CRHSP-28 Regulates Ca\(^{2+}\)-stimulated Secretion in Permeabilized Acinar Cells*

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CRHSP-28 is a Ca\(^{2+}\)-regulated heat-stable phospho-protein, abundant in the apical cytoplasm of epithelial cells that are specialized in exocrine protein secretion. To define a functional role for the protein in pancreatic secretion, recombinant CRHSP-28 (rCRHSP-28) was introduced into streptolysin-O-permeabilized acinar cells, and amylase secretion in response to elevated Ca\(^{2+}\) was determined. Secretion was enhanced markedly by rCRHSP-28 over a time course that closely corresponded with the loss of the native protein from the intracellular compartment. No effects of rCRHSP-28 were detected until ~50% of the native protein was lost from the cytosol. Secretion was enhanced by rCRHSP-28 over a physiological range of Ca\(^{2+}\) concentrations with 2–3-fold increases in amylase release occurring in response to low micromolar levels of free Ca\(^{2+}\). Further, rCRHSP-28 augmented secretion in a concentration-dependent manner with minimal and maximal effects occurring at 1 and 25 \(\mu\)M, respectively. Covalent cross-linking experiments demonstrated that native CRHSP-28 was present in a 60-kDa complex in cytosolic fractions and in a high molecular mass complex in particular fractions, consistent with the slow leak rate of the protein from streptolysin-O-permeabilized cells. Probing acinar lysates with rCRHSP-28 in a gel-overlay assay identified two CRHSP-28-binding proteins of 35 (pp35) and 70 kDa (pp70). Interestingly, preparation of lysates in the presence of 1 mM Ca\(^{2+}\) resulted in a marked redistribution of both proteins from a cytosolic to a Triton X-100-insoluble fraction, suggesting a Ca\(^{2+}\)-sensitive interaction of these proteins with the acinar cell cytoskeleton. In agreement with our previous study immunohistochemically localizing CRHSP-28 around secretory granules in acinar cells, gel-overlay analysis revealed pp70 copurified with acinar cell secretory granule membranes. These findings demonstrate an important cell physiological function for CRHSP-28 in the Ca\(^{2+}\)-regulated secretory pathway of acinar cells.

Exocrine cells specializing in protein secretion release a variety of factors necessary for normal function of the digestive, urogenital, respiratory, and ocular systems. Activation of these epithelia by neural and humoral agents stimulates the exocytosis of secretory granules at the apical plasma membrane in a process that is largely controlled by cellular Ca\(^{2+}\). In pancreatic acinar cells, Ca\(^{2+}\) release is initiated in the apical pole and then propagates through the cell periphery to the basal cytoplasm. The cyclic reuptake and release of Ca\(^{2+}\) from intracellular stores create an oscillatory mode of signaling with spatial and temporal characteristics that are unique to the specific type and concentration of physiological stimulus (for review, see Refs. 1–3). Although the high concentrations of Ca\(^{2+}\) generated in the apical cytoplasm are necessary for secretory granule trafficking and exocytosis to occur, a comprehensive understanding of the molecular events elicited by this ion is lacking.

The secretory pathway in acinar cells is a multifactorial process beginning with the microtubule-directed transport of newly formed zymogen granules (ZGs)† to the apical cytoplasm. To reach the plasma membrane ultimately, ZGs are released from microtubules and must penetrate a prominent subapical cytoskeletal web composed of actin and intermediate filaments (4). ZG movement along microtubules was shown to involve motor proteins from both the microtubule minus end-directed dyneine/dynactin complex (5) as well as the plus end-directed kinesin family (6, 7). Similarly, a potential role for myosin motor proteins in facilitating ZG exocytosis has also been described based on the localization of myosin I to ZG membranes (8), myosin II to the apical cytoplasm (8, 9), and the acute activation of myosin light chain kinase in response to secretagogues (10). In addition to ZG movement via cytoskeletal motor proteins, a selective reorganization of subapical actin filaments in response to acinar cell stimulation has been described (9, 11, 12). Muallem et al. (11) demonstrated that partial depolymerization of actin filaments in permeabilized acini with the enzyme thymolysin independently stimulated exocytosis in the absence of Ca\(^{2+}\). On the other hand, complete depolymerization of filaments fully arrested agonist-induced secretion, indicating that distinct alterations in these structures are necessary for normal secretion to occur (11). Valentinij et al. (13) recently reported that just prior to reaching the plasma membrane, ZGs release the small GTP-binding protein Rab3D and become coated with filamentous actin in a process that may facilitate the movement of granules across the terminal web. Interestingly, other studies utilizing actin filament destabilizing agents in pancreatic lobules demonstrated an integral role for the

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The abbreviations used are: ZG(s), zymogen granule(s); SNARE, soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor; SLO, streptolysin-O; CRHSP-28, calcium-regulated heat-stable protein; rCRHSP-28, recombinant CRHSP-28 protein; TPDP52, tumor protein D52 family; pp35 and pp70, 35- and 70-kDa CRHSP-28-binding proteins, respectively; BS\(^{1}\), bis(sulfofuccinimidy) suberate; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; E-64, trans(epoxy)succinyl-l-t-leucylamido-(4-guanidino)butane; PIPES, 1,4-piperazinediethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; SNAP, soluble NSF attachment protein; CLIP, cytoplasmic linker protein.

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subapical web in controlling the compensatory retrieval of ZG membranes back into the cell after exocytosis (14, 15). Actin filament destabilization not only inhibited membrane retrieval, but also led to the inhibition of secretion by sequestering granule contents into large vacuolar structures that appeared continuous with the apical plasma membrane (14).

Similar to nerve and endocrine cells, the final step in ZG fusion with apical plasma membrane is mediated by protein components of the SNARE apparatus (for review, see Ref. 16). Acinar cells possess a number of isoforms of the SNARE protein family members (16, 17). Use of clostridial neurotoxins to cleave SNARE proteins selectively in streptolysin-O (SLO)-permeabilized acini (18) and in an in vitro membrane fusion system (17) indicates a direct role for these proteins in ZG membrane fusion; however, the major ZG-associated SNARE protein that mediates this process is currently unknown. Although it is clear from these studies that a considerable overlap of regulatory proteins exists between acinar cells and other secretory cell types, it is also evident that acini express unique regulatory proteins to direct the cytoskeletal and membrane fusion events that coordinate ZG exocytosis.

Utilizing a proteomic approach to identify proteins that are functionally regulated by Ca$^{2+}$ in exocrine pancreas, we previously purified a regulated phosphoprotein, termed CRHSP-28 (19, 20). Also known as D52 (21), N8 (22, 23), R10 (24), and CSPP-28 (25), this protein has been identified based on its overexpression in transformed epithelial cells (21, 22, 24) and Ca$^{2+}$-sensitive phosphorylation in gastric mucosa (25). Moreover, Byrne et al. (26) demonstrated that CRHSP-28 belongs to a novel tumor protein D52 family (TPD52) comprised of at least three homologous genes that may undergo alternative splicing to create different protein products (27). Using polyclonal specific antibodies, we recently demonstrated that CRHSP-28 is abundant in digestive epithelial cells specializing in protein secretion including acinar cells from pancreas, salivary, and lacrimal glands, as well as chief cells, paneth cells, and goblet cells present throughout the gastrointestinal mucosa (20).

Despite a lack of hydrophobic membrane-association motifs, CRHSP-28 partitions between soluble and particulate fractions after cell lysis. Further, immunohistochemical localization indicates a high concentration of CRHSP-28 surrounding secretory granules in the apical cytoplasm of acini (20). These findings together with the acute Ca$^{2+}$ sensitivity of CRHSP-28 phosphorylation suggest that the protein may be involved in modulating acinar cell secretion. To test this hypothesis, the present study takes advantage of the high solubility of purified recombinant CRHSP-28 protein (rCRHSP-28), allowing its introduction into SLO-permeabilized pancreatic acinar cells. The ability of rCRHSP-28 to restore partially the Ca$^{2+}$-dependent secretory activity after the leakage of soluble proteins from the intracellular compartment directly demonstrates an important role for CRHSP-28 in the later steps of the secretory pathway. Investigation of the molecular interactions of CRHSP-28 led to the identification of a 70-kDa CRHSP-28-binding protein (pp70) that is present in ZG membranes. It is proposed that CRHSP-28 functions in acinar cell secretion by modulating the delivery of secretory granules to the apical plasma membrane via dynamic interactions with the pp70 protein.

**EXPERIMENTAL PROCEDURES**

**Materials**— Soybean trypsin inhibitor, benzamidine, phenylmethylsulfonyl fluoride, Triton X-100, and Percoll were purchased from Sigma, and a protease mixture containing leupeptin, AEBSF, and E-64 was from Calbiochem. Bovine serum albumin and phosphodiester-conjugated secondary antibody were from Amersham Pharmacia Biotech. Bis-(sulfofuccinimidyl) suberate (BS$^3$) cross-linker, and protein A beads were from Pierce; SLO was from Difco, a Phadebas amylase assay kit was from Bio-Rad. The anti-rat cysteine string protein polyclonal antibody was purchased from StressGen (Victoria, British Columbia). Bacterial expression and purification of human rCRHSP-28 protein and characterization of the affinity-purified anti-CRHSP-28 polyclonal antibodies have been detailed previously (19, 20).

**Preparation of Rat Acini—** Pancreatic acinar cells were isolated from adult male Harlan Sprague-Dawley rats by collagenase digestion as described previously (28, 29). Acini were suspended in a 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 d-glucose, 2 l-glutamine, and an essential amino acid solution. The buffer was supplemented with 0.1 mg/ml soybean trypsin inhibitor, 1 mg/ml bovine serum albumin, gassed with 100% O$_2$, and adjusted to pH 7.4. Cells were maintained at 37 °C for 1 h prior to performing assays.

**Acinar Cell Permeabilization—** Acini were suspended in a permeabilization buffer containing (in mM) 20 PIPES (pH 6.6), 139 K$^+$-glutamate, 4 EGTA, 1.78 MgCl$_2$, 2 Mg-ATP, 0.1 mg/ml soybean trypsin inhibitor, 1 mg/ml bovine serum albumin, and 0.5 unit/ml SLO. The SLO was allowed to bind to the cells on ice for 10 min and was then solubilized to 4% in the same buffer without SLO. Equal aliquots into microcentrifuge tubes (200 μl/tube) containing the indicated amounts of rCRHSP-28. The cell suspension was then diluted with an equal volume of the same buffer containing enough CaCl$_2$ to create the desired final concentration of free Ca$^{2+}$. The quantity of Ca$^{2+}$ added to the buffer was calculated based on dissociation constants using a computer program as described (30). Cell suspensions were maintained at 37 °C for 30 min and incubated in an Sp6000 shaker at the indicated times. At the end of the incubation period, cells were cooled in an ice bath and then centrifuged at 12,000 × g for 1 min. The content of amylase in the medium was determined using a Phadebas assay kit. Data were calculated as the percent of total cellular amylase present in an equal amount of cells measured at the start of the experiment.

**CRHSP-28 Leakage from SLO-permeabilized Cells—** SLO-permeabilized acini were exposed to basal or stimulatory concentrations of free Ca$^{2+}$, and at indicated times, cells were pelleted in a microcentrifuge. Proteins present in the medium were precipitated in 15% trichloroacetic acid and then solubilized in SDS sample buffer. Cell pellets were sonicated in a lysis buffer containing (in mM) 50 Tris (pH 7.4), 25 NaF, 10 tetrasodium pyrophosphate, 5 EDTA, 0.2% Triton X-100, 1 phenylmethylsulfonyl fluoride, 2 benzamidine, and a protease inhibitor mixture. Total protein was measured using a Bio-Rad assay reagent. Equal amounts of cellular protein (50 μg/sample) and equal volumes of culture medium were analyzed for CRHSP-28 content by immunoblotting using enhanced chemiluminescence. The intensity of the CRHSP-28 signal was quantified using a PDI model DNA35 scanner interfaced with the Protein and DNA Imageware system (Huntington Station, NY).

**Cross-linking studies—** Acini were lysed in phosphate-buffered saline containing 100 μM phenylmethylsulfonyl fluoride and 10 μM leupeptin by repeated freeze/thawing in liquid nitrogen. The BS$^3$ cross-linking reagent was fresh prepared in H$_2$O and added to lysates at room temperature for 30 min. The cross-linking reaction was quenched by the addition of 20 mM glycine at 4 °C for 10 min. Soluble and particulate fractions were obtained by centrifugation at 12,000 × g for 30 min. Equal amounts of protein were analyzed by immunoblotting after SDS-PAGE.

**Gel-overlay Assays—** Proteins were separated by SDS-PAGE, immobilized to nitrocellulose membrane, and blocked in Tris-buffered saline containing 0.3% Tween 20 and 3% non-fat milk for 1 h at room temperature. Membranes were incubated sequentially with 2–4 μg/ml rCRHSP-28 and 0.5 μg/ml anti-CRHSP-28 antibody for 1 h at room temperature. CRHSP-28-binding proteins were then detected using a horseradish peroxidase-conjugated secondary antibody (15,000).

For pp70 cell fractionation experiments, acini were sonicated in lysis buffer without Triton X-100 and containing 1 μM CaCl$_2$ or 5 μM EGTA. Cytosolic and particulate fractions were prepared by centrifugation (100,000 × g). Particulate fractions were sonicated further in the same buffer containing 0.2% Triton X-100 and centrifuged again to produce a membrane- and Triton-insoluble fraction. The detergent-insoluble protein was resolved directly in SDS buffer. Equal cytosolic and membrane protein (40 μg) and equal volumes of detergent-insoluble protein (1/10 of total volume) were separated by SDS-PAGE and analyzed by gel-overlay or immunoblotting.

**Other Methods—** CRHSP-28 was immunoprecipitated from equal amounts of lysate (0.7 mg) from [32P]-labeled acini as described previously (31). ZGs, granule membranes, and granule content were prepared as detailed previously (32, 33).
CRHSP-28 Regulates Secretion in Acinar Cells

RESULTS

CRHSP-28 Increases Ca\(^{2+}\)-stimulated Secretion in Permeabilized Acini—A role for CRHSP-28 in pancreatic secretion was examined utilizing a well characterized SLO-permeabilized acinar cell preparation (11, 18, 30, 34) (Fig. 1). Isolated acini were permeabilized with SLO and exposed to basal (\(\leq 10 \text{nM}\)) or stimulatory (10 \(\mu\text{M}\)) concentrations of free Ca\(^{2+}\). Secretion of the digestive enzyme, amylase, into the medium was measured at various times over 30 min. Consistent with previous studies utilizing SLO-permeabilized acini (30, 34), the highest rate of Ca\(^{2+}\)-stimulated secretion occurred during the first 10 min of incubation when 6.1% of total cellular amylase was released into the medium (filled squares). A considerably slower rate of secretion occurred during the following 10-min intervals adding up to 9.5 and 11.9% of total cellular amylase at 20 and 30 min, respectively. Basal secretion in low Ca\(^{2+}\) represented 3.2% of total over the entire 30 min incubation (open squares). To illustrate the diminished secretory response caused by SLO permeabilization of cells, secretion from intact acini stimulated with the calcium ionophore ionomycin is included on the graph for comparison (triangles). Intact and SLO-treated acini showed similar rates of secretion over the first 10 min of stimulation; however, secretion at later times was diminished significantly in the permeabilized cells.

Introduction of rCRHSP-28 into permeabilized acini had no effect on secretion during the first 10 min of Ca\(^{2+}\)-stimulation (filled circles); however, rCRHSP-28 augmented secretion by greater than 160% of untreated cells when measured at 20 and 30 min. Basal secretion in low Ca\(^{2+}\) was not altered by rCRHSP-28 at any time (open circles), indicating that the ability of the protein to modulate secretion was dependent on increased cellular Ca\(^{2+}\). The addition of rCRHSP-28 to cells that had been pre-permeabilized for 10 min, washed, and resuspended in incubation buffer containing the protein had no significant effect on amylase secretion (not shown). Rather, it was necessary for rCRHSP-28 to be present in the medium throughout the entire incubation period to elicit a significant effect. For control measures, rCRHSP-28 was introduced into the SLO-permeabilized cells in the presence of 1 mg/ml bovine serum albumin and 0.1 mg/ml soybean trypsin inhibitor. Accordingly, rCRHSP-28 represented ~2% of the total protein in the incubation medium, demonstrating the specificity of the CRHSP-28 effect.

CRHSP-28 Leakage from SLO-permeabilized Acini—Previous studies indicate that SLO permeabilization of acini is complete within 2–5 min at 37 °C and is not altered by micromolar concentrations of free Ca\(^{2+}\) (11, 30, 34). To ensure complete permeabilization of cells in our preparations, diffusion of the 12-kDa cytosolic protein cyclophilin A from acini was analyzed by immunoblotting following SLO treatment (Fig. 2A, top gel). The majority of cyclophilin A was lost rapidly from permeabilized acini within 2 min and was completely absent from the intracellular compartment by 5 min. Moreover, elevation of Ca\(^{2+}\) in the medium did not influence the rate or extent of cyclophilin A leakage.

To examine the loss of native CRHSP-28 from permeabilized acini, the content of the protein both inside and outside the cells was measured over time following SLO treatment (Fig. 2, A–C). In contrast to the rapid and complete diffusion of cyclophilin A, a much smaller proportion of CRHSP-28 was lost from the intracellular compartment over 15 min when cells were incubated in basal Ca\(^{2+}\). With prolonged exposures of the blots, it was estimated that 50% of CRHSP-28 remained in the cells after 15 min, consistent with the levels of the protein found in the particulate fraction after cell lysis (see Fig. 5). Interestingly, elevation of Ca\(^{2+}\) in the medium enhanced CRHSP-28 leakage from acini significantly at all time points (Fig. 2B). Complementary results were obtained when measuring CRHSP-28 levels in the extracellular medium (Fig. 2C). Again, CRHSP-28 slowly diffused from the cells over the 15-min incubation, and its appearance in the medium was clearly enhanced at each time point when Ca\(^{2+}\) was elevated.

Characterization of CRHSP-28-enhanced Secretion—Corresponding with previous studies utilizing SLO-permeabilized acini (30, 34), amylase secretion in these preparations was stimulated by physiological levels of free Ca\(^{2+}\) with maximal release occurring between 3 and 10 \(\mu\text{M}\) Ca\(^{2+}\) (Fig. 3). The addition of rCRHSP-28 to cells enhanced secretion significantly over the entire range of Ca\(^{2+}\) concentrations. The effects of the protein were most pronounced at low stimulatory levels of Ca\(^{2+}\) (0.1–1 \(\mu\text{M}\)) when secretion in the absence of rCRHSP-28 was at a minimum. Indeed, rCRHSP-28 augmented secretion greater than 3-fold when stimulating cells with 0.3 \(\mu\text{M}\) Ca\(^{2+}\). In addition, rCRHSP-28 enhanced secretion in a concentration-dependent manner with a minimal response detected at 1 \(\mu\text{g/ml}\).
CRHSP-28 Regulates Secretion in Acinar Cells

Fig. 3. CRHSP-28 increases amylase secretion over a range of calcium concentrations. SLO-permeabilized acini were stimulated with the indicated concentrations of Ca\(^{2+}\), and the effect of 25 \(\mu\)g/ml rCRHSP-28 on amylase secretion was determined after 30 min. Data are the mean ± S.E. of five experiments performed in duplicate. The basal secretion in low Ca\(^{2+}\) was less than 3% of the total cellular amylase in each experiment and was therefore subtracted from the stimulated values.

Fig. 4. Concentration-dependent effects of rCRHSP-28 on Ca\(^{2+}\)-stimulated secretion. SLO-permeabilized acini were incubated with the indicated concentrations of rCRHSP-28 and stimulated with 1 \(\mu\)M free Ca\(^{2+}\). Control cells received no rCRHSP-28. Data are the mean ± S.D. of two independent experiments performed in duplicate.

Fig. 5. Chemical cross-linking identifies CRHSP-28-protein interactions. Acinar cell lysates were incubated with BS\(^3\) cross-linker, and soluble (S) and particulate (P) fractions were separated by centrifugation. The CRHSP-28 content of each fraction (50 \(\mu\)g/lane) was analyzed by immunoblotting after 8% SDS-PAGE. This experiment was performed three times with identical results.

Detection of CRHSP-28-binding Proteins in Acini—Immunoprecipitation of CRHSP-28 under nondenaturing conditions from \(^{32}\)P-labeled acini demonstrated a large increase in CRHSP-28 phosphorylation after treatment with cholecystokinin (Fig. 6A). Two additional phosphoproteins of ~35 and 70 kDa (pp35 and pp70, respectively) were also reproducibly precipitated with the CRHSP-28 from the lysate. Neither protein was detected by immunoblotting, with the same antibody, suggesting that they were bound to CRHSP-28 in the lysate. To verify these results, a gel-overlay analysis was conducted by probing the membrane with 2 \(\mu\)g/ml rCRHSP-28 protein followed by 0.5 \(\mu\)g/ml anti-CRHSP-28 antibody. A CRHSP-28 immunoblot is shown on the right for comparison.
with the single 28-kDa signal seen when immunoblotting acinar proteins, gel-overlay analysis revealed two additional bands at 35 and 70 kDa, consistent with the coimmunoprecipitation experiments. The band at ~45 kDa did not remain upon further washing of the membrane. These same results were obtained when probing with radiolabeled rCRHSP-28 protein (not shown).

Localization of CRHSP-28-binding Proteins in Membrane and Detergent-insoluble Fractions in Acini—Gel-overlay analysis of subcellular fractions indicated that pp 35 was a mostly soluble protein, whereas pp70 was equally partitioned between soluble and membrane (Triton X-100-soluble) fractions when isolated in the absence of Ca²⁺ (Fig. 7). Interestingly, preparation of lysates in the presence of 1 mM Ca²⁺ resulted in a striking redistribution of both proteins from the soluble to membrane and Triton-insoluble fractions. Because of the difficulty of measuring the protein content of detergent-insoluble fractions, these samples were dissolved directly in 2% SDS. Coomassie staining was conducted to ensure equal loading of each sample and also demonstrated that Ca²⁺-sensitive translocation of CRHSP-28-binding proteins to membrane/cytoskeletal fractions in acini. Acini were sonicated in lysis buffer without Triton X-100 and containing either 2 mM EGTA or 1 mM CaCl₂. Soluble (Sol) and particulate fractions were prepared by centrifugation. Particulate fractions were sonicated further in the same buffer containing 0.2% Triton X-100. Detergent-soluble or membrane (Mem) fractions and Triton-insoluble (TX100-insol) fractions were obtained by a second centrifugation. Triton-insoluble proteins were dissolved directly in SDS buffer. Equal amounts of soluble and membrane protein (40 μg/lane) and equal volumes of Triton-insoluble proteins (1/10 of total volume) were separated by SDS-PAGE. Panel A: top section, Coomassie-stained proteins demonstrating the relative distribution of cellular protein isolated under each condition; middle and bottom sections, gel-overlay assay showing CRHSP-28-binding proteins. Panel B, CRHSP-28 immunoblot.

DISCUSSION

In rat (34) and mouse (30) acini the highest rates of Ca²⁺-stimulated secretion occur during the first 10 min after SLO permeabilization. The diminished secretory activity at later times was shown to result from the loss of soluble proteins diffusing from the cells because the addition of cytosolic extracts from lacrimal gland or brain were able to restore Ca²⁺-stimulated secretion (34). Introduction of rCRHSP-28 into SLO-permeabilized acini markedly enhanced Ca²⁺-stimulated amylase release with a time course that corresponded to the loss of the native protein from the intracellular compartment (compare Figs. 1 and 2). No effects of rCRHSP-28 were detected until ~50% of the native protein had diffused from the cytoplasm. Further, it was necessary that rCRHSP-28 be added to the cells during the initial incubation period. The addition of protein to cells that had been pre-permeabilized and “run down” for 10 min had little effect, indicating that CRHSP-28 must function in concert with other soluble regulatory proteins in modulating secretion. Because the secretory pathway in acini encompasses multiple regulatory steps coordinating cytoskeletal and membrane fusion events, the specific point at which CRHSP-28 modulates this pathway is uncertain. However, the inability of CRHSP-28 alone to reconstitute acinar secretion presents a likelihood that the protein acts at a step preceding the final stage of ZG fusion with the plasma membrane.
CRHSP-28 Regulates Secretion in Acinar Cells

Secretion from acini was augmented by rCRHSP-28 over a micromolar range of Ca\(^{2+}\) concentrations, well within the physiological levels detected in secretagogue-stimulated acini using digital imaging and microfluorimetry (1–3). Interestingly, a biphasic concentration response for rCRHSP-28 was seen, with high levels of the protein (\(\geq 50 \mu g/ml, -2.6 \mu M\)) having a markedly diminished effect on secretion. Morgan and Burgoyne (38) reported almost identical effects of the soluble N-ethylmaleimide-sensitive factor attachment protein α-SNAP on catecholamine secretion from digitonin-permeabilized chromaffin cells. In regulating secretion, α-SNAP is transiently associated with the SNARE complex and released during membrane fusion. Subsequent studies showed that excess levels of the yeast homologue of α-SNAP (Sec17p) arrested membrane fusion in vitro by stabilizing SNARE complexes and preventing α-SNAP dissociation (39). Our results may support an analogous on/off role for CRHSP-28 protein interactions in mediating secretion. Cell fractionation and cross-linking demonstrated CRHSP-28 was bound to a large protein complex in acini, in agreement with the slow release of the protein from SLO-permeabilized acini. The Ca\(^{2+}\)-enhanced release of CRHSP-28 from permeabilized acini suggests that cell stimulation increased the dissociation of CRHSP-28 from this complex. Presumably, flooding the cells with high concentrations of recombinant protein may saturate CRHSP-28 binding sites, leading to a loss of the regulatory activity of the protein.

By expressing the three known members of the TPD52 family in the yeast two-hybrid system, Byrne et al. (35) demonstrated both homo- and heteromeric interactions between the D52 proteins which were dependent on an intact coiled-coil motif. Similarly, cross-linking of CRHSP-28 in acinar lysates produced a 60-kDa protein in soluble fractions, consistent with a CRHSP-28 dimer. Gel-overlay analysis also identified an interaction between rCRHSP-28 and the native protein on the blot (Fig. 6). Whether or not other members of the TPD52 family or alternatively spliced isoforms of CRHSP-28 are expressed in acinar cells is unknown. TPD52 mRNAs were shown to be present alone and together in different carcinoma cell lines, indicating that CRHSP-28 may be expressed independently of other family members (26). The polyclonal antibody used in the present study was raised against full-length CRHSP-28 and did not cross-react with any other acinar proteins present on one- or two-dimensional SDS gels, suggesting that no alternatively spliced isoforms of CRHSP-28 were present.

Similar to CRHSP-28, the 70-kDa binding protein pp70, also partitions between cytosolic and membrane fractions of acinar cells prepared in the absence of Ca\(^{2+}\), indicating that it is unlikely to be an integral membrane protein. Translocation of pp70 to detergent-insoluble-fractions occurs after the addition of Ca\(^{2+}\) to cell lysates, suggesting that the ion interacts directly with pp70 in promoting this effect. Identically, pp35, which was found only in soluble fractions, also translocated to detergent-insoluble fractions in the presence of Ca\(^{2+}\). These results may suggest that pp70 is a dimer of the 35-kDa protein. However, the distribution of pp35 and pp70 was unchanged when cell lysates were prepared under reducing conditions in 2% SDS (\(\times\) boiling) or 8 M urea. Alternatively, the large difference in molecular mass between the two proteins may indicate that pp35 represents a proteolytic fragment of pp70 rather than a homologous form of the same molecule.

A potential cytoskeletal role for CRHSP-28 in epithelial cell function was described previously based on the finding that its expression is induced dramatically after activation of an epithelial gene program in mesenchymal cells by the adenoviral E1a protein (23). The E1a protein induces the expression of a number of epithelial specific cytoskeletal regulatory proteins including desmoplakin, desmogelin, desmocollin, E-cadherin, and the cytokeritins K8/K18 (40). Similarly, ectopic expression of CRHSP-28 in NIH3T3 cells was shown to convert them to a spheroid shape under low serum conditions (23), suggesting that CRHSP-28 modulates cytoskeletal elements related to epithelial cell shape and function. In addition, CRHSP-28 shares some limited homology throughout its coiled-coil region with cytoskeleton-related proteins including myosin heavy chain and CLIP-170/arrestin (41). In particular, CRHSP-28 is ~30% identical and ~45% similar to the Drosophila cytoplasmic linker protein, CLIP-190 (41) over a 107-amino acid region. CLIP-190 was reported to coordinate vesicle interactions between microtubule and actin filaments by binding to both intact microtubules and the class VI unconventional myosin protein. In preliminary experiments we have been unable to demonstrate CRHSP-28 binding to taxol-stabilized microtubules in vitro. Further, the microtubule binding motif in CLIP-190 is absent in CRHSP-28. A potential interaction between CRHSP-28 and non-muscle myosin is currently being investigated.

Contrasting a potential association of CRHSP-28 with actin filaments in acini, we reported previously that although the protein is localized around ZGs in the apical cytoplasm, it was absent in the subapical actin web (20). Further, little or no CRHSP-28 was detected in actin-rich detergent-insoluble fractions after cell lysis, suggesting, at best, a weak association with intact filaments. One possibility is that under conditions of basal Ca\(^{2+}\), CRHSP-28 is associated with ZGs via an interaction with pp70. Upon an increase in cell Ca\(^{2+}\), CRHSP-28 dissociates from this complex promoting the translocation of the ZG-bound pp70 to actin filaments. This would explain the Ca\(^{2+}\)-enhanced leakage of CRHSP-28 from SLO-permeabilized acini and Ca\(^{2+}\)-sensitive translocation of pp70 to cytoskeleton-rich detergent-insoluble fractions. The importance of Ca\(^{2+}\) in coordinating these events may entail the acute phosphorylation of CRHSP-28, which occurs on serine residues within seconds of acinar cell stimulation (19). Parente et al. (25) reported that CRHSP-28 is a substrate for the multifunctional Ca\(^{2+}\)/calmodulin-dependent protein kinase II in vitro. Interestingly, CaM kinase II was recently localized to the subapical region of acinar cells by immunofluorescence microscopy (42), indicating that the kinase is positioned precisely at the site of ZG entry into the terminal web.

Collectively, the (a) Ca\(^{2+}\)-dependent secretory effects of rCRHSP-28 on digestive enzyme secretion, (b) Ca\(^{2+}\)-sensitive translocation of the CRHSP-28-binding proteins to detergent-insoluble fractions of cell lysates, (c) localization of pp70 to ZG membranes, and (d) acute Ca\(^{2+}\)-regulated phosphorylation of CRHSP-28 clearly implicate this protein as a major regulatory factor in the acinar cell secretory pathway. Identification of the molecular identity of pp70 and characterization of its interactions with CRHSP-28 will likely provide key insight into the biochemical mechanisms by which these molecules regulate ZG exocytosis.

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