This study reports the purification and identification of a novel 28 kDa phosphoprotein from rat pancreatic acini, previously described as being highly regulated by calcium-mobilizing secretagogues, which we have designated calcium-regulated heat-stable protein 28 (CRHSP-28). Internal amino acid sequences of purified CRHSP-28 were obtained following trypsin digestion and found to match with >95% identity the predicted amino acid sequence of a novel cDNA recently identified as being highly expressed in human breast carcinomas. Verification that this cDNA codes for human CRHSP-28 was demonstrated by the ability of antisera raised against purified rat CRHSP-28 to recognize the recombinant human protein when expressed in bacteria. Furthermore, this antibody was found to specifically react with CRHSP-28 in rat acini following one- and two-dimensional electrophoresis and underwent a marked acidic shift in mobility after cholecystokinin stimulation, a phenomenon indicative of an increase in its phosphorylation. CRHSP-28 is predicted to be extremely hydrophilic, is phosphorylated entirely on serine residues, and bears little homology to any known proteins. Finally, the distribution of the CRHSP-28 protein in various rat tissues revealed that although it was present at low levels in almost all tissues, it was most highly expressed in pancreas, followed by the gastric, intestinal, and colonic mucosa. In view of its relative abundance throughout the digestive system and its apparent regulation by calcium-mobilizing agents, this protein may provide valuable insight into the mechanism(s) of calcium signaling in these tissues.

Stimulation of pancreatic acinar cells with calcium-mobilizing secretagogues, such as cholecystokinin (CCK) or acetylcholine, regulates a number of diverse cellular functions, including digestive enzyme secretion, growth, and metabolism. Intracellularly, calcium regulates a number of divergent cellular functions, including calcium-regulated heat-stable protein with a molecular mass of 28 kDa; calcium signaling in these tissues.

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mobilizing secretagogues, as well as JMV-180, a high affinity CCK receptor agonist (10). Heat stability refers to the finding that it remains soluble when heating acinar cell lysates to 90 °C, a property that has greatly facilitated its purification. Given that the amino acid sequence of CRHSP-28 is novel, having little or no homology to any other known proteins, and also that it is most highly expressed in the pancreas in comparison with other tissues, an elucidation of its function may provide valuable insights into the mechanism(s) by which CCK regulates acinar cell function.

EXPERIMENTAL PROCEDURES

Materials—Isofluror-β-thiogalactoside, phosphoamino acids, soybean trypsin inhibitor, reduced glutathione, benzamidine, phenylmethylsulfonyl fluoride, thrombin, and ninyhdrin were purchased from Sigma. Glutathione-agarose and a Mono-Q column were purchased from Pharmacia Biotech Inc.; phosphocellulose thin layer plates were from Eastman Kodak Co.; Protoblot membrane was from Applied Biosystems (Foster City, CA); leupeptin and pepstatin were from Boehringer Mannheim (Indianapolis, IN); 32P orthophosphate (9000 Ci/mmol) was from DuPont NEN; peroxidase-conjugated goat antiguinea pig IgG antibody was from Chemicon (Temecula, CA); Affi-Gel 15 resin was from Bio-Rad; and Centricon 10 and Centricon 100 tubes were from Amicon (Beverly, MA). Frozen rat pancreases used for purification were obtained from Harlan Bioproducts (Indianapolis, IN). CCK octapeptide was a gift from Sellocon Corporation. Expression vector was a gift from Dr. J. E. Dixon (University of Michigan). Clone 132820 was a gift from the Washington Squibb. The pGEX-KG expression vector was a gift from Dr. J. E. Dixon (Lawrence Livermore National Laboratory). P. aurophilus.

Production of Anti-rat CRHSP-28 Antiserum—Rat pancreatic CRHSP-28 was purified from several purification runs (see below) and visualized by Coomassie Brilliant Blue staining following two-dimensional electrophoresis. The protein was excised from multiple gels, ground, partially solubilized in Freund’s adjuvant, and injected subcutaneously into a guinea pig. Antibody production was conducted at the Pocono Rabbit Farm and Laboratory Inc. (Canadensis, PA).

Isolation of Rat Acini—Pancreatic acinar cells were isolated from adult male Sprague Dawley rats (150–200 g) by collagenase digestion and as described previously (11). Acini were suspended in Hepes Ringer buffer (10 mM Hepes, 137 mM NaCl, 4.7 mM KCl, 0.56 mM MgCl2, 1.2 mM CaCl2, 5.5 mM t-glucose, and 2 mM L-glutamine) and an essential amino acid solution. 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. Acinar cells were incubated in Hepes Ringer buffer containing 0.3 M NaCl (32P) for 2 h prior to initiating experiments.

Phosphorylation Studies—To measure the time course of CRHSP-28 phosphorylation, 32P-labeled acinar cells were treated with 1 nM CCK, which activates CRHSP-28 with a latency that precedes heat stability before homogenization. All homogenates were kept on ice for 1 h and centrifuged at 150,000 × g for 1 h, and heat-stable protein was made from the soluble fraction. Total protein was measured using a Bio-Rad assay, and samples were diluted and boiled in SDS sample buffer containing a final concentration of 62.5 mM Tris-RCI, pH 6.8, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, and 10% (v/v) glycerol.

Heat-stable protein from 32P-labeled acinar cells that had been treated with 1 mM CCK for 5 min was separated by 2D-PAGE, transferred to a polyvinylidene difluoride membrane, and autoradiographed. The area of the membrane corresponding to both CRHSP-28-phosphorylated isoforms was excised with a razor, washed once with 100% methanol and then with 3 times with H2O, and placed in 1 mL of 0.1 M glycine, pH 2.3, and 500 μL of 6 M HCl was hydrolyzed at 110 °C for 90 min in a vacuum oven. Following extensive vortexing, the sample was centrifuged for 5 min, and the membrane was discarded. The sample was lyophilized and washed with H2O three times to remove residual acid and then suspended in 5 μL of H2O containing 1 μg each of phosphoserine, phosphothreonine, and phosphotyrosine. The digest was spotted on a phosphocellulose plate, and thin-layer phosphorin analysis was run at 20 mA for 45 min in an H2O:CH2Cl2:ACN (9:1:1) running buffer consisting of (v/v) 5.9% acetic acid, 0.8% formic acid (88%), 0.3% pyridine, and 0.3 mM EDTA, as described previously (12). Standards were visualized by ninhydrin staining, whereas 32P-labeled amino acids were detected by autoradiography.

Purification of CRHSP-28—Pancreases from 100 Sprague Dawley rats were homogenized, frozen in liquid nitrogen, and suspended at 24 h at ~40 °C before use. Frozen pancreases were homogenized in 5 volume of lysis buffer using a Polytron homogenizer and allowed to sit on ice for 30 min. Following a 150,000 × g centrifugation for 1 h, heat-stable protein was prepared from the soluble fraction as described above, yielding approximately 125 mg of total protein. To identify CRHSP-28 throughout the purification, 32P-labeled heat-stable protein prepared from acinar cells of two rat pancreases was added to the unlabeled heat-stable material. The combined extracts were adjusted with 100% trichloroacetic acid to a final concentration of 0.25% (w/v) and kept on ice for 1 h, and the precipitated material was removed by centrifugation at 3,000 × g for 20 min. The soluble fraction was then further adjusted to 2.5% trichloroacetic acid for 1 h. The precipitated protein (approximately 20 mg total) containing CRHSP-28 was recovered by a second centrifugation. To remove residual trichloroacetic acid, the pellet was washed in 100% ethanol at 4 °C and then suspended in approximately 10 mL of 20 mM Tris buffer, pH 7.8, creating a final pH of 6.8 at room temperature. Following filtration through a 2-μm syringe filter, the extract was loaded on a Mono-Q anion exchange column equilibrated in 20 mM Tris, pH 7.8, using a Pharmacia fast protein liquid chromatographic apparatus and then eluted with a linear 0–200 mM NaCl gradient at a flow rate of 0.5 ml/min. CRHSP-28 was recovered in the 160–200 mM NaCl fraction (containing approximately 2 mg of total protein), concentrated in a Centricon 10 concentrating tube, and analyzed by 2D-PAGE (0.3 mg protein/gel). Proteins were transferred to a polyvinylidene difluoride membrane, stained with 0.1% Coomassie Brilliant Blue in 40% methanol and 1% acetic acid, destained in 50% methanol, washed extensively with water, and autoradiographed. CRHSP-28, identified by Coomassie Brilliant Blue staining and autoradiography, was excised from the membrane with a razor and submitted to the Michigan State University Protein Sequencing Facility for tryptic peptide amino acid sequencing. The protein was digested with trypsin as described (13), and peptides were separated by reversed phase HPLC using an LC Packings 0.8 × 250-mm C18 column equilibrated in 0.1% trifluoroacetic acid and a linear gradient (0–70% in 135 min) of acetonitrile at a flow of 0.1 mL/min. Acidic amino acid sequencing of peptides was conducted using an Applied Biosystems 494 automated protein sequencer.

CRHSP-28 Fusion Protein—A 1,172-base pair cDNA subclone (clone 132820) from a human placenta cDNA library was obtained from the Washington University-Merck EST project. The first 600 base pairs of this subclone were sequenced by conventional techniques and contained the entire 552-base pair predicted open reading frame coding for CRHSP-28. To obtain a glutathione S-transferase (GST) fusion protein of CRHSP-28, plasmids corresponding to both the 5′- and 3′-ends of the coding sequence were designed (5′-GGGCTGGAGC-CATGG-3′ and 5′-GCTGGAGGAAACTCAGAGG-3′) containing restriction sites to NcoI and XhoI, respectively. These primers were used to amplify the CRHSP-28 coding sequence from subclone 132820 by a polymerase chain reaction, and the resulting 576-base pair polymerase chain reaction product was extracted from an agarose gel and

Phosphoamino Acid Analysis—To isolate gastrointestinal proteins, the entire stomach and 15 cm of proximal small and large intestine were removed, opened longitudinally, cleared of luminal contents, washed with 9.9% NaCl, and scraped with a spatula before homogenization. All homogenates were kept on ice for 1 h and centrifuged at 150,000 × g for 1 h, and heat-stable protein was made from the soluble fraction. Total protein was measured using a Bio-Rad assay, and samples were diluted and boiled in SDS sample buffer containing a final concentration of 62.5 mM Tris-RCI, pH 6.8, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, and 10% (v/v) glycerol.
subcloned into bacterial expression vector pGEX-KG (14) using conventional techniques. Nucleotide sequencing was performed to verify that the coding sequences for GST and subclone 132820 were in frame. Fusion protein was produced in BL21/D3 Escherichia coli by induction with 0.2 mM isopropyl-β-D-thiogalactoside for 4 h at room temperature. Cells were pelleted, suspended in PBS (150 mM NaCl, 16 mM Na₂HPO₄, and 4 mM NaH₂PO₄) containing 1% Triton X-100, 2 mM EDTA, 0.1% 2-mercaptoethanol, 5 mM benzamidine, and 0.2 mM phenylmethylsulfonyl fluoride and lysed using a French press. The GST-CRHSP-28 fusion protein was purified by incubating the lysate with glutathione-agarose beads for 30 min at room temperature, followed by a series of washes in PBS and elution in a 50 mM Tris-HCl, pH 8.0, buffer containing 10 mM reduced glutathione. Thrombin cleavage of CRHSP-28 was performed by incubating the glutathione-agarose-immobilized fusion protein with 500 units of thrombin in 0.5 ml of PBS for 2 h at room temperature. Protein concentrations were determined using a Bio-Rad assay, and samples were boiled in SDS sample buffer for use in SDS-PAGE.

**Western Blotting**—Protein samples were separated on 12% polyacrylamide gels and then transferred to a nitrocellulose membrane overnight at 30 V in a transfer buffer containing 24 mM Tris base, 0.2 M glycine, 0.1% SDS, and 20% methanol. Membranes were blocked in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.3% Tween 20, and 10% nonfat dry milk for 1 h at room temperature, incubated with anti-rat CRHSP-28 antiserum (1:1,500) for 1 h at room temperature, and visualized by enhanced chemiluminescence using a goat anti-guinea pig IgG horseradish peroxidase-conjugated secondary antibody (1:10,000). To preabsorb enhanced chemiluminescence using a goat anti-guinea pig IgG horseradish peroxidase-conjugated secondary antibody (1:10,000), affinity-purified antibody was used at a concentration of 1:1,500 final dilution of the antiserum. This mixture was incubated for 30 min at room temperature and then used directly to probe the membrane as described above. As a control, antiserum was incubated under identical conditions in the absence of the GST-CRHSP-28 fusion protein. Antibodies to GST-CRHSP-28 were also purified using a GST-CRHSP-28 affinity column produced by covalently immobilizing the fusion protein to an Affi-Gel 15 resin. Briefly, the crude serum was diluted 1:10 with PBS and then passed over the column three times at 4 °C. The column was washed extensively with PBS, and antibodies were eluted using 100 mM citrate, pH 2.5, and immediately neutralized by dilution in 1 x Tris, pH 8.5. Antibodies were washed and concentrated in PBS using Centricon 30 tubes. Affinity-purified antibody was used at a concentration of approximately 1 μg/ml for probing Western blots.

**RESULTS**

**CRHSP-28 Phosphorylation in Acini**—Using ³²P labeling of isolated acinar cells and 2D-PAGE, CRHSP-28 was previously shown to be phosphorylated in response to concentrations of CCK as low as 10 pM, with a maximal increase occurring at 1 nM following 5 min of incubation with the agonist (10). Using this same technique, a time course of CRHSP-28 phosphorylation in response to 1 nM CCK revealed the protein to undergo a maximal 2-fold increase in phosphate content as early as 30 s after the addition of agonist (Fig. 1). Phosphorylation remained elevated for 10 min and then declined to basal levels by 30 min in the continued presence of CCK.

Previous work had indicated that phosphorylated CRHSP-28 was found predominantly in the soluble fraction following a 100,000 × g centrifugation, and that the protein remained soluble after heating acinar cell lysates to 90 °C for 7 min (10). Under these conditions, approximately 95% of the total cell protein is precipitated, greatly enriching the CRHSP-28 content of the lysates. In the heat-stable protein of control cells, ³²P-labeled CRHSP-28 was present as a single isoform (pI 5.4) of relatively low intensity (Fig. 2). On stimulation with CCK, there was a large increase in the phosphate content of this isoform, as well as the appearance of a second, more acidic phosphorylated form (pI 5.2). An identical pattern of phosphorylation was seen in both whole cell (10) and cytosolic lysates (data not shown) of acinar cells, indicating that heat treatment does not effect the mobility of this protein on 2D gels. Thus, using this method to enhance the CRHSP-28 signal on two-dimensional autoradiographs, a phosphoamino acid analysis of CRHSP-28 was performed following treatment of acini with 1 nM CCK for 5 min (Fig. 2). Thin layer electrophoresis of acid-hydrolyzed amino acids from CRHSP-28 demonstrated the protein to be phosphorylated entirely on serine residues. The lack of phosphoformosine was confirmed by Western blotting with antiphosphoformosine antibodies (data not shown).

**Purification and Identification of CRHSP-28**—The thermostability of CRHSP-28 was exploited to purify it from batches of 100 rat pancreases in a procedure involving heat and trichloroacetic acid precipitation, anion exchange chromatography, and 2D-PAGE. When identified by Coomassie Brilliant Blue staining, purified CRHSP-28 was found as three isoforms with pl values of 5.6, 5.4, and 5.2 (Fig. 3, A, B, and C, respectively). However, only the two more acidic isoforms were seen by autoradiography (Fig. 3, B and C), suggesting that the protein existed in a nonphosphorylated and two phosphorylated states. Coomassie Brilliant Blue staining indicated that CRHSP-28 was present predominantly in the nonphosphorylated state, a result consistent with the protein being purified from unstimulated pancreases. Internal amino acid sequence was obtained using CCK as low as 10 pM, with a maximal increase occurring at 1 nM CCK for 5 min. Arrows, position of CRHSP-28. Right, phosphoamino acid analysis of acid-hydrolyzed CRHSP-28 obtained from ³²P-labeled heat-stable protein following treatment of cells with 1 nM CCK for 5 min.
CRHSP-28 Purification in Exocrine Pancreas

**Fig. 3.** Purification of CRHSP-28 by two-dimensional electrophoresis. Top, portion of a Coomassie Brilliant Blue-stained gel (molecular mass, 20–40 kDa; pl, 4–6) indicating three isoforms of CRHSP-28 obtained following anion exchange chromatography and two-dimensional electrophoresis. Bottom, corresponding autoradiograph showing CRHSP-28-phosphorylated isoforms (B and C). A, non-phosphorylated form.

**Fig. 4.** Separation of CRHSP-28 peptides by HPLC following trypsin digestion. Purified CRHSP-28 was transferred to a polyvinylidenefluoride membrane and digested with trypsin, and the resulting peptides were resolved by HPLC. Microsequence analysis was obtained from peaks 18 (GEQDVATNAYKK), 19 (SFEERVENLK), 22 (ASAAPSSVGSVITK).

**Fig. 5.** Amino acid sequence of human CRHSP-28. The sequence is based on the open reading frame of a cDNA originally described by Byrne et al. (16), termed D52. Purified rat CRHSP-28 tryptic peptides are shown below the full-length sequence. Boxes, two amino acids that differ.

**Fig. 6.** Anti-rat CRHSP-28 antiserum recognizes recombinant human CRHSP-28 protein. Left, A glutathione S-transferase fusion protein of human CRHSP-28 was constructed in a pGEX-KG expression vector. Protein expression was induced in bacteria using 0.2 mM isopropyl-β-D-thiogalactoside. Bacterial cell lysates (20 µg/lane) were separated by 12% SDS-PAGE and stained with Coomassie Brilliant Blue. Lanes 1 and 2, bacteria transformed with the pGEX vector alone; lanes 3–6, bacteria transformed with the GST-CRHSP-28 construct; lane 5, GST-CRHSP-28 purified using a glutathione-agarose affinity resin; lane 6, purified, recombinant CRHSP-28 obtained following thrombin cleavage of the fusion protein. Right, Western analysis of same samples using anti-rat CRHSP-28 antiserum (1:1,500) generated in guinea pig by injection of pancreatic protein isolated following 2D-SDS-PAGE. Top arrow, presence of recombinant GST-CRHSP-28; bottom arrow, CRHSP-28 following thrombin cleavage.

**Table 1.** Amino acid sequence of human CRHSP-28. The sequence is based on the open reading frame of a cDNA originally described by Byrne et al. (16), termed D52. Purified rat CRHSP-28 tryptic peptides are shown below the full-length sequence. Boxes, two amino acids that differ.

CRHSP-28 Western Analysis in Rat Tissues—To investigate the ability of the anti-rat antiserum to recognize CRHSP-28 in the rat pancreas, heat-stable protein from acinar cell lysates was separated by SDS-PAGE and Western blotted (Fig. 7, left). Although the antiserum reacted with a number of proteins in the lysate, a very prominent band was seen at the expected size of 28 kDa. Specificity of the antiserum toward the 28-kDa protein was indicated by the absence of this band when probing with preimmune serum. Moreover, absorption of CRHSP-28-specific antibodies from the serum by a preincubation with an excess of purified GST-CRHSP-28 fusion protein, which was added to the Western blotting buffer, completely abolished the presence of the 28-kDa signal, but it had no effect on the other nonspecific proteins. Finally, Western blotted was also conducted using CRHSP-28-specific antibodies, which were purified from the crude serum using a GST-CRHSP-28 affinity column. Use of the affinity-purified antiserum resulted in a large decrease in the intensity of the nonspecific bands but maintained the CRHSP-28 signal. However, some of these nonspecific bands were still present, precluding the use of this antibody for immunohistochemical studies. Thus, these data further establish the specificity of this antiserum toward CRHSP-28 in the rat pancreas.
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Fig. 7. Western analysis of CRHSP-28 in acinar cells. Heat-stable protein from acinar cell cytosolic lysates was separated by one- or two-dimensional electrophoresis and Western blotted using anti-rat CRHSP-28 antiserum. Left, heat-stable protein (30 µg/lane) was probed using lane 1, preimmune serum (1:1,000); lane 2, immune serum (1:1,500), which was preincubated for 30 min with 60 µg of GST-CRHSP-28 protein before probing the blot; lane 3, immune serum (1:1,500); and lane 4, affinity-purified CRHSP-28 antibodies (1 µg/ml). Arrow, CRHSP-28-specific band. Right, Western analysis of heat-stable protein (200 µg/lane) from control or CCK-treated (1 nM, 5 min) acini following two-dimensional electrophoresis.

In agreement with the observation that CRHSP-28 exists in three isoforms on purification (Fig. 3), Western blotting following 2D-PAGE also indicated the presence of three forms of the protein of appropriate sizes and charges in lysates of unstimulated cells (Fig. 7, right). Furthermore, treatment of acinar cells with 1 nM CCK for 5 min stimulated a marked acidic shift in the protein. This was indicated by an almost complete loss in the intensity of the most alkaline, nonphosphorylated isofrom and corresponding increases in the intensity of to the two more acidic, phosphorylated isoforms. These data are consistent with an increase in CRHSP-28 phosphorylation in response to CCK.

To begin to elucidate a potential role for CRHSP-28 in exocrine pancreatic function, tissue distribution of the protein in various rat tissues, with a special emphasis on the gastrointestinal tract, was conducted (Fig. 8). Western blotting of heat-stable protein from whole cell homogenates indicated CRHSP-28 to be present in nearly all tissues tested. Strikingly, the highest level of expression was found in gastrointestinal tissues, in which it was most abundant in the pancreas, followed by the mucosa of the stomach and small and large intestines, respectively. Relatively high levels were also seen in the lacrimal gland, a tissue morphologically similar to the exocrine pancreas. Intermediate amounts of the protein were found in the spleen, kidney, testis, and maxillary gland; low levels were found in the brain, liver, and skeletal muscle; and no detectable signal was seen in the heart.

DISCUSSION

The present study describes the purification and identification of a novel pancreatic phosphoprotein, which we designate CRHSP-28. This protein was initially described by 32P-metabolic labeling studies in mouse and guinea pig (8) and more recently in rat acini (10) as a predominantly cytosolic, heat-stable protein that is markedly phosphorylated by calcium-mobilizing secretagogues. CRHSP-28 phosphorylation has been shown to be stimulated in response to picomolar concentrations of CCK and also following treatment of cells with the high affinity CCK receptor agonist JMV-180 (10). Phosphorylation of the protein appears to occur exclusively through a calcium-dependent mechanism, as maximal increases in phosphate content can be demonstrated by treatment of cells with the calcium ionophore A23187 (8, 10). Furthermore, it does not appear to be regulated via protein kinase A (10) or protein kinase C (8) activation. Based on its molecular mass, acidic pI value, and regulation by calcium, CRHSP-28 appears to be very similar to two other phosphoproteins previously described in gastrointestinal tissues using 2D-gel electrophoresis and 32P metabolic labeling of cells. In isolated rabbit parietal cells, Brown and Chew (17) demonstrated a calcium-regulated phosphoprotein of 28 kDa and pI of approximately 5.0, which was transiently phosphorylated in response to carbachol. This protein was also phosphorylated following treatment of cells with the calcium ionophore but was unaffected by phorbol esters (17). In addition, Cohn et al (18) also showed the presence of a cytosolic protein with a molecular mass of 29 kDa and acidic pI value, which was rapidly phosphorylated following treatment of colonic mucosal T84 cells with histamine or the calcium ionophore ionomycin. Further resembling CRHSP-28, this protein was unaffected by treatment of T84 cells with vasoactive intestinal peptide, an agent that exerts its effects through cAMP. At present it seems quite possible that these phosphoproteins are related to CRHSP-28, given the fact that we have found this protein to be highly expressed in mucosa of both the stomach and intestine, as well as the pancreas.

Internal amino acid sequence analysis of three tryptic peptides of purified CRHSP-28 revealed the protein to be highly homologous to the predicted amino acid sequence of a novel cDNA isolated from human breast carcinomas, designated D52 (16). Verification that D52 codes for the human CRHSP-28 protein was established by demonstrating the ability of anti-rat CRHSP-28 antibodies to specifically recognize the human recombinant protein. Human CRHSP-28 is composed of 184 amino acids and bears little or no homology to any known proteins. Its predicted molecular mass of 19.8 kDa is somewhat lower than its 28-kDa mobility on SDS-PAGE. This appears to be a common characteristic of heat-stable proteins, as other such proteins, such as PHAS-I (19), inhibitors I and II, DARPP-32 (20), and stathmin (21), all have been reported to undergo anomalous mobilities on denaturing gels. In addition, human CRHSP-28 has a calculated acidic pI value of 4.75, reasonably close to the measured pI of 5.6 seen by isoelectric focusing in 10 M urea gels. Finally, the molecule is predicted to be highly hydrophilic with very little hydrophobic character, consistent with its presence in cytosolic extracts by 32P labeling.

Because CRHSP-28 focuses as two 32P-labeled isoforms on isoelectric focusing gels, and this modification occurs entirely on serine, it seems likely the protein is phosphorylated on two separate serine residues following CCK treatment. Human CRHSP-28 contains 17 serine residues with consensus sites for phosphorylation by casein kinase II (Ser-31) and glycogen synthase kinase 3 (Ser-100, Ser-111, Ser-115 and Ser-131), neither of which are known to be directly regulated by a calcium-dependent mechanism. Interestingly, the transient time course of CRHSP-28 phosphorylation by CCK is similar to what has been demonstrated for calcium- and calmodulin-dependent protein kinase II activation in rat acini (22). Furthermore, although the molecule does not appear to possess an obvious calcium- and calmodulin-dependent protein kinase II consensus sequence (RXXS) (23), a full evaluation of the ability of the
enzyme to phosphorylate CRHSP-28 can only be made empirically. Clearly, more direct studies are necessary to establish what kinases and phosphatases are responsible for regulating CRHSP-28 phosphorylation in acini.

The D52 mRNA is 3.3 kilobases in length with an extensive 3'-untranslated region, containing multiple copies of an ATTGA motif, which are suggested to have an mRNA-stabilizing effect (16). Similar to the distribution of the CRHSP-28 protein in the rat, D52 transcripts were also reported to be differentially expressed in normal human tissues (16). Curiously, although the CRHSP-28 protein was most abundant in the rat gastrointestinal tract, the mRNA was not detected in the human stomach but was reported at moderate levels (in comparison with carcinoma cells) in the colon (16). In addition, the message was shown to be abundantly expressed in a variety of carcinoma cell lines, yet was found at comparatively low levels in fibroadenomas (16). A preliminary analysis using CRHSP-28 antiserum revealed the presence of the CRHSP-28 protein in the ZR-75 breast carcinoma cell line shown to express the mRNA (data not shown). From these findings, it is tempting to speculate that the high protein expression in gastrointestinal mucosa, pancreas, and lacrimal gland, taken together with the high mRNA expression in carcinomas, may potentially suggest a specific role for this protein in cells of epithelial origin. Although rich in epithelial cells, the mucosal scrapings prepared under these conditions may contain multiple cell types. However, it seems likely that CRHSP-28 is localized to the epithelial cells, as in the pancreas it was first identified in isolated acini. In preliminary experiments, we have determined by Western blotting that CRHSP-28 is abundant in the particulate fraction following a 150,000 X g centrifugation and is released by low concentrations of nonionic detergent (data not shown), suggesting that the protein may be loosely associated with the cell membrane or cytoskeleton. Thus, the function of CRHSP-28 is unclear at this time. However, given its relatively high level of expression in the pancreas and other gastrointestinal tissues, as well as the fact that its phosphorylation is tightly regulated by calcium-mobilizing digestive hormones and neurotransmitters, an elucidation of its function should provide valuable insight into the mechanism(s) by which these secretagogues regulate acinar cell function.

While this manuscript was under review, Parente et al. (24) described the purification of the rabbit homologue of CRHSP-28 from gastric gland mucosa. These findings are consistent with our results demonstrating a relatively high abundance of the protein in the rat gastric mucosa.

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