished annexin VI staining, demonstrating the specificity of this localization (Fig. 7).

Colocalization of CRHSP-28 and Annexin VI in Acinar Cells—The subcellular distribution of CRHSP-28 and annexin VI was examined using dual immunofluorescence microscopy (Fig. 8). As previously reported (9), CRHSP-28 was highly localized to the apical cytoplasm of acini extending from the supranuclear region to the apical plasma membrane (Fig. 8A). Low levels of CRHSP-28 staining were present in basal regions of cells. As indicated above (Fig. 7), annexin VI immunofluorescence was detected in a punctate pattern throughout the cytoplasm, including apical regions of acini just below the acinar lumen (Fig. 8B, arrows). Overlay of the CRHSP-28 and annexin VI images demonstrated a pronounced overlapping of the proteins throughout the supranuclear and apical cytoplasm (Fig. 8C). Little or no overlap of CRHSP-28 and annexin VI occurred in the basal cytoplasm where annexin VI staining was evident. Incubation of lobules for 5 min with CCK promoted the accumulation of both CRHSP-28 annexin VI staining to regions immediately below the apical membrane. Although quantitative immunofluorescence was not conducted, this effect was seen in multiple experiments and was also evident upon ionomycin stimulation of lobules. The co-localization of CRHSP-28 and annexin VI in control and secretagogue-stimulated acini supports the biochemical data demonstrating a Ca2+ sensitive interaction between these proteins and is consistent with an important role for these molecules in acinar cell membrane trafficking.
Rat pancreatic lobules were fixed in 4% formaldehyde, and the acinar lobules. A postnuclear supernatant (PNS) was prepared and subjected to differential centrifugation to isolate fractions that were enriched in zymogen granules (ZG), mitochondria (Mito), lysosomes (Lyso), crude microsomes (Micro), and a 100,000 × g supernatant (Cyto). Crude microsomes from control animals were further separated into smooth (SM) and rough microsomes (RM) by sucrose gradient centrifugation. Anti-CRHSP-28 and anti-annexin VI antibody k80102r were used for immunoblotting each fraction (40 μg/lane).

**FIG. 6. Subcellular fractionation of CRHSP-28 and annexin VI in rat pancreas.** Rat pancreases were obtained from animals treated as control or infused with a secretory dose (300 pmol/min/kg body weight) of cholecystokinin for 40 min. A postnuclear supernatant (PNS) was prepared and subjected to differential centrifugation to isolate fractions that were enriched in zymogen granules (ZG), mitochondria (Mito), lysosomes (Lyso), crude microsomes (Micro), and a 100,000 × g supernatant (Cyto). Crude microsomes from control animals were further separated into smooth (SM) and rough microsomes (RM) by sucrose gradient centrifugation. Anti-CRHSP-28 and anti-annexin VI antibody k80102r were used for immunoblotting each fraction (40 μg/lane).

**FIG. 7. Immunofluorescence localization of annexin VI in pancreatic lobules.** Rat pancreatic lobules were fixed in 4% formaldehyde, and annexin VI localization was analyzed in 0.5-μm-thick optical sections by confocal microscopy. Anti-annexin VI antibody s1390 (1:50) was detected using a fluorescein isothiocyanate-conjugated anti-goat secondary antibody (1:200). A, immunofluorescence localization of annexin VI and corresponding differential contrast image (B) demonstrating a punctate pattern of annexin VI immunoreactivity throughout the acinar cell cytoplasm. C, the annexin VI antibody was preincubated with a 10-fold molar excess of antigen prior to incubation with the tissue. D, differential contrast image of field shown in panel C.

**DISCUSSION**

Annexin VI is a member of an extended family of Ca\textsuperscript{2+}-dependent phospholipid-binding proteins reported to function in diverse cellular processes related to signaling, membrane trafficking, and cytoskeletal dynamics (24, 26). Annexin proteins are characterized by the presence of at least four conserved tandem repeats of 70 amino acids that mediate their interaction with negatively charged phospholipids in a Ca\textsuperscript{2+}-dependent manner. Annexin VI is unique in that it contains eight such repeats arranged in a two-lobed configuration that is separated by a linker region (27). The flexibility of the linker region supports both parallel and perpendicular orientations of the two lobes and, as such, is thought to impart the many dynamic properties of the annexin VI protein (27).

Annexin VI has been implicated as an important regulatory component of clathrin-mediated endocytosis (15–17, 28), although the primary significance of the protein in this process remains unclear (29). Lin et al. (28) used an in vitro system to demonstrate that annexin VI is required for the Ca\textsuperscript{2+}- and ATP-dependent budding of clathrin-coated pits from membranes. Subsequent studies in intact fibroblasts indicated that annexin VI functions by directing the remodeling of membrane-bound spectrin during vesicle budding (15). In addition to modulating endocytosis at the plasma membrane, annexin VI was shown to be present in a late endocytic compartment of epithelial cells (30–34), where it is thought to direct the delivery of endosomal vesicles to lysosomes (16, 17). Interestingly, in smooth muscle, annexin VI plays a dynamic role in regulating reversible interactions between actin-cytoskeletal components and the caveolar fraction of the sarcolemma during muscle contraction (35, 36). Collectively, these studies suggest that annexin VI plays a generalized role in Ca\textsuperscript{2+}-dependent cellular processes involving transient interactions between membrane and cytoskeletal proteins.

The binding of CRHSP-28 with annexin VI required low micromolar concentrations of free Ca\textsuperscript{2+} in vitro, indicating the likelihood that these molecules interact under physiological conditions (1–3). A specific interaction between CRHSP-28 and annexin VI was supported by dual immunofluorescence microscopy colocalizing these proteins within the apical cytoplasm of acinar cells. Furthermore, the apparent recruitment of CRHSP-28 and annexin VI to the cell apex upon secretagogue stimulation also supports a Ca\textsuperscript{2+}-regulated interaction. The Ca\textsuperscript{2+} dependence of CRHSP-28/annexin VI binding is remarkably similar to the Ca\textsuperscript{2+}-dependent interaction of annexin VI with negatively charged phospholipids and suggests that these molecules interact by a similar molecular mechanism. CRHSP-28 contains three clusters of negatively charged acidic residues (amino acids 16–20, 34–39, and 48–50) present within the amino half of the protein (4). These concentrated regions of negative charge may potentially support Ca\textsuperscript{2+}-dependent interactions of CRHSP-28 with either of the phospholipid binding domains of annexin VI.

Our previous study (5) demonstrating a role for CRHSP-28 in acinar cell secretion strongly suggests that CRHSP-28 be associated with zymogen granules. Although CRHSP-28 was highly localized around zymogen granules in the apical cytoplasm, the protein was not detected when immunoblotting purified fractions of these organelles. Conversely, annexin VI was clearly detected in purified zymogen granules (Fig. 6) and zymogen granule membranes (5) prepared in the absence of added Ca\textsuperscript{2+}. A Ca\textsuperscript{2+}-independent association of annexin VI with membranes has previously been described in a number of tissues including liver and mammary epithelial cells (22, 32, 34). Therefore, it is possible that CRHSP-28 is recruited to zymogen granules via an interaction with annexin VI during periods of elevated cellular Ca\textsuperscript{2+}. Indeed, numerous studies have documented that the highest levels of free Ca\textsuperscript{2+} achieved following secretagogue stimulation occur immediately below the apical membrane and are believed to be necessary to trigger the exocytosis of zymogen granules (1–3). Zymogen granules have been shown to be enriched in cholesterol- and sphingolipid-containing microdomains, and, further, these structures are essential for granule maturation and apical secretion in acini (37). In smooth muscle, annexin VI has been shown to regulate reversible interactions of the actin cytoskeleton with cholesterol- and glycosphingolipid-rich membrane.
Annexin VI Is a CRHSP-28-binding Protein

FIG. 8. Colocalization of CRHSP-28 and annexin VI in pancreatic lobules. Cryostat sections were prepared from formaldehyde-fixed pancreatic lobules treated as control or with 10 nm CCK for 5 min. Affinity-purified CRHSP-28 and anti-annexin VI si1930 antibodies were detected using Alexafluor 594- and fluorescein isothiocyanate-conjugated secondary antibodies, respectively. CRHSP-28 and annexin VI antibodies were used at final concentrations of 1 μg/ml and 1:50, respectively. A and D, CRHSP-28 localization is shown in red. B and E, annexin VI localization is shown in green. C and F, overlay of the CRHSP-28 and annexin VI images showing that CCK stimulation enhances the accumulation of both proteins below the apical membrane. Asterisks indicate location of nuclei. Arrows indicate the apical plasma membrane just below the acinar lumen.

microdomains (35, 36). Thus, similar to its role in smooth muscle contraction, annexin VI may regulate interactions between zymogen granules and actin-cytoskeletal components of the subapical web in acini. The actin-rich subapical web in acinar cells has been shown to play an integral role in regulating both the exocytosis of zymogen granules and the subsequent retrieval of granule membranes from the apical plasma membrane (38-40).

CRHSP-28 and annexin VI were diffusely localized in the apical cytoplasm of acini under both basal and CCK-stimulated conditions. The diffuse nature of the immunofluorescence staining precluded a precise localization of the proteins to specific membrane compartments. Because significant cytosolic pools of both CRHSP-28 and annexin VI exist, these data may reflect a dynamic and reversible association of these molecules with membranes. Cell fractionation studies indicated CRHSP-28 was equally present in both soluble and particulate fractions of acinar lysates. Further, CRHSP-28 was peripherally associated with membranes, as NaCO₃ treatment efficiently released the protein from pancreatic microsomes. We recently showed that the leakage of CRHSP-28 from streptolysin-O-permeabilized acini was significantly enhanced over 15 min under conditions of elevated cellular Ca²⁺, suggesting that cell stimulation promoted a translocation of CRHSP-28 within the cytoplasm (5). Interestingly, we have determined that phosphorylated CRHSP-28 is primarily released from permeabilized acini following CCK stimulation, whereas the nonphosphorylated protein is associated exclusively with membrane fractions. These findings are consistent with the current study showing that the recovery of CRHSP-28 in cytosolic fractions of pancreas was significantly enhanced following a 40-min infusion of CCK in vivo. Collectively, these findings support a dynamic association of CRHSP-28 with membranes that is significantly altered by secretagogue stimulation.

The question remains as to the Ca²⁺-independent association of CRHSP-28 and annexin VI with endosome-enriched microsomal fractions of pancreas. One possibility is that CRHSP-28 binds to annexin VI during periods of elevated Ca²⁺ to support the movement of zymogen granules across the actin-rich subapical web. CRHSP-28 and annexin VI would then enter the plasma membrane during zymogen granule fusion and be retrieved from the apical membrane during endocytosis. The finding that the association of CRHSP-28 with endosome-enriched microsomes was detected in the absence of added Ca²⁺ further suggests CRHSP-28 entered a stable complex with additional membrane-bound proteins. Supporting this, we recently demonstrated by covalent cross-linking experiments that CRHSP-28 is part of a large molecular mass complex (>300 kDa) in acini that partitions into membrane fractions following cell lysis (5). The large mass of this complex is consistent with an association of CRHSP-28 with a multiprotein complex. Further, in accordance with the CCK-enhanced cytosolic localization of CRHSP-28 in vivo and the Ca²⁺-enhanced release of the protein from permeabilized cells (5), CRHSP-28 may be dissociated from this complex via its Ca²⁺-mediated phosphorylation. CRHSP-28 phosphorylation occurs on at least two serine residues and is a transient event, reaching maximal levels within 2 min and fully dephosphorylating over 60 min in the continued presence of secretagogues (4). The transient phosphorylation of CRHSP-28 is clearly consistent with the movement of the protein into the cytoplasm, where it may be dephosphorylated by constitutively active serine/threonine protein phosphatases. Once dephosphorylated, CRHSP-28 would be available to support subsequent zymogen granule trafficking events.

Interestingly, it was recently reported that CRHSP-28 specifically interacts with a member of the MAL protein family by yeast two-hybrid analysis and in vitro pull-down assays (12). MAL proteins are known to regulate apical targeting of vesicles in renal epithelial cells and, similar to annexin VI, are targeted to cholesterol and sphingolipid-rich microdomains on secretory granules (13, 14). Moreover, MAL proteins have been shown to

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2 D. D. H. Thomas and G. E. Groblewski, unpublished observation.
cycle from the apical plasma membrane back to the Golgi compartment on endocytic vesicles (41). Although MAL proteins are essential for apical membrane targeting in polarized epithelia, the precise molecular function of these proteins has not been described. Clearly, further experimentation directly examining the role of CRHSP-28 and its associated proteins in zymogen granule trafficking and membrane retrieval is necessary to understand how this molecule modulates acinar cell function.

Acknowledgments—We extend a special thanks to Dr. Stephen Ernst for helpful suggestions on performing immunofluorescence microscopy on pancreatic tissue and Dr. Joseph Leykam and staff at the Michigan State University Microsequence Facility for help in sequencing the annexin VI protein.

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