Immunolocalization of CRHSP28 in exocrine digestive glands and gastrointestinal tissues of the rat

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Groblewski, Guy E., Mutsumi Yoshida, Hongren Yao, John A. Williams, and Stephen A. Ernst. Immunolocalization of CRHSP28 in exocrine digestive glands and gastrointestinal tissues of the rat. Am. J. Physiol. Liver Physiol. 39: G219–G226, 1999.—The 28-kDa (on SDS-PAGE) Ca2+-regulated heat stable protein (CRHSP28) was recently purified as a novel phosphoprotein in exocrine pancreas, since it undergoes an immediate increase in serine phosphorylation when acini are stimulated with Ca2+-mobilizing agonists. Examination of CRHSP28 protein expression in rat revealed that most was highly expressed in pancreas and other morphologically related exocrine tissues, including the parotid, lacrimal, and submandibular glands. Immunofluorescence staining in pancreas indicated that CRHSP28 was specifically concentrated in zymogen granule-rich areas in the apical cytoplasm of acinar cells. Lack of colocalization with pancreatic lipase in dual immunofluorescence studies confirmed localization of CRHSP28 to the area immediately surrounding the granules. Western analysis of pancreatic zymogen granule membrane proteins indicated CRHSP28 was not associated with the granules following their purification. A similar pattern of apical cytoplasmic secretory granule staining was noted in lacrimal and submandibular glands. CRHSP28 protein was also expressed at relatively high levels in mucosal epithelial cells of the stomach and small intestine. CRHSP28 was found in the supra-nuclear apical cytoplasm of cells lining the small intestinal crypts, including Paneth cells, and was abundant in the cytoplasm of goblet cells. In the stomach, strong CRHSP28 staining was seen in mucus-secreting cells in the upper portion of the gastric glands and in the apical, granule-rich cytoplasm of chief cells located in the lower portions of the glands. Dual labeling with anti-\(^{3}H\)-\(^{3}K\)-ATPase demonstrated a comparatively lower expression of CRHSP28 in parietal cells. Collectively, the high relative expression of CRHSP28 in various secretory cell types within the digestive system, together with its intracellular localization surrounding the acinar cell secretory granules, strongly supports a role for CRHSP28 in Ca\(^{2+}\)-mediated exocrine secretion.

D52; N8; calcium; phosphoprotein; pancreas; 28-kDa calcium-regulated heat stable protein

A ROLE FOR CALCIUM as an intracellular messenger in pancreatic acinar cell function is well established (reviewed in Ref. 20). However, few of the molecular mechanisms by which this ion exerts its effects on digestive enzyme secretion have been elucidated. Recently, one of the most prominent Ca\(^{2+}\)-regulated phosphoproteins in acini, CRHSP28 (calcium-regulated heat stable protein with a molecular mass of 28 kDa on SDS-PAGE), was purified from rat pancreas and its primary sequence was determined (12). This acidic phosphoprotein was described over a decade ago in mouse and guinea pig (3) and later in rat acini (21) as a predominantly cytosolic, heat stable protein that is markedly phosphorylated by treatment with physiological concentrations of Ca\(^{2+}\)-mobilizing secretagogues such as CCK, ACh, and bombesin. With the use of the same approach of \(^{32}P\) metabolic labeling and two-dimensional electrophoresis, a phosphoprotein with similar characteristics was also reported in isolated rabbit parietal and chief cells (2). Indeed, this homologous protein was recently purified and cloned from rabbit gastric mucosa as a Ca\(^{2+}\)-sensitive phosphoprotein with a molecular mass of 28 kDa on SDS-PAGE (CSPP28) (15).

The nucleotide sequence encoding CRHSP28 was first reported by Byrne et al. (7) as a cDNA, termed D52, that is overexpressed in human breast carcinomas. Later, Chen et al. (8) described an essentially identical cDNA in lung and colon carcinomas, designated N8, as well as a second clone, NL8, encoding a protein with a 64-amino acid \(^{N}H_{2}\)-terminal extension (8). More recently, Byrne et al. cloned two additional D52 homologous, D53 (5) and D54 (6), both of which are potentially expressed as multiple protein isoforms due to alternatively spliced variations in their nucleotide sequences. These studies established that these D52-like sequences belong to a novel gene/protein family. Finally, Proux et al. (16) identified an avian homologue, R10, that is 87% identical to D52 within a 121-amino acid span. In general, these clones code for novel homologous proteins whose mRNAs are differentially expressed in various cell types; furthermore, their expression may be highly induced on growth transformation.

CRHSP28 is a relatively small 184-amino acid molecule that bears little identity to any known proteins. In rat acini, CRHSP28 undergoes an immediate increase in phosphorylation on serine residues when agonist stimulated (12). Consistent with this Ca\(^{2+}\) sensitivity, the rabbit protein was shown to be a substrate for the multifunctional Ca\(^{2+}\)/calmodulin-dependent protein kinase II in vitro (15). In the initial characterization of CRHSP28, Western analysis using a low-affinity antibody prepared against gel-purified protein showed that CRHSP28 was highly expressed in pancreas and throughout the gastrointestinal mucosa.

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MATERIALS AND METHODS

Materials. Soybean trypsin inhibitor, benzamidine, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma (St. Louis, MO); leupeptin and pepstatin were from Boehringer Mannheim (Indianapolis, IN); [32P]orthophosphate (9,000 Ci/mmol) was from NEN (Boston, MA); protein A beads were from Pierce (Rockford, IL); and CCK octapeptide was from Research Plus (Bayonne, NJ). Peroxidase-conjugated goat anti-guinea pig IgG antibody was from Chemicon (Temecula, CA); peroxidase-conjugated donkey anti-rabbit IgG was from Amersham (Arlington Heights, IL); and the Affigel-15 resin and protein determination reagent were from Bio-Rad (Hercules, CA). The FITC-conjugated anti-rabbit and Cy3-conjugated anti-mouse antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). The anti-human pancreatic lipase monoclonal antibody was from Chemicon (San Leandro, CA). The anti-h+K+-ATPase monoclonal antibody 12-18 (17) was a gift from Dr. A. Smolka. The pGEX-KG expression vector was a gift from Dr. J. Dixon. Clone 132820 was a gift from the WashU-Merk EST Consortium (Lawrence Livermore National Laboratory). Pocono Rabbit Farms & Laboratory (Canadensis, PA) conducted the antibody production.

Antibody production and purification. The entire 552-nt CRHSP28 coding sequence was expressed as a glutathione-S-transferase (GST) fusion protein in bacteria and purified by glutathione affinity chromatography as previously described (12). Purified GST-CRHSP28 was cleaved with thrombin to obtain the CRHSP28 portion of the protein, which was used as an antigen. Female New Zealand White rabbits received 200 µg of antigen in complete Freund’s adjuvant by intradermal injection. Animals were injected again 28 and 42 days later with 100 and 50 µg, respectively, of antigen in incomplete Freund’s adjuvant. High antibody titers were produced following 3–6 weekly bleeds. All methods were carried out in accordance with Institutional Animal Care and Use Committee guidelines. CRHSP28 antibodies were purified from rabbit serum using an affinity column that was produced by covalently binding GST-CRHSP28 to an Affi-gel support resin (Bio-Rad). Alternatively, in a second rabbit, GST-CRHSP28 was used as an antigen and CRHSP28-specific antibodies were isolated by first removing the anti-GST antibodies using a GST affinity resin, followed by affinity purification using a CRHSP28 affinity resin that did not contain the GST portion of the protein. Similar results were obtained using both antibodies.

Isolation of rat acini. Pancreatic acinar cells were isolated from adult male Sprague-Dawley rats as previously described (4, 21). Acini were suspended in a buffer consisting of (in mM) 10 HEPES, 137 NaCl, 4.7 KCl, 0.56 MgCl₂, 1.28 CaCl₂, 0.6 Na₂HPO₄, 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 BSA, gassed with 100% O₂, and adjusted to pH 7.4. For ³²P metabolic labeling studies, acinar cells were incubated in the same buffer without Na₂HPO₄ and containing 0.3 mCi/ml [³²P]orthophosphate for 2h before experiments were initiated.

Tissue preparation. Organs (pancreas, parotid, lacrimal, and submandibular glands, liver, heart, lung, kidney, spleen, testis, and brain) were dissected from rats and placed in ice-cold isotonic saline. Stomach and small and large intestinal mucosa were separated from the underlying smooth muscle by scraping the tissues with a glass microscope slide. Tissues were suspended in 5 vol of lysis buffer containing 50 mM Tris (pH 7.4), 5 mM EDTA, 25 mM NaF, 10 mM pyrophosphate, 1% Triton X-100, 1 mM benzamidine, 0.5 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin, and 0.01 mg/ml soybean trypsin inhibitor and homogenized with a Polytron homogenizer. Homogenates were centrifuged at 150,000 × g for 15 min. Protein concentrations of the resulting supernatants were determined (1), and lysates were dissolved in 4× concentrated SDS sample buffer, creating a final concentration of 62.5 mM Tris·HCl, pH 6.8, 2% (wt/vol) SDS, 5% (vol/vol) 2-mercaptoethanol, and 10% (vol/vol) glycerol and then boiled for 4 min.

Acinar cell soluble and particulate fractions were prepared by sonicating acini in lysis buffer with the Triton X-100 omitted. After centrifugation at 100,000 g for 30 min, the soluble fraction was removed and the particulate fraction was again sonicated in the same buffer containing 0.2% Triton X-100. Whole cell lysates were obtained by directly sonicating cells in lysis buffer containing 0.2% Triton X-100. Protein concentrations were determined (1), and samples were prepared for SDS-PAGE. A heat stable extract of acinar cell-soluble proteins was prepared as previously described (12). Immunoprecipitations were conducted using the above lysis buffer containing, in addition, 150 mM NaCl and 10 mM β-glycerophosphate as previously described (11). Two-dimensional gel electrophoresis was performed as previously described (21).

Western blotting. Western analysis was performed as previously described (12). Affinity-purified CRHSP28 antibodies were used at a concentration of 1 µg/ml. Immunoreactive proteins were detected using a donkey anti-rabbit IgG horse-radish peroxidase-conjugated secondary antibody (1:5,000). The protein A-purified anti-rat CRHSP28 antibody (12) was used at a concentration 1 µg/ml and detected using a horseradish peroxidase-conjugated goat anti-guinea pig secondary antibody (1:10,000).

Purification of parietal and chief cells from canine gastric mucosa. Chief and parietal cell fractions were purified from canine gastric mucosa as previously described (18, 19). This procedure entailed isolation of a crude fraction of gastric epithelial cells by exposure to collagenase and EDTA and then further purification by elutriation centrifugation and Percoll gradient centrifugation. Whole cell lysates were prepared for SDS-PAGE as described above.

Immunofluorescence studies. Rat pancreas, submandibular and lacrimal glands, stomach, and small intestine were fixed on ice for 2h in PBS containing either 4% formaldehyde, prepared from paraformaldehyde, or 2% formaldehyde plus 0.25% glutaraldehyde. Subsequently, tissue blocks were rinsed in PBS, cryoprotected in sucrose, and frozen in a mixture of...
20% sucrose and Tissue-Tek OCT embedding medium (Miles, Elkhart, IN) as described previously (14). Procedures for immunofluorescence microscopy with 5-µm-thick cryostat sections were as described previously in detail (10, 14). Primary and secondary antibodies were diluted in PBS containing 2% normal goat serum and 0.2% Triton X-100. Affinity-purified rabbit polyclonal antibodies to CRHSP28 were used at dilutions ranging from 0.5 to 10 µg/ml. In some experiments with pancreas, sections were double labeled with a mixture of anti-CRHSP28 and monoclonal antibody to human pancreatic lipase (diluted 1:200) and then exposed to a corresponding mixture of anti-rabbit and anti-mouse secondary antibodies labeled with FITC and Cy3, respectively. Similar double-labeling studies were carried out with sections of stomach using CRHSP28 antibody and a monoclonal antibody to H⁺-K⁺-ATPase (diluted 1:500). After antibody incubations and rinsing, sections were mounted under coverslips with a 3:1 mixture of glycerol and PBS containing 4 mg/ml p-phenylenediamine and viewed by conventional epifluorescence microscopy with a Leitz Aristoplan microscope (Leica, San Jose, CA) and laser scanning confocal fluorescence microscopy (Noran OZ, Middleton, WI). Images from 35-mm film were digitized and processed similarly to the confocal images using Photoshop 3.0 software (Adobe, Mountain View, CA).

RESULTS

Western analysis of CRHSP28 in rat pancreas. Polyclonal antiserum was produced in rabbits using recombinant human CRHSP28 protein as an antigen, and antibodies were purified by affinity chromatography. Western analysis of a lysate from rat acini indicated that the antibodies strongly reacted with a 28-kDa protein (Fig. 1A, lane 1). Immunoreactivity was predominantly associated with the soluble fraction following a 100,000 g centrifugation of a lysate prepared in the absence of detergent (lane 2) and was also detected at significant levels in the particulate fraction (lane 3). Treatment of acini with CCK had no effect on the relative distribution of the 28-kDa band between these fractions (not shown). Although the 28-kDa protein was partially associated with the particulate fraction, no immunoreactivity was detected in either purified zymogen granules, prepared by Percoll density gradient centrifugation (14), or purified granule membranes (lanes 4 and 5). Furthermore, no signal was seen in the zymogen granule content or rat pancreatic juice collected from the pancreatic duct (data not shown). Consistent with purified rat CRHSP28 (12), the 28-kDa signal was markedly enhanced in acinar cell heat stable extracts (lane 6).

CRHSP28 antibodies were also used to immunoprecipitate the protein from rat acinar cell lysates (Fig. 1B). After immunoprecipitation, CRHSP28 was detected by Western analysis using polyclonal antibodies that were raised against purified rat pancreatic CRHSP28 protein (12) (lane 1). Alternatively, phosphorylated CRHSP28 was identified by autoradiography.

A

B

C

Fig. 1. Characterization of an anti-human 28-kDa (on SDS-PAGE) Ca²⁺-regulated heat stable protein (CRHSP28) antibody in rat pancreatic acini. A: acinar cell proteins (60 µg/lane) prepared from the indicated fractions were separated by SDS-PAGE. A smaller amount of heat stable material (10 µg) was analyzed because of the high intensity of the signal following partial purification of the protein. Proteins were analyzed by Western blotting using affinity-purified CRHSP28 antibody (1 µg/ml). zym, zymogen; zym. gran., zymogen granule. B: CRHSP28 was immunoprecipitated from acinar cell lysates using anti-human CRHSP28 antibody and then detected by Western blotting (lane 1) using an anti-rat CRHSP28 antibody (1 µg/ml). Arrow indicates the position of the IgG heavy chain. Alternatively, CRHSP28 was immunoprecipitated from 32P-labeled acinar cells and the phosphorylated protein was detected by autoradiography. Acini were treated as control (lane 2) or with 1 nM CCK for 2 min (lane 3). C: Western analysis following two-dimensional electrophoresis of proteins (300 µg/gel) prepared from control and CCK-stimulated (1 nM, 2 min) acini using the anti-human CRHSP28 antibody (1 µg/ml).
Figure 2. Relative amounts of CRHSP28 protein in whole tissue homogenates from rat. Proteins (50 µg/lane) from tissue homogenates prepared in the presence of 1% Triton X-100 were separated by SDS-PAGE and analyzed by Western blotting using anti-human CRHSP28 antibody (1 µg/ml).
tive epithelial cells in the villi were more poorly stained (Fig. 5, C and D). Of particular interest was the high-intensity staining noted in goblet cells throughout both crypt (Fig. 5, A and B) and villar epithelium (Fig. 5, C and D), where CRHSP28 was present in the cytoplasm at the base of the cells but was absent from the large mucus-containing granules of the apical region.
CRHSP28 distribution (green fluorescence) was also analyzed in sections of rat stomach following double labeling (Fig. 6) with the parietal cell marker, $\text{H}^+\text{-K}^+\text{-ATPase}$ (red fluorescence). CRHSP28 was highly expressed in the mucous cells of the gastric surface and pits (Fig. 6A). Similarly, intense staining was noted in the granule-rich region of chief cells at the base of gastric glands (Fig. 6C). In contrast, CRHSP28 was more weakly expressed in the parietal cell-rich midregion of the gastric glands, where it was restricted primarily to cells that were negative for $\text{H}^+\text{-K}^+\text{-ATPase}$ (Fig. 6B). However, some parietal cells exhibited small amounts of punctate staining (yellow in Fig. 6B), which likely represents a low level of CRHSP28 expression in these cells. The relative CRHSP28 content of chief and parietal cells was confirmed by Western blotting purified cell fractions from canine gastric mucosa (Fig. 7). In accordance with the immunofluorescence studies, CRHSP28 content was highly enriched in lysates from purified chief cells, whereas the protein was expressed at considerably lower levels in the purified parietal cell fractions.

**DISCUSSION**

In addition to the human D52 sequence, cDNAs from mouse (5) and rabbit (15) have been reported that code for proteins that are 86 and 95% identical to human CRHSP28, respectively. Consistent with these high homologies between species, polyclonal antibodies raised against full-length recombinant human CRHSP28 reacted with the protein on immunoblots from rat and canine tissues. Recent evidence indicating that the CRHSP28 protein likely belongs to a novel D52 gene/protein family (5, 6) makes it possible that other CRHSP28-like proteins may also be expressed in pancreas. However, the presence of a single immunoreactive molecule on the one- and two-dimensional gels suggests that the current antibodies did not cross-react with any related proteins. This result is not unexpected, as the D53 homologue codes for a protein that shares only ~52% identity with D52 at the amino acid level.

CRHSP28 contains a 49-amino acid region (Ala22-Leu71) displaying a leucine zipperlike repeat with a seven residue periodicity of apolar amino acids. Prediction analysis of the leucine zipper motif indicates a high probability for CRHSP28 to adopt a coiled-coil structure (5, 8). Coiled-coil domains are amphipathic $\alpha$-helical regions that promote protein-protein interactions (13). Using the yeast two-hybrid system, Byrne et al. (6) recently demonstrated that the D52-like proteins were capable of forming both homo- and heteromeric interactions when expressed in yeast. These associations were further confirmed using GST fusion protein pull-down assays with in vitro-translated proteins. As predicted, formation of these protein complexes depended on the presence of an intact coiled-coil motif, as removal of this region abolished protein binding (6). Similar results were reported for the N8 protein (9) and the avian neuroretina R10 protein (16). The physiological significance of these coiled-coil interactions and the influence that serine phosphorylation may have on this process remain to be elucidated.

Hydropathy profile analysis of CRHSP28 predicts an abundantly hydrophilic protein exhibiting a high degree of surface charge. These predictions are in agreement with the solubility of CRHSP28 following heat and acid treatment of acinar lysates (12) as well as its predominant association with the soluble fraction following cell lysis in the absence of detergent. Contrasting this apparently high solubility was the immunolocalization of CRHSP28 to the secretory granule region of acinar cells. These data, as well as the presence of small amounts of CRHSP28 in acinar cell particulate fractions, imply that the protein is likely to be associated with cell membranes, other integral membrane
proteins, or cytoskeletal components within cells. The absence of a signal with Western blotting of purified zymogen granule fractions suggests that CRHSP28 is at best loosely attached to the granule membrane. The primary structure of human, rabbit, and mouse CRHSP28 contains no cystine residues for potential isoprenylation or an NH₂-terminal glycine residue to support a myristylation of the protein. Although it is possible that the fourth amino acid, glycine, could be myristylated following posttranslational processing of the NH₂ terminus, the protein’s heat and acid stability, coiled-coil motif, and discrete cytoplasmic localization and the fact that it is highly expressed following growth transformation of cells suggest CRHSP28 is involved in modulating cytoskeletal dynamics.

Chen et al. (9) previously postulated an epithelial cell-specific role for N8/D52, based primarily on findings that the N8 mRNA and protein are most highly expressed in cultured epithelial cell lines and that its ectopic expression in NIH/3T3 fibroblast cells converts them to a more spheroid shape. In the present study, Western analysis of CRHSP28 protein expression demonstrated that the protein is highly concentrated in the mucosal layer of the rat gastrointestinal tract and other exocrine glands. Closer examination using immunofluorescence microscopy fully supported these findings and further demonstrated an epithelial cellspecific expression of the protein with little or no CRHSP28 being present in other support tissues. Consistent with its characterization (2) and purification (15) from rabbit gastric gland mucosa, CRHSP28 was present in both chief and parietal cell types from rat and canine stomach, although expression at the protein level, as judged by immunofluorescence and Western blotting, was much more prominent in chief cells. With the exception of gastric mucus-secreting cells (where CRHSP28 was abundant throughout the entire cytoplasm) and goblet cells (where staining was distributed basally), the protein was consistently concentrated within the apical regions of polarized epithelia involved in regulated protein secretion. This latter distribution included pancreatic acinar cells, salivary gland cells, chief cells, and Paneth cells. Together with the observation that CRHSP28 is discretely localized to the area immediately surrounding secretory granules in acinar cell types such as pancreas, these results not only imply a specialized role for CRHSP28 in epithelial cells but further support the hypothesis that it functions in Ca²⁺-stimulated zymogen secretion.

Fig. 6. Localization of CRHSP28 in stomach. Confocal fluorescence stainings of gastric mucosa double labeled with CRHSP28 (green fluorescence) and a monoclonal antibody to the parietal cell marker, H⁺-K⁺-ATPase (red), are shown. CRHSP28 is present in a diffuse staining pattern in the cytoplasm of mucous cells lining the gastric surface (arrows in A and B) and pits (asterisks in A) in the upper region of the mucosa (A) but is poorly expressed in the parietal cell-rich midportion of the gastric glands (B). At the base of the gastric glands (C), near the muscularis mucosa (mm), CRHSP28 is concentrated in the supranuclear and subapical regions of chief cells (arrows in C). Expression of CRHSP28 in parietal cells (arrowheads; stained red in A–C) is difficult to resolve above background levels of FITC fluorescence; some of the punctate yellow staining in parietal cell cytoplasm in B may represent low expression of CRHSP28. Scale bar = 20 µm.

Fig. 7. CRHSP28 is highly expressed in purified chief cell fractions of canine gastric mucosa. Parietal cells and chief cells were purified from canine gastric mucosa by elutriation centrifugation followed by Percoll density gradient centrifugation. Proteins (50 µg/lane) from whole cell lysates were separated by SDS-PAGE and Western blotted using anti-human CRHSP28 antibody (1 µg/ml).
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