Purification, Cloning, and Expression of a Novel, Endogenous, Calcium-sensitive, 28-kDa Phosphoprotein*

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In gastric parietal cells, cholinergically induced increases in intracellular free calcium concentrations have been well characterized, but little is known about the signaling events beyond the initial rise in intracellular calcium. In the present study, we report the isolation of a 28-kDa protein, which is rapidly phosphorylated in intact, enriched parietal cells in response to both the cholinergic agonist, carbachol, and the calcium ionophore, ionomycin. A combination of in situ ³²P labeling and one- and two-dimensional gel electrophoresis was used to acquire sufficient quantities of protein to obtain partial amino acid sequence. Cloning of the pp28 cDNA revealed a novel protein which we have named CSP2P28 based on its calcium-sensitive phosphorylation. There are three CSP2P28 mRNA species (1.7, 2.2, and 3.3 kilobases) that are widely distributed throughout a variety of rabbit tissues. Recombinant CSP2P28 was phosphorylated by both crude parietal cell homogenate and purified CaM kinase II in a calcium/calmodulin-dependent manner. We propose that CSP2P28 may play an important and ubiquitous role in the calcium signaling pathway.

Intracellular signaling is generally mediated by activation of specific receptors leading to alterations in intracellular concentrations of different second messengers, including calcium, inositol phosphates, diacylglycerol, and cyclic AMP. These second messengers modulate many physiological processes that involve the phosphorylation of enzymes, receptors, and substrates by multifunctional protein kinases, namely calcium/calcmodulin-dependent protein kinase II (CaM kinase II), calciumphospholipid-dependent protein kinase, and cyclic AMP-dependent protein kinase (for reviews, see Refs. 1-3). Although there is an abundance of information about second messengers and second messenger-dependent protein kinases, much less is known about the specific protein kinase substrates in these signaling pathways.

In many secretory cells, cholinergic stimulation of muscarinic receptors activates phospholipase C, which hydrolyzes phosphoinositide 4,5-bisphosphate to liberate inositol 1,4,5-bisphosphate and diacylglycerol (2). Similarly, in HCl-secreting gastric parietal cells, cholinergic agonists elevate inositol 1,4,5-bisphosphate concentrations which, in turn, stimulate the rapid release of calcium from internal stores (4-8). The cascade of signaling events following the rise in intracellular free calcium concentrations in parietal cells and in other cell types remains obscure. It is clear, however, that protein phosphorylation is a critical component of second messenger-dependent cascades. In parietal cells, at least three different proteins with molecular masses of 28, 36, and 66 kDa are phosphorylated in response to cholinergic stimulation (4, 8, 9). These phosphorylation events appear to occur by way of different protein kinase activating mechanisms. Since the 36- and 66-kDa phosphoproteins are phosphorylated in isolated intact parietal cells following addition of phorbol ester under calcium-chelating conditions, it appears that these phosphoproteins are protein kinase C substrates (4, 8, 9). In contrast, the 28-kDa (pp28) protein is not phosphorylated in response to protein kinase C activators, but is phosphorylated in response to either cholinergic agonists or the calcium ionophore, ionomycin (9). Furthermore, pp28 phosphorylation is inhibited by chelation of intracellular calcium with the cell permeant form of BAPTA (9).

Until now, none of these phosphoproteins have been identified or characterized.

The present study describes the purification of pp28 and the isolation and initial characterization of a full-length cDNA clone. Since previous work determined that the 28-kDa protein was present in parietal cells in low abundance, a novel purification strategy was developed that utilized a combination of in situ ³²P labeling and preparative two-dimensional gel electrophoresis. These techniques allowed for the isolation of sufficient quantities of highly purified protein to obtain partial amino acid sequence information which was then used to clone the open reading frame of pp28. Recombinant pp28 was phosphorylated in a calcium-dependent manner by crude parietal cell homogenate and purified CaM kinase II. Our data suggest that pp28 represents a novel phosphoprotein. We have designated this protein as CSP2P28, a calcium-sensitive phosphoprotein of 28 kDa (10).

**MATERIALS AND METHODS**

Isolation of Parietal Cells and Gastric Glands—Parietal cells were prepared from fundic mucosa of male 2-3-kg New Zealand White rabbits as described previously (5, 11). This method yields approxi-
Identification of a 28-kDa Calcium-sensitive Phosphoprotein

Maturely 20–30 million >95% pure parietal cells (5). Gastric glands were isolated from gastric mucosa as described previously (9, 12).

In situ 32P Labeling and Isolation of pp28 Marker from Parietal Cell Extracts—Since pp28 was in low abundance, a novel methodology was developed to purify sufficient quantities for sequencing. In situ 32P-labeled pp28 from enriched parietal cells was used as an internal marker throughout the purification. Protein for pp28 purification was extracted from gastric glands rather than parietal cells because this phosphoprotein was previously detected in both chief and parietal cells (9) and protein yields were substantially higher in glands as compared to extracts from similar numbers of parietal cells (10). 32P-labeled pp28 (100 pmol) was heated at 95°C for 5 min, then pooled and used as markers for further purification.

Isolated glands (2 mg dry weight/ml) were stimulated with 3 mM ionomycin for 5 min, then disrupted using an Omni 5000 Polytron (4 × 30 s bursts at 70% power). Following a low speed centrifugation (50 × g, 10 min), supernatants were precipitated with 50% ammonium sulfate. Precipitated protein was dialyzed (Amicon Centripreps, Amicon Inc., Beverly, MA), then pooled with the radiolabeled pp28 spots and subjected to preparative two-dimensional-PAGE (Millipore InvestigatorTM 2-D Electrophoresis System, Bedford, MA). 32P-labeled pp28 (100 pmol) was electrophoresed at 200 volts. Gels were stained (0.025% Coomassie, 6–8, and 0.4%pH 3.5–9.5)). Samples were then resolved by preparative two-dimensional polycrylamide gel electrophoresis (two-dimensional-PAGE) (Millipore InvestigatorTM 2-D Electrophoresis System, Bedford, MA) (13). 32P-labeled pp28 (3 mm diameter, 18 cm length) were focused at 400 volts for 17 h and then 1000 volts for 1 h. Following a 20-min equilibration with equilibration buffer (0.05% bromphenol blue, 3% (w/v) SDS, and 62.5 mM Tris-HCl, pH 6.8). 32P-labeled pp28 (not loaded) into gels were focused (14.5% acrylamide, 0.128% cross-linker piperazine diacrylamide stacking, 12% acrylamide, 0.32% piperazine diacrylamide resolving) and electrophoresed at 200 volts. Gels were stained (0.025% Coomassie, 25% isopropl alcohol, 10% acetic acid), dried, and pp28 detected by autoradiography. Radiolabeled pp28 spots were excised from 5 gels, pooled, and used as markers for further purification.

Microsequencing of pp28—Peptides for sequencing were prepared by two different procedures: in-gel V8 protease digest (14) and in-gel trypsic digest (15, 16). The in-gel V8 protease digest was prepared by a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). An 18-kDa peptide fragment was isolated from the membrane and microsequenced at the Emory University Core facility (Atlanta, Georgia). The V8 digest was then digested with in-gel trypsin (11) and the tryptic digest was subjected to a second in-gel V8 digestion. The tryptic fragments were dried down and identified by mass spectrometry using a MALDI-TOF mass spectrometer. The peptide sequence was identified by searching the NCBInr database using the MASCOT software. Microsequencing of the identified peptide was performed with an Applied Biosystems 470A Protein Sequencer or the fMol DNA Sequencing System (Promega, Madison, WI). The primary sequence of pp28 was confirmed by the addition of an oligo(dC) tail to the 5‘ end of the cDNA with subsequent amplification by PCR with degenerate sense primers (800 nM), a manufacturer supplied (3‘-RACE System) antisense adapter primer (200 nM), 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 3 mM MgCl2, 1% Triton X-100, and 1 unit of Taq DNA polymerase (Promega).

After amplification of the cDNA, a range of PCR products was generated. These products were reamplified with the same degenerate sense primer SP-2 plus a degenerate antisense primer that was designed from a tryptic digest-derived amino acid sequence 5FEEKE (TCACRTTTCRTCYAAIGA, ASP-1). Upon reamplification, a 294-bp product was generated, subcloned into pBluescript, and sequenced.

PCR Screening—PCR screening was performed as described by Friedman and colleagues (17) using a subdivided Lambda Zap II cDNA Library (Stratagene, La Jolla, CA) prepared from >95% pure rabbit parietal cells. To facilitate screening, the amplified library was divided into smaller aliquots of 50,000 plaques each and screened using a 5-gal Euromax II filter (10). Five microliter from each library aliquot were diluted with 30 μl of water, heated (70°C, 5 min), and immediately placed on ice. Sense (CAGAGGAT- GCAAGATGTAAC, SP5) and antisense (GTCCTGGAGTTTGGGAT, ASP-5) primers were designed from the 294-bp sequence. Because the product size was small (130 bp) and to decrease nonspecific annealing, the cycling times were shortened (95°C for 30 s, 55°C for 10 s and 72°C for 10 s, 35 cycles). Ethidium bromide-stained (0.5% w/v) agarose gels (3% w/v) were used to detect the correct product size. Two aliquots from the rabbit cDNA library were found to contain product appropriately sized.

Library Screening—The 294-bp fragment obtained in the initial PCR amplification of cDNA with SP-2 and ASP-1 was PCR labeled with digoxigenin-dNTPs (Life Technologies Inc.) and used as a probe to screen 50,000 plaques from each of the two positive library aliquots. Membranes (MagnaGraph, MSI, Westboro, MA) were hybridized with the 294-bp digoxigenin-labeled probe in a solution of 5 × SSC, 0.02% SDS, and 1% blocking reagent (Boehringer, Mannheim, Germany) at 65°C for 18 h. Membranes were washed twice in 1 × SSC, 0.5% SDS (15 min, 65°C) and three times in 0.2 × SSC, 1% SDS (15 min, 65°C). Positive plaques were identified using the Genius System (Boehringer Mannheim). Briefly, membranes were blocked with 100 ml Tris-HCl (pH 7.5), 150 mM NaCl, and 2% blocking reagent (blocking solution) (1 h, 23°C), incubated with primary antibody (1:5000 anti-digoxigenin-Fab fragments, blocking solution, 30 min, 23° C), washed three times in 0.2× SSC, 1% SDS, material was pre hybridized and hybridized, membranes were washed twice in 1 x SSC, 0.5% SDS (15 min, 65°C) and 3 times (0.2× SSC, 1% SDS). Positive plaques were identified by chemiluminescence (Lumiphos 530 reagent, Boehringer Mannheim). The two longest inserts were selected for sequencing.

5‘-RACE—5‘-RACE was performed using a Life Technologies 5‘-RACE System (Gaithersburg, MD) according to manufacturer instructions with modifications. An anchored antisense primer (ASP-4, CCTCCAGGTCGTAGCTAC) (200 nM) was excised, pooled, and used as a reverse primer. Microsequenced antisense primer was then reamplified with an nested antisense primer (ASP-5) yielding a band of 383 bp. All DNA sequencing was performed with T7 DNA polymerase and either an Applied Biosystems 377 Automated Sequencer or the fmD DNA Sequencing System (Promega, Madison, WI). Recombinant Protein Expression—Recombinant protein was expressed in a prokaryotic system using the pET19b expression vector (Novagen, Madison, WI). The 5551 bp open reading frame of pp28 was amplified by PCR from parietal cell cDNA with a sense primer (GGCATATGGACCGCGGCGAGCAAGGTCGT) containing a 5‘ NdeI restriction site and an antisense primer (CCCGATCTCAGGCGGCGCTCAGTCG) containing a 3‘ BamHI restriction site. The resulting full-length sequence was inserted into pET19b in-frame with the 5‘-polyhistidine (His-tag) sequence. Ligated plasmids were transformed into the Escherichia coli strain BL21 cells, and recombinant protein expression was optimized. The recombinant protein was secreted into the culture supernatant, purified by protein A chromatography, and used for in vivo experiments.
Identification of a 28-kDa Calcium-sensitive Phosphoprotein

RESULTS

Purification and Protein Sequencing—Based on our previous knowledge that: 1) pp28 was a low abundance, acidic phosphoprotein that was present in both parietal cells and chief cells; 2) pp28 was phosphorylated in response to elevated calcium; and 3) pp28 exhibited a characteristic migratory pattern on analytical two-dimensional-gels (9), we designed new strategic approaches to obtain sufficient protein for microsequencing. Thus, parietal cell proteins were radiolabeled in situ with carrier-free \( ^{32} \)P orthophosphate, then stimulated with ionomycin. Relatively large amounts of radiolabeled proteins were resolved using preparative two-dimensional SDS-PAGE. pp28 spots were located by autoradiography, pooled with ammonium sulfate-precipitated proteins from ionomycin-stimulated glands and resolved by molecular mass to a single radio-labeled peak detected by Cerenkov counting. Peak fractions were pooled then resolved according to molecular mass and pl using preparative two-dimensional-PAGE. By combining one- and two-dimensional preparative electrophoresis protocols and using radiolabeled pp28 as an internal marker, sufficient amounts of Coomassie Blue-stained pp28 spots were obtained for initial microsequencing (Fig. 1, top left).

Initial attempts to microsequence intact protein transferred to polyvinylidene difluoride membranes were unsuccessful, apparently because pp28 was N terminally blocked. In-gel V8 protease (14) and in-gel tryptic digest protocols (15-16) were used to obtain internal amino acid sequence for these digestes. Six to eight spots of radiolabeled pp28 (~1-2 \( \mu \)g/gel) prepared as described above (Fig. 1, top) were used for each protocol. The V8 protease (not shown) digest yielded a major 18-kDa peptide from which 25 amino acids were sequenced. Fragments from the in-gel tryptic digest were resolved with a Pharmacia SMART system and selected peptide peaks were sequenced as shown in (Fig. 1, bottom). Amino acid sequence information

Fig. 1. Purification of CSPP28. Top left, Coomassie Blue-stained sheet of a two-dimensional gel of \(^{32} \)P-labeled protein that was separated on a Bio-Rad Prep cell (see "Materials and Methods"). Both Coomassie Blue-stained spots (arrow) were used for sequencing because initial amino acid composition analyses of the two spots indicated that they were identical (C. S. Chew and A. C. Petropoulos, unpublished observations). Top right, autoradiograph of Coomassie Blue-stained two-dimensional gel depicted on the left. The arrow identifies \(^{32} \)P labeled CSPP28. Bottom, chromatogram of peptides resolved with a Pharmacia Smart system after in-gel tryptic digest of CSPP28. Letters indicate peaks that were used for amino acid sequencing.
Identification of a 28-kDa Calcium-sensitive Phosphoprotein

V8 Digest Fragment

Tryptic Digest Fragments

A) TSETLS
B) NSPTF (Anti-sense primer)
C) SFEKEVKNL (Anti-sense primer)
D) GWQDVTATSAY (Anti-sense primer)
E) ASAALFSVG
F) VGGTPKPDGFGEYLNSTANASATSEGPV

Derived from both protocols is summarized in Fig. 2.

Molecular Cloning of CSPP28—Based on the amino acid sequence of the V8 digest, two degenerate oligonucleotides were designed and used as sense primers for the initial 3' RACE. PCR products ranging in size from 1.5 kb to 200 bp were isolated and re-amplified with the degenerate sense primers along with a degenerate antisense oligonucleotide designed from peptides obtained from the tryptic digest (Fig. 2, bottom). This re-amplification generated a specific 294-bp fragment (Fig. 3A). The deduced amino acid sequence from the 294-bp product contained 5 of the 6 sequenced tryptic fragments (Fig. 3A).

The 294-bp fragment was then used to screen a parietal cell cDNA library, resulting in the identification of CSPP28 clones of 1400 and 1860 bp. These clones were identical from their 5' ends through the open reading frame to the first polyadenylation signal sequence site. The 1400-bp clone was polyadenylated after the first polyadenylation signal sequence, whereas the 1863-bp clone contained an additional polyadenylation sequence which was 460 bp beyond the first sequence (Fig. 3A). Since the clones did not contain an initiating methionine, additional sequence was obtained using a 5' RACE strategy. The resulting additional 219 bp contained a putative start codon and an additional 5 nucleotides upstream of the start codon, which contained a Kozak consensus sequence. The deduced amino acid sequence from the 294-bp fragment, obtained from the initial PCR cloning, is in bold text.

In Vitro Phosphorylation—Analysis of the CSPP28 amino acid sequence did not detect any consensus phosphorylation sites. However, since all available evidence suggested that CSPP28 phosphorylation was calcium dependent (4, 9), we used in vitro phosphorylation analyses to determine whether...
Identification of a 28-kDa Calcium-sensitive Phosphoprotein

CSPP28 phosphorylation could be increased in a calcium/calmodulin-dependent manner. Fig. 6 shows that recombinant CSPP28 was phosphorylated by gastric gland extracts in a calcium-dependent manner and its phosphorylation was enhanced by addition of calmodulin. In addition, purified CaM kinase II phosphorylated recombinant CSPP28 in a calcium- and calmodulin-dependent manner. CSPP28 phosphorylation by both cell extracts and purified CaM kinase II was strongly inhibited by a CaM kinase II pseudosubstrate inhibitor peptide (40 μM) (Fig. 6).

DISCUSSION

Although receptor-mediated elevation of intracellular free calcium concentrations is a universal second messenger signaling event and a number of calcium-dependent protein kinases have been identified, little is currently known about the downstream substrates for these kinases. This is particularly so in gastric parietal and chief cells, in which only the calcium dependence for cholinergically modulated secretion of HCl has been well characterized (4, 5, 8, 20–24). In this work, we define a novel combination of methodologies, based on preparative one- and two-dimensional gel electrophoresis in conjunction with in situ 32P labeling, which allows for the isolation of sufficient amounts of low abundance agonist-responsive phosphoproteins for microsequencing and cDNA cloning. Through the use of such strategies we have successfully identified and partially characterized CSPP28, a novel acidic phosphoprotein member of the calcium signaling cascade. Northern analyses indicate that CSPP28 mRNA is widely distributed throughout the gastrointestinal tract as well as in brain. Thus, CSPP28 may serve an important and ubiquitous function in calcium signaling cascades in a variety of cell types.

Previous work in our laboratory demonstrated that CSPP28 is rapidly and transiently phosphorylated in parietal and chief cells upon cholinergic stimulation. CSPP28 is also strongly phosphorylated when intracellular free calcium concentrations are elevated by calcium ionophores. The calcium dependence of CSPP28 phosphorylation was further demonstrated by a complete inhibition of this response upon chelation of intracellular and extracellular calcium using a combination of EGTA and the cell-permeant form of BAPTA (9). A phosphoprotein with similar molecular weight and calcium sensitivity has also been detected in two-dimensional gel analyses of extracts of other cell types from several species, as well as in cultured cell lines. For example, Williams and colleagues have reported calcium-dependent phosphorylation of a pancreatic acinar cell protein in mice and guinea pigs with properties similar to those of CSPP28 (25). Also in a colonic epithelial cell line (HT-29), Cohn and colleagues (26) have detected a similar protein which is phosphorylated upon activation of H1 receptors.

A potential role for CaM kinase II in parietal cell secretion was proposed by Tsunoda and colleagues (27) who found that the CaM kinase II inhibitor, KN-62, strongly suppressed cholinergic stimulation, but not histaminergic stimulation of parietal cell accumulation of [14C]aminopyrine, an indirect measure of HCl secretion (28). In addition, we were unable to detect either phorbol ester- or cAMP-induced increases in CSPP28 phosphorylation in intact parietal cells (9). On the basis of these data and observations that CaM kinase II is present in parietal cells (29–31), we hypothesized that CSPP28 is a CaM kinase II substrate. Our results thus far support this hypothesis that both purified CaM kinase II and parietal cell extracts increase phosphorylation of recombinant CSPP28 in that this phosphorylation exhibited calcium/calmodulin dependence. In addition, CSPP28 phosphorylation was strongly inhibited by a specific CaM kinase II pseudosubstrate inhibitor peptide.

Although the cDNA and deduced amino acid sequence of CSPP28 was initially not found to have significant homology to any protein in the GenBank (10), more recent searches have detected a 95% amino acid sequence identity (Fig. 3B) between CSPP28 and the predicted amino acid sequence of an overexpressed cDNA transcript (D52) initially isolated from an infiltrating ductal breast carcinoma (19). A similar transcript was shown to be present in eight breast carcinoma cell lines (BT-20, BT-474, HBL-100, MCF7, MDA-MB-231, SK-BR-3, T-47D, and...
Identification of a 28-kDa Calcium-sensitive Phosphoprotein

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