Coronin Family Member, Coronin<sub>se</sub>, Which Is Regulated within the Protein Kinase C Signaling Pathway*  

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In order to understand the regulatory role of protein kinase C (PKC) in secretory epithelia, it is necessary to identify and characterize specific downstream targets. We previously identified one such protein in studies of gastric parietal cells. This protein was referred to as pp66 because it migrated with an apparent molecular mass of 66 kDa on SDS-polyacrylamide gels. The phosphorylation of pp66 is increased by the cholinergic agonist, carbachol, and by the PKC activator, phorbol-12-myristate-13-acetate, in a calcium-independent manner. In this study, we have purified pp66 to homogeneity and cloned the complete open reading frame. GenBank™ searches revealed a 45% homology with the Dictyostelium actin-binding protein, coronin, and 67% homology with the previously cloned human and bovine coronin-like homologue, p57. pp66 appears to be most highly expressed in the gastrointestinal mucosa and in kidney and lung. Confocal microscopic studies of an enhanced green fluorescent protein fusion construct of pp66 in cultured parietal cells and in Madin-Darby canine kidney cells indicate that pp66 preferentially localizes in F-actin-rich regions. On the basis of our findings, we propose that pp66 may play an important, PKC-dependent role in regulating membrane/cytoskeletal rearrangements in epithelial cells. We have tentatively named this protein coronin<sub>se</sub> because it appears to be highly expressed in secretory epithelia.  

Actin-binding proteins play important roles in regulating diverse activities in nonmuscle cells including, for example, cytoskeletal remodeling, signal transduction, cell adhesion, migration, and motility (see Refs. 1–3 for reviews). Coronin, which is expressed by the unicellular eukaryote, Dictyostelium discoideum (4), is one such protein. There is compelling evidence that coronin is involved in the regulation of variety of actin-associated activities in Dictyostelium, including phagocytosis, cytokinesis, and cellular locomotion (5, 6). Coronin translocates into the phagocytic cup region during phagocytosis and is recruited to the leading edge of newly formed cell processes in migrating cells (4, 5). Moreover, in coronin null mutants, the rate of phagocytosis is reduced by ∼70% (6), and these mutants also exhibit reduced chemotaxis and motility (7). Since Dictyostelium takes up all nutrients by endocytosis and since coronin has been found to be present in the actin-coated, endosomal phagocytic compartment that also contains V-type H<sup>-</sup> -ATPase subunits and Rab proteins, it has been suggested that coronin also plays a role in regulating endocytotic processes (8).  

Recently, a mammalian homologue of coronin, p57, was isolated from calf thymocytes as a 57-kDa contaminant of phospholipase C-containing cellular fractions (9). The deduced amino acid sequence of cDNA from both the human peripheral blood leukocyte HL60 cell line and bovine spleen indicated ∼40% homology between p57 and coronin. p57, which is also referred to as mammalian coronin, appears to be highly conserved among mammalian species, with 95% identity between the human and bovine homologues (9). Although the function of p57 has not been defined, the homology between coronin and p57 and the observation that p57 co-precipitates with F-actin in vitro has prompted Suzuki and colleagues (9) to propose that this protein may also play a role in regulating cell migration and motility. Western blot analyses have shown that p57 is highly expressed in immune tissues but weakly expressed in lung and intestine. There is also little to no detectable expression of p57 in stomach, kidney, liver, and skeletal muscle (9). More recently, a 57-kDa protein in neutrophils was found to bind to the COOH-terminal region of the oxidase-specific protein, p40<sub>phox</sub>, and to accumulate around phagocytic cups. On the basis of partial peptide sequence analysis, this protein was identified as the human coronin homologue, p57 (10). Thus, the mammalian p57 protein may, like coronin in Dictyostelium, play an important role in regulating phagocytosis in the mammalian immune system.  

In the present study, we report the isolation, cloning, and characterization of a new mammalian coronin family member, which we have named coronin<sub>se</sub> because, in contrast to p57, it is highly expressed in a variety of secretory-type epithelial tissues including stomach, intestine, kidney, and lung. Coronin<sub>se</sub> contains numerous PKC phosphorylation consensus sites as well as a putative membrane-spanning region, characteristic of type 1b membrane proteins, near the amino terminus. Interestingly, although this latter region was not previously identified in either coronin or p57, it is highly conserved in all three protein sequences.  

Coronin<sub>se</sub> was initially identified as pp66, a cholinergically regulated, PKC-dependent phosphoprotein in the HCl-secreting gastric parietal cell (11, 12). In this study, we present evidence that pp66/coronin<sub>se</sub> is primarily located in an intracellular canalicular region of parietal cells, which is the site of active HCl secretion. In the parietal cell, the onset of HCl secretion is correlated with dramatic morphological transformations in which internal tubulovesicles containing the proton-
transporting H⁺K⁺-ATPase appear to fuse, by an exocytotic-like process, with the canalicular membrane to form elongated microvilli that extend into the canalicular lumen (13). The cessation of secretion is correlated with the endocytotic-like retrieval and return of the H⁺K⁺-ATPase to subcanaliculuar tubulovesicles (13). Because coronin appears to localize in an intracellular region associated with active membrane fusion/retrieval events and because the phosphorylation of pp66/coromin is increased by the secretory agonist carbachol, we hypothesize that this novel phosphoprotein may play a role in directing membrane/cytoskeletal rearrangements in the gastric parietal cell. Coronin might also play a similar role in other ion-transporting secretory epithelial cells such as kidney and lung in which secretion is regulated by exocytotic/endocytotic cycles that direct ion transporters and channels to and from the active sites (14–16). In addition, other data presented in this study indicate that coronin translocates to the leading edge of actively developing cellular processes. Thus, this novel PKC-dependent phosphoprotein may also play a role in regulating epithelial cell adhesion.

MATERIALS AND METHODS

Cell Isolation and Culture—Parietal cells were isolated from the gastric mucosa of pathogen-free, male New Zealand White rabbits (2–4 kg) using the partial Pronase/collagenase digestion (17). These cells were enriched to 70–85% purity by density gradient separation (Accudenz, Accurate Chemical, Westbury, NY) or to 95–98% purity using combined density gradient separation followed by centrifugal elutriation (17). Freshly isolated cells were used for metabolic labeling experiments and for pp66 isolation and purification. For transfection experiments, cells were placed in primary culture by plating on Matrigel-coated glass coverslips or glass-bottomed 35-mm culture dishes (Insert System 1000. Madison, WI). This mRNA was then used in a 3’-rapid amplification of cDNA ends system (Life Technologies, Inc.) to synthesize a tagged cDNA as described previously (19, 20). To prepare a specific probe for pp66, two degenerate primers were synthesized using peptide sequences obtained from the trypptic digest as a template and using OligoPrimer Analysis software (version 5.0; National Biosciences, Inc., Plymouth, MN) to assist in primer design. The sense primer (24-fold degeneracy) was based on the amino acid sequence IVTHWPT (ATHTGATCTTTGYCCCNAC, SP-406). The antisense primer (64-fold degeneracy) was based on the amino acid sequence CEIARFYK (TTRTARAAICKGGCNATCTCRCA, ASP-997). Approximately 40 ng of cDNA were amplified (95 °C for 20 s, 48 °C for 20 s, and 72 °C for 45 s; 35 cycles) in a reaction mixture containing these primers, 10 μM tri-HF Tris-HCl (pH 8.8), 1 mm dNTPs, 10 μM (NH₄)₂SO₄, 0.1% Triton X-100, and 0.5 units of Pfu DNA polymerase (Stratagene, La Jolla, CA). After amplification of the cDNA, a 620-base pair PCR product was generated, subcloned into pCR-Script SK+ (Stratagene). Sequencing was performed by the Molecular Biology Core Facility at the Medical College of Georgia using an ABI Prism 377 automated DNA sequencer and ABI Prism Cycle Sequencing Dye Terminators Ready Reaction kit (Applied Biosystems). Parietal Cell cDNA Library Screening—To obtain the complete open reading frame of pp66, the 620-base pair fragment obtained in the initial PCR amplification of cDNA with SP-406 and ASP-997 was PCR-labeled with digoxigenin dNTPs (Life Technologies) and used as a probe to screen 50,000 plaques from a Uni-ZAP cDNA library (Stratagene; derived from rabbit mRNA isolated from 98% pure parietal cells) as described previously (19, 20). Four positive plaques were identified by chemical biosecreening (CSFD® bioassay; GenHunter, Men-heim). The two longest inserts were selected for sequencing.

Molecular Cloning of Calf Spleen p57—Total RNA was isolated from calf spleen, and tagged cDNA was synthesized by 3’-rapid amplification of cDNA ends as described above for pp66 cloning. Sense (amino acid positions 1–6; ATGAGCAGCGAGTGGTGTCG) and antisense (amino acid positions 457–462; CTTGGCCTGGACTGTCTCC) primers were designed based on the cDNA sequence (GenBank™ accession number D44496) and synthesized in the Medical College of Georgia Molecular Biology Core Facility. p57 double-stranded DNA was generated using PCR amplification with Pfu DNA polymerase (Stratagene). The PCR-generated band of the expected size was subcloned into pCR-Script. Positive plaques were detected by blue-white screening (21), and cDNA from plasmids was sequenced as described above.

Molecular Cloning of Rabbit Spleen p57—Tagged cDNA was synthesized from total RNA isolated from rabbit spleen by the same methods used to prepare bovine spleen cDNA. Highly conserved regions of p57 cDNA were identified by comparing the bovine and human sequences in GenBank (accession numbers D44496 and D44497, respectively) to the rabbit p57 sequence (GenBank™ accession number D44496) and the Medical College of Georgia Molecular Biology Core Facility. p57 double-stranded DNA was generated using PCR amplification with Pfu DNA polymerase (Stratagene). The PCR-generated band of the expected size was subcloned into pCR-Script. Positive plaques were detected by blue-white screening (21), and cDNA from plasmids was sequenced as described above.

Northern Blot Analyses of pp66 and p57 mRNA—A 32P-labeled probe for pp66 mRNA was prepared by radiolabeling the cDNA encoding the entire open reading frame of pp66 by random priming with phospho[y]labeled dCTP (Amersham Pharmacia Biotech) using a Ready-To-go kit (Amersham Pharmacia Biotech). Total RNA was isolated from various tissues using an RNA STAT-60 kit (Tel-Test, Friendswood, TX), separated on 1.25% formaldehyde-agarose gels containing 1 μg/ml ethidium bromide, and transferred to Magnagraph (Micron, Inc., Westboro MA) nylon membranes and probed as described previously (19, 20). Membranes were washed under high stringency conditions (0.1X · SSPE, 1% · SDS, 65 °C) and then autoradiography. Co-migrating Coomassie Blue-stained spots were excised, pooled, and concentrated to a single band on an 8% SDS-polyacrylamide gel electrophoresis minigel. This band was then excised and subjected to “in gel” tryptic digestion (19, 20). Peptides from tryptic digests were resolved on a reversed phase high pressure liquid chromatography column, and sequenced as described at the Emory University Microsequencing Facility (Atlanta, GA).

Molecular Cloning of pp66 from Parietal Cell mRNA—Initially, a reverse transcription-PCR strategy, employing peptide sequence information obtained from tryptic digests of the purified pp66 protein, was used to generate a specific cDNA product. Messenger RNA was prepared from >95% pure gastric parietal cells, using biotinylated oligo[dT] beads to purify mRNA. PolyA⁺ particles from the Poly(A)tract System 1000 (Promega, Madison, WI). This mRNA was then used in a 3’-rapid amplification of cDNA ends system (Life Technologies, Inc.) to synthesize a tagged cDNA as described previously (19, 20). To prepare a specific probe for pp66, two degenerate primers were synthesized using peptide sequences obtained from the trypptic digest as a template and using OligoPrimer Analysis software (version 5.0; National Biosciences, Inc., Plymouth, MN) to assist in primer design. The sense primer (24-fold degeneracy) was based on the amino acid sequence IVTHWPT (ATHTGATCTTTGYCCCNAC, SP-406). The antisense primer (64-fold degeneracy) was based on the amino acid sequence CEIARFYK (TTRTARAAICKGGCNATCTCRCA, ASP-997). Approximately 40 ng of cDNA were amplified (95 °C for 20 s, 48 °C for 20 s, and 72 °C for 45 s; 35 cycles) in a reaction mixture containing these primers, 10 μM tri-HF Tris-HCl (pH 8.8), 1 mm dNTPs, 10 μM (NH₄)₂SO₄, 0.1% Triton X-100, and 0.5 units of Pfu DNA polymerase (Stratagene, La Jolla, CA). After amplification of the cDNA, a 620-base pair PCR product was generated, subcloned into pCR-Script SK+ (Stratagene). Sequencing was performed by the Molecular Biology Core Facility at the Medical College of Georgia using an ABI Prism 377 automated DNA sequencer and ABI Prism Cycle Sequencing Dye Terminators Ready Reaction kit (Applied Biosystems). Parasitology—Parietal cells—

1 The abbreviations used are: PKC, protein kinase C; EGFP, enhanced green fluorescent protein; PCR, polymerase chain reaction.
subjected to autoradiography (Hyperfilm-MP; Amersham Pharmacia Biotech) at -70 °C with intensifying screens.

In Vitro Phosphorylation of Native pp66 with Recombinant PKC—Parietal cells were extracted with Triton X-100 and Empigen BB as described above. The detergent-insoluble portion of these extracts (50 μg/g tissue) was resuspended in phosphorylation buffer (final concentration: 25 mM HEPES, pH 7.4, 10 mM Mg(C2H3O2), 0.3 mM dithiothreitol, 10 μg of phosphatidylserine, 2 μg of 1,2-dioleyl-sn-glycerol, and 0.1 mM [γ-32P]ATP) and incubated with or without (control) recombinant PKC (50 units; PanVera, Madison, WI) for 5 min at 30 °C. Reactions were stopped by the addition of hot SDS/buffer and subjected to analytical two-dimensional gel electrophoresis and autoradiography as described above. pp66 was identified based on co-migration of in vitro radiolabeled spots with authentic pp66 labeled in vivo by stimulation of intact 32P-labeled parietal cells with carbachol.

Transfection and Expression of EGFP-pp66 Fusion Protein in Cultured Parietal Cells—Parietal cells plated on Matrigel®-coated 35-mm glass-bottomed dishes were cultured for 24 h as described previously (18) and then transiently transfected with either pEGFP-N2 NH2-terminal protein fusion vector (CLONTECH) or the same vector into which the complete open reading frame for pp66 containing the initiator methionine was inserted (Qiagen, Valencia, CA). Transfections were performed with either the cat-ionic lipid Effectene™, and a manufacturer-supplied enhancer (Qiagen) at a ratio of 1.8 (DNA to enhancer) and 1.25 (DNA to Effectene) or DMRIE-C (Life Technologies) using 0.8 μg of DNA per 5 μg DMRIE-C. Transfection efficiencies with these reagents ranged from 5 to 20% in different cell preparations.

Localization of EGFP-pp66 Fusion Protein in Living and Fixed Cells: Comparison with Localization of Selected Cytoskeletal and Organelle Markers—EGFP-pp66 fusion protein was localized by confocal microscopy in living and paraformaldehyde-fixed cells. In the latter case, cells were rinsed in phosphate-buffered saline and fixed for 15 min at room temperature in freshly prepared 4% paraformaldehyde. For F-actin localization, fixed cells were permeabilized with Triton X-100 (0.15%, 4 min) and then stained with Alexa Fluor 586 phallidin (Molecular Probes, Inc., Eugene, OR) as per manufacturer’s instructions. Cells containing the fluorescent EGFP-pp66 fusion protein were optically sectioned using a krypton/argon laser at 488-nm excitation wavelength, and fluorescence was detected with a photomultiplier at a 530-nm (emission filter, long pass) emission wavelength. Cells counterstained for F-actin were scanned at 488–568-nm excitation using a 565-nm beam splitter and 530 ± 30-nm (short pass) and 590-nm (long pass) wavelength emission filters to detect the EGFP and Alexa 586 phallidin signals, respectively. For each dual label experiment, laser power, gains, and photomultiplier tube settings were adjusted so that there was no cross-over between the signals. Three-dimensional image reconstructions (0.2–0.4-μm sections) were performed using Molecular Dynamics ImageSpace™ software running on a Silicon Graphics O2 computer platform. For Golgi localization, living cells were loaded with the fluorescent Golgi marker, Bodipy FL C5 ceramide (1 μM), for 30 min at 37 °C and then scanned at 488-nm excitation and 530-nm emission wavelengths. Mitochondria were localized in living cells using the potential-sensitive fluorescent dye, Mitotracker (Molecular Probes; 500 nM, 30 min, 37 °C; 586-nm excitation and 600-nm emission wavelengths).

RESULTS

Characterization of pp66 Phosphorylation Response in Vivo and in Vitro—We previously demonstrated that carbachol increases pp66 phosphorylation in parietal cells and that this response is 1) mimicked by the PKC activator, phorbol-12-myristate-13-acetate; 2) calcium-independent; and 3) inhibited by the bisindolylmaleimide PKC inhibitor, Ro 318220 (11, 12). Since these studies focused solely on the 5-min time point, we expanded the analysis in this study to determine more precisely the temporal pattern of carbachol-dependent pp66 phosphorylation. The graph from experiments shown in Fig. 1A shows that pp66 phosphorylation in rat parietal cells reached a maximum within 5 min of stimulation. In addition, although the level of phosphorylation declined after approximately 15 min of stimulation, it remained significantly elevated above control for at least 30 min. Fig. 1B shows the autoradiographic localization of pp66 in analytical two-dimensional gels before and after carbachol stimulation of 32P-labeled parietal cells, and data in Fig. 1C show that carbachol stimulation leads to a strong phosphorylation of pp66 on serine residues with a barely detectable level of phosphorylation on threonine residues and no detectable phosphorylation on tyrosine residues.

Purification, Sequencing, and Cloning of pp66: Sequence Comparisons with Human and Bovine p57 Coronin-like Protein and Coronin—Several experimental approaches were undertaken in the attempt to identify pp66. In situ 32P-labeling studies indicated that the phosphorylated form of pp66 was enriched in a Triton X-100-insoluble cell fraction; however, because pp66 was not only NH2-terminally blocked but also highly resistant to extraction from polyvinylidene fluoride membranes, it was not possible to isolate a sufficient amount of pp66 from this fraction to obtain reliable sequence information (not shown). Subsequently, a combined detergent extraction (0.5% Triton X-100 plus 1% Empigen BB) was found to yield a detergent-insoluble fraction that was highly enriched in pp66. pp66 was identified as a row of two major and two minor Coomassie Blue-stained spots with the major spots migrating immediately basic to the minor spots that co-migrated with endogenously 32P-labeled pp66 (Fig. 2). Amino acid analyses of the major spots confirmed that they were isoforms of the same protein (not shown). At least two additional minor 32P-labeled spots that were more acidic than the two major phosphorylated spots would be detected in preparative gels (Fig. 2, arrows) as well as in analytical gels with longer exposure times (not shown).

Of the seven peptides sequences from these tryptic digests, a GenBank™ search indicated that these peptides possessed varying degrees of homology with the predicted amino acid sequences of previously cloned human and bovine p57 actin-
binding proteins (GenBank accession numbers X89109, U34690, and D44497; Figs. 3 and 4). The p57 protein from human and bovine immune tissues (9) bears ~64% similarity and 42% sequence identity with coronin, an actin-binding protein in D. discoideum (GenBank accession number X61480).

Interestingly, coronin has also been detected in Triton X-100-insoluble cell fractions (4).

Since one of the sequenced pp66 peptides bore no apparent homology to either p57 or coronin sequences and since there was strong identity (95%) between the human and bovine p57 sequences, the rabbit cDNA was cloned to determine whether or not the pp66 protein was indeed the rabbit homologue of the mammalian p57 coronin-like protein. As shown in Fig. 3, the predicted amino acid sequence of rabbit pp66 shares 67% identity to the predicted human sequence. In contrast, pp66 shares a somewhat higher identity with coronin than does p57 (Fig. 4) (45 versus 40%, respectively). Interestingly, in comparing the pp66 and p57 sequences, it is apparent that the COOH-terminal region of pp66 is significantly more degenerate than the NH2-terminal region. Moreover, the predicted amino acid sequence for pp66 is 22 amino acids longer than p57. A search of the nonredundant data base of the GenBank expressed sequence tag division further revealed that the highest scoring expressed sequence tags for both human and bovine p57 were completely different from those for rabbit (Table 1). Finally, a Blast sequence alignment (BLASTP, version 2.0.4) indicated that the rabbit pp66 protein shares greater homology with the putative human IR10 protein (GenBank accession number Z31590) than does the human p57 protein (49 versus 45% homology). The function of the IR10 protein is unknown. However, the fact that the IR10 sequence has been mapped to a candidate genomic region of the nevoid basal cell carcinoma syndrome (9q22.3 chromosomal region (23)) may prove to be of future importance in unraveling the function(s) of pp66.

Potential Phosphorylation Sites and Other Motifs Present in pp66—Although a standard Prosite Pattern search detects only two WD (or G-b) repeats in the human p57 sequence and only a single such repeat in the rabbit pp66 and coronin sequences, further analysis with the more powerful Prosite Proflscan (Swiss Institute for Experimental Cancer Research server) detects three WD repeats within a single WD repeat region in all three proteins (Fig. 3). The WD repeat region, which was first identified in the β-subunit of heterotrimeric GTP-binding proteins, has since been detected in a number of proteins involved in the regulation of a variety of functions including signal transduction, vesicular trafficking, cytoskeletal function, gene regulation, and RNA processing (24). In contrast to p57 and coronin, pp66 does not possess a dileucine zipper motif, which is present both in the human and bovine p57 sequences (Fig. 3, amino acid residues 433–454). There are also a number of potentially important motifs in pp66 that do not appear in p57 based on a Blocks analysis using Prosite Motifinder, version 15 (25). These include 1) a WNN motif (70% identity; residues 182–191) that is present in the ephrin protein family, members of which have been shown to associate with receptor tyrosine kinases; 2) a DGR motif that is highly conserved in all actin isoforms found in vertebrates and invertebrates (67% identity; residues 209–263); 3) A YGR motif found in clathrin adapter complex proteins, which have been shown to be involved in linking clathrin to receptors present in coated vesicles (58% identity; residues 275–293); 4) an LRL-like motif that is present in the dynamin family, members of which have been associated with endocytic processes (70% identity; residues 392–417); and 5) a motif found in the AAA family of ATPases, one member of which is the vesicular fusion protein, NSF (72% identity; residues 366–402). Finally, a Prosite Prints analysis detected a potential fibronectin type III finger (residues 162–180). The fibronectin superfamily consists of 45 different families of proteins, many of which are involved in cell adhesion or are associated with receptor protein-tyrosine kinases or cytokine receptors.

Based on further analyses with Prosite Motif, there are 12 potential PKC phosphorylation sites within the predicted pp66 amino acid sequence, seven of which are also present in the human p57 sequence (Fig. 3). Seven of the 11 PKC consensus sequences in pp66 contain serine residues, and, of these, three are conserved as compared with the human sequence (amino acid residues 243–245, 291–293, and 391–393). There is also a single putative threonine-containing cyclic AMP-dependent protein kinase phosphorylation consensus sequence (residues 206–209) that is conserved between pp66 and the human and bovine sequences. Since this motif may also serve as a PKC phosphorylation site (26) and since there is no evidence for cyclic AMP-dependent pp66 phosphorylation in rabbit parietal cells, it is unlikely that this site is phosphorylated by cyclic AMP-dependent protein kinase upon activation of the cholinergic signaling pathway.

Within the deduced pp66 protein sequence, there are a number of other motifs of unknown significance including six potential casein kinase II phosphorylation sites (amino acid residues 100–103, 164–167, 311–314, 330–333, 381–384, and 391–394), six N-myristoylation sites (residues 96–101, 101–106, 188–193, 208–213, 326–331, and 443–448), and two glycosylation sites (residues 181–184 and 187–190). If, in addition to phosphorylation, pp66 undergoes additional post-translational modifications such as myristoylation, this may explain why this protein, which has a predicted Mr of 53,609, migrates on SDS-polyacrylamide gel electrophoresis gels with and apparent molecular mass of 66 kDa.

Based on further analysis with PSORT, version 6.4 (27), pp66 contains a putative transmembrane region (residues 47–63) typical of a type 1b membrane protein with a long cytoplasmic tail (residues 64–486). The presence of such a transmembrane region might explain the detergent insolubility of pp66, since interconnected cytoskeletal proteins and tightly associated integral membrane proteins are known to co-pellet as Triton X-100-insoluble structures. Secondary structure analyses using both the Paircoil algorithm of Berger et al. (28) and the coiled-coil algorithm of Lupas (29) and both weighted and unweighted MTK and MTIDK matrices with a window size of 21 amino acids predicted the presence (p > 0.9) of a highly coiled region at the end of the cytoplasmic tail (residues 444–484). Interestingly, all of these structural features are conserved in the human p57 protein (Fig. 3). There are also two conserved dileucine residues within the putative cytoplasmic tails of pp66 and p57 (Fig. 3). There are several lines of evidence that dileucine motifs can serve as endocytic targeting signals (30).

Northern Blot Analyses of pp66 and p57 mRNA Expression—In high stringency Northern blot analyses of total RNA from a variety of tissues, the pp66 message was found to be

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**Fig. 2.** Purification of pp66 isoforms on preparative two-dimensional gels. Individual spots were identified as pp66 isoforms by amino acid analysis. Boxes surround pp66 isoforms. Representative of a total of 15 such gels used to obtain amino acid sequence from tryptic digests of excised pp66 spots. A, Coomassie Blue stain. B, autoradiograph. C, overlay of autoradiograph and gel.

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widely distributed and of a similar size (1.8 kilobases) (Fig. 5, A and C). The level of pp66 expression was relatively high in the fundic mucosa of rabbit stomach, intestinal mucosa, kidney, and lung as well as in spleen and adrenal. In contrast, pp66 mRNA expression was low in heart, smooth muscle, and brain and almost undetectable in liver, pancreas, and skeletal muscle. Within the gastric epithelium, more detailed Northern blot analyses of mRNA from parietal cells, chief cells, and gastric glands (which contain 50% parietal and 50% chief cells plus a few endocrine-like cells) indicated that pp66 mRNA expression was higher in parietal cells as compared with chief cells (Fig. 5D). Since our chief cell preparations contained approximately 20% parietal cells, it is unclear whether or not the apparent expression of pp66 message in this cell type is the result of parietal cell contamination. In contrast, parietal cells used for mRNA isolation were 95–98% enriched. Thus, it is highly unlikely that the apparent high level of expression in parietal cells is the result of contamination by some other cell type.

In parallel Northern blot analyses using a full-length p57 probe generated as described under “Materials and Methods,” a similarly sized message of approximately 1.6 kilobases was readily detected in bovine and rabbit spleen but not in rabbit parietal cells even after extended exposure times (Fig. 5B). Thus, pp66 but not p57 appears to be expressed in parietal cells, whereas both messages are present in spleen.

Partial Molecular Cloning of a Rabbit p57 Homologue—To provide further confirmation that pp66 and p57 are different coronin isoforms, primers specific for bovine p57 were used to partially clone the p57 isoform from rabbit spleen RNA as described under “Materials and Methods.” DNA sequencing of the resultant PCR product (accession number AF100414) indicated 89% identity between the predicted bovine p57 amino acid sequence and the putative rabbit p57 sequence as compared with 67% identity between the putative rabbit p57 sequence and the rabbit pp66 sequence (not shown).

Expression and Localization of pp66-EGFP Fusion Protein in Parietal Cells—In order to define the subcellular localization of pp66, parietal cells were transfected with plasmids containing cDNA encoding for pp66-EGFP fusion protein. Within 24 h after transfection, a vesicular pattern of pp66-EGFP expression was readily detected in living parietal cells (Fig. 6A). In contrast, in control experiments in which plasmids containing EGFP alone were transfected, the resulting EGFP expression pattern was diffusely cytosolic, as expected (Fig. 6B). Incubation of transfected cells with Triton X-100 had no effect on the pp66-EGFP signal (Fig. 6C) but led to the rapid disappearance (within 2 min) of the EGFP signal in controls (Fig. 6D). Thus, the pp66-EGFP fusion protein appears to behave like the native pp66 protein in that both the expressed and native proteins are highly resistant to Triton X-100 solubilization. Since the pattern of
pp66-EGFP distribution was different from both Golgi and mitochondrial staining (not shown), it is unlikely that there is significant pp66-EGFP expression within these cellular organelles. In other experiments with paraformaldehyde-fixed parietal cells, pp66-EGFP was found to be highly expressed in close proximity to the F-actin-rich intracellular canaliculus both before (Fig. 7A) and after carbachol (100 μM) stimulation (Fig. 7B). Although less prominent, pp66-EGFP expression was also detected immediately below the F-actin-containing plasma membrane in these cells (Fig. 7). In contrast, in polarized Madin-Darby canine kidney cells in which F-actin is predominately localized at the plasma membrane, pp66-EGFP expression appeared to be localized exclusively in this region (not shown). As shown in Fig. 7B, a moderate level of pp66-EGFP expression over a period of 24 h does not suppress the rapid morphological changes known to be induced by carbachol, including the expansion of the intracellular canaliculus (18).  

During the course of these latter experiments, we serendipitously discovered that carbachol also induces a time-dependent formation of actin-rich filopodia and membrane ruffles in parietal cells (Fig. 8). The confocal microscopic images depicted in Fig. 8A were obtained by scanning paraformaldehyde-fixed cells stained for F-actin at the level of extracellular matrix attachment. F-actin staining is strong at the cell membranes and within intracellular canaliculi in unstimulated cells in which no filopodia were detected (Fig. 8, left panel). In contrast, 1 h after carbachol addition, a time when the acid secretory response to this agonist has been shown to have declined to near basal levels (22), a number of newly formed filopodia are apparent (Fig. 8, right panel). In parietal cells transfected with pp66-EGFP, there was also a movement of this fluorescent fusion protein into the actin-rich filopodia and an apparent enrichment at the leading edges of these cell processes (Fig. 8B). Carbachol induced the formation of cell processes within 15 min; however, most of the processes formed within this time frame were relatively short. After a 1-h exposure to carbachol, the majority of the newly formed processes were longer, and some cells also formed curtain-like lamelopodia and exhibited

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**Fig. 4. Multiple sequence alignment comparing the predicted amino acid sequences of bovine p57, rabbit pp66, and coronin with human p57.** Shaded areas indicate regions of identity. Note the high degree of identity between the human and bovine p57 sequences (95%) as compared with pp66 (67%). Note also that the degree of homology between pp66 and p57 is substantially lower near the COOH-terminal end as compared with the NH2 terminus. GenBank accession numbers for the human p57, bovine p57, and coronin sequences used in this analysis were X89109, D44496, and X61480, respectively.

**Table I**

Comparison of expressed sequence tags (ESTs) with best fits from the NH2 terminus to the COOH terminus for pp66 and human and bovine p57

| Rabbit pp66 | Bovine p57 | Human p57 |
|-------------|------------|-----------|
| Origin EST  | Origin EST | Origin EST |
| Mouse myotubes AA691936 | Jurakat T cells AA310078 | Same Same |
| Mouse myotubes AA636245 | Fetal liver/spleen W01981 | Same Same |
| H. sapiens CDNA clone T57897 | Mouse heart | AA221613 |
| NCI_CGAP_CO3 (H. sapiens) AA471051 | Fetal heart | A0047578 |
| NCI_CGAP_GC4 (H. sapiens) AA903739 | NCI_CGAP_Br2 A1005103 | Same Same |

* The closest matches at the COOH terminus were weaker than those at the NH2 terminus.

* Also similar to AA755181 and AAC912262.
membrane ruffling (see legend to Fig. 8). Similar results were obtained with the PKC activator, phorbol-12-myristate-13-acetate. However, with phorbol-12-myristate-13-acetate the formation of filopodia was more dramatic, and a high percentage of cells formed curtain-like lamelopodia and exhibited membrane ruffling (not shown).

DISCUSSION

In this study, we report the isolation, partial sequencing, and cloning of a novel cholinergically regulated, coronin-like 66-kDa signaling phosphoprotein, which we have tentatively named coroninse. The high degree of predicted secondary structure conservation in coroninse as compared with p57 and coronin strongly suggests that these proteins might serve similar functions within different cell types. For example, the recruitment of coronin into the actin network of the leading edge of migrating cells (5) offers intriguing parallels with our finding that EGFP-coroninse translocates to F-actin-rich, nascent cell processes formed in response to carbachol stimulation. Thus, one role for coroninse may be an involvement in the cytoskeletal reorganization of epithelial cells, which is similar to the role of coronin in the leading edge of migrating cells. Further experiments are needed to determine the precise role of coroninse in epithelial cell biology.
played by coronin in Dictyostelium. The uniqueness of our findings with coronin$_{se}$ lies in the fact that this protein is regulated in vivo by PKC-dependent changes in the state of phosphorylation, an observation that has not been reported for either coronin or p57. Although not yet proven, we hypothesize that coronin$_{se}$ serves as a direct substrate for PKC in vivo because of the large number of PKC phosphorylation consensus sequences present in this protein and our observations that coronin$_{se}$ is phosphorylated by a cholinergically activated serine/threonine kinase in intact parietal cells. Because the phosphorylation of coronin$_{se}$ in intact cells is calcium-independent and because coronin$_{se}$ can serve as an in vitro substrate for PKC, it is possible that coronin$_{se}$ is a direct in vivo substrate for this specific calcium-independent PKC isozyme. The unequivocal demonstration of these latter points will require further detailed studies to identify and characterize the specific in vivo phosphorylation sites.

The presence of proton pumps in the contractile vacuole of Dictyostelium (31) as well as the presence of V-H$^\pm$ ATPase subunits and Rab proteins in the endocytic vesicle compartment of this organism offer intriguing parallels with the gastric parietal cell in which Rab proteins are present within internal tubulovesicular membranes containing the H$^\pm$K$^-$$ $ATPase (32, 33) and in which activation of this proton pump appears to depend upon reversible fusion of tubulovesicles with the apically oriented canalicular membrane (13).

The proposal that vesicular acidification regulates the association of coronin with endosomal vesicles (8) has a precedent in that the WD repeat motif-containing peptide component of intra-Golgi transport (COP1) vesicles, $\beta'$-COP, undergoes reversible, pH-dependent associations with Golgi membranes (34). Since $\beta'$-COP has recently been shown to be a PKC-selective receptor for activated protein kinase C (35), this interaction could have potential relevance to our findings that coronin$_{se}$, which is also a WD repeat-containing protein, is regulated within the PKC signaling pathway. Another potentially relevant finding is that there is a fairly well conserved clathrin adaptors motif in coronin$_{se}$. Since clathrin and clathrin adaptors have recently been localized to tubulovesicular membranes in parietal cells (36), it is possible that there is a regulated interaction between coronin$_{se}$ and clathrin or a related protein that serves to direct, for example, the endocytic retrieval of the H$^\pm$K$^-$$ $ATPase from the apical membrane to subapical tubulovesicular membranes.

In contrast to coronin, no definite function has yet been assigned to the human or bovine p57 proteins. However, a protein that may be p57 has recently been found to accumulate around phagocytic cups and to be associated with a p67$^{phox}$ and p40$^{phox}$ protein-containing complex in human neutrophils (10). However, unlike coronin, the p57/coronin-like protein in neutrophils does not appear to disperse from around the phagocytic vacuole. Grogan and colleagues (10) have speculated that the sustained localization of p57/coronin near this vacuole may be important for the translocation of the p40$^{phox}$ and p67$^{phox}$ proteins to their site of action. Since we have also not detected any movement of coronin$_{se}$-EGFP around F-actin-rich intracellular canaliculi in actively secreting parietal cells, coronin$_{se}$, like the neutrophil protein, may serve as a stable but regulated anchor for other translocating proteins involved in ion transport in this cell type. It should be emphasized, however, that this apparent lack of translocation of coronin$_{se}$ within the canalicular region is in direct contrast to the movement of coronin$_{se}$ into nascent filopodia formed in response to carbachol stimulation. Thus, it remains to be determined whether there is a more subtle relocation of coronin$_{se}$ that is associated with secretion that was not detected by our methodology.

The conserved, highly coiled tail region at the far carboxyl terminus of coronin has been identified as an actin-binding region (4). Given the conservation of this predicted secondary structure in coronin$_{se}$ as well as in p57, this region may well serve a similar function in these mammalian proteins. Moreover, since all three proteins have a putative transmembrane region near the amino terminus, they are all likely to be inserted into membrane vesicles with the long tails projecting into the cytoplasm. This structural arrangement would clearly allow for interactions with F-actin as well as with other proteins. The presence of WD repeat motifs, which have been shown to be involved in a wide range of protein-protein interactions (24), further supports this assumption. In contrast, the degeneracy in the carboxyl terminus of p57 and coronin$_{se}$ could allow for different protein-protein interactions. Given such a scenario, the changes in the state of phosphorylation of coronin$_{se}$ could serve, for example, to regulate protein associations within vesicles.

The localization of two dileucine sequence motifs within the putative cytosolic tail of coronin$_{se}$ provides additional support for the assumption that coronin$_{se}$ is targeted to specific internal membrane compartments, since there is evidence that the dileucine motif can serve as the internal functional equivalent to tyrosine-containing targeting signals within coated pits of the plasma membrane (30). The presence of these and other potential endocytosis motifs in coronin$_{se}$ is particularly intriguing given recent findings that alteration of a tyrosine-based endocytosis motif within the $\beta$-subunit of the H$^\pm$K$^-$$ $ATPase leads to constitutive acid secretion in transgenic mice (37). Finally, since PKC activation has been associated mainly with the inhibition of HCl secretion (22, 38), increases in coronin$_{se}$ phosphorylation might also serve to increase the endocytic retrieval of the ion-transporting H$^\pm$K$^-$$ $ATPase or some other...
secretion-related ion channel or transporter from the apical membrane.

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