CaM kinase II regulation of CRHSP-28 phosphorylation in cultured mucosal T84 cells

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APICAL MEMBRANE TRAFFICKING in digestive epithelia is necessary for a variety of cellular processes regulating nutrient digestion and absorption, water and electrolyte secretion, and mucosal defense. Membrane proteins and secretory cargo are packaged in specialized vesicles in the Golgi compartment and are vectorially transported on microtubule networks to the cell apex. Transport vesicles must penetrate a prominent actin-cytoskeletal web before fusing with the apical membrane (18). Membrane fusion occurs by both constitutive and regulated pathways and involves protein components of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor complex (SNARE) (14). Coupled with vesicle fusion at the cell apex is the compensatory retrieval of vesicle membrane from the apical plasmalemma by endocytosis. This concomitant recycling of membrane by exocytotic and endocytotic events dictates the protein composition of the apical membrane and also prevents its overexpansion with excess phospholipid (29).

Ca2+-regulated heat-stable protein of 28 kDa (CRHSP-28, also known as D52) is a member of the tumor protein D52 family (6) that is highly expressed in the apical cytoplasm of acinar cells from pancreas, salivary, lacrimal, and gastric glands (16). We previously reported (27) that CRHSP-28 plays an important role in regulating digestive enzyme exocytosis from pancreatic acinar cells. Corresponding with its Ca2+-dependent effects on secretion, we recently found CRHSP-28 to interact in a Ca2+-sensitive manner with the phospholipid and actin cytoskeleton-binding protein annexin VI in vitro and in intact acinar cells (26). Further supporting a role for CRHSP-28 in apical membrane trafficking, Wilson et al. (30), using yeast two-hybrid screening, identified MAL2 as a CRHSP-28/D52 binding protein. MAL2 is an integral membrane protein that plays an essential role in mediating the apical delivery of transport vesicles in polarized epithelia (12). Additionally, another member of the TPD52 family, D53, was recently shown to interact with and stabilize the association of synaptobrevin 2 and syntaxin 1 in vitro, implicating D53 in the promotion of SNARE complex formation and the facilitation of membrane fusion in cells (22). Thus, although the precise molecular function of CRHSP-28 has yet to be elucidated, a wide body of evidence has recently accumulated implicating this protein as an important regulatory factor in apical membrane trafficking events in epithelial cells.

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Cohn et al. (10), using 32P labeling and two-dimensional electrophoresis, previously reported the presence of a Ca2+-sensitive phosphoprotein of 29 kDa in T84 cell cultures. The acidic isoelectric point, molecular mass, and Ca2+-sensitive phosphorylation were remarkably similar to reported phosphoproteins in gastric (4) and pancreatic (5) epithelial cells. These proteins were later identified as Ca2+-sensitive phosphoprotein (CSPP) 28 in gastric mucosa (21) and CRHSP-28 in acinar cells (15), both of which are highly homologous to D52 (7). Coinciding with the high expression of CRHSP-28 in acinar cells (15), both of which are highly modulating the phosphorylation of this protein.

The current study describes the expression of CRHSP-28 in T84 cells and further elucidates the intracellular localization and signaling mechanisms modulating the phosphorylation of this protein.

METHODS

Materials. Leupeptin, benzamidine, and protein A beads were purchased from Pierce (Rockford, IL); peroxisome-conjugated donkey anti-rabbit IgG secondary antibody and amphoteries were from Amersham Pharmacia Biotech (Piscataway, NJ); α-toxin, 12-O-tetradecanoylphorbol 13-acetate (TPA), 8-(4-chlorophenyl)-cAMP (CPT-cAMP), carbachol (CCh), ionomycin, di-phospho-l-serine, di-phospho-l-threonine, d-phospho-l-tyrosine, EDTA, ninhydrin, phenylmethylsulfonyl fluoride, the catalytic subunit of protein kinase A (PKA), and casein kinase II (CKII) were from Sigma (St. Louis, MO); casein kinase I (CKI) was from Promega (Madison, WI); 8-bromo-cGMP (8-Br-cGMP) and thapsigargin (Thapsig) were from Biomol (Plymouth Meeting, PA); glycogensynthase kinase 3 (GSK3), 2-[N-(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)]amino-N-methyl-l-tyrosyl]-4-phenylpiperazine (KN93), and BAPTA-AM were from Calbiochem (La Jolla, CA); [γ-32P]ATP and [γ-32P]orthophosphoric acid were from New England Nuclear (Boston, MA); 0.45-μm polyvinylidene difluoride membrane was from Osmonics Laboratory Products (Minnetonka, MN); and chymotrypsin was from Worthington Biochemicals (Lakeewood, NJ). The human adenoma cell line T84 was obtained from the American Type Culture Collection (Rockville, MD). Affinity-purified anti-human CRHSP-28 antibodies were previously characterized (16). The anti-Ca2+/calmodulin-dependent kinase II (CaMKII) antibody G301 was kindly provided by Dr. Angus Nairn and was previously characterized for immunofluorescence measurements in gastrointestinal epithelial cells (19).

Cell culture. T84 cells were grown in DMEM/F-12 (50:50) supplemented with 10% fetal bovine serum and containing penicillin, streptomycin, and gentamicin. Stock cultures were maintained in a 37°C and 5% CO2 humidified atmosphere and were passaged by using trypsin-EDTA. Media were changed the day after seeding and every 3–4 days thereafter. For experiments, cells were plated at a density of 2.5 × 10⁴ cells/cm² on 6- or 12-well plates. Unless otherwise noted, experiments were conducted on confluent cell monolayers. T84 cells were used between passages 4 and 20.

Cell treatments. Cells were incubated with various agents for the indicated time periods at 37°C unless otherwise noted. For agents dissolved in DMEM [ionomycin, TPA, BAPTA-AM, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide·HCl (W7), and thapsigargin], control cells received an equal volume of vehicle; the final concentration of vehicle did not exceed 0.1% (vol/vol). For the BAPTA-AM study, cells were washed twice with medium containing 0.5 mM EGTA, then incubated in medium containing 0.5 mM EGTA and 50 mM BAPTA-AM for 1 h. After BAPTA-AM pretreatment, the medium was removed and replaced with either normal medium or low-Ca2+ medium containing EGTA. Cells were then stimulated with CCh. For growth factor treatments, cells were maintained in serum-free media for 48 h before treatment.

Immunoblotting. After indicated treatments, cells were scraped in isoelectric focusing (IEF) buffer containing 9 M urea, 4% Nonidet P-40, 1% 2-mercaptoethanol, and 2% amphoteries (pI range 3–10). Cell lysates were sonicated, and the protein concentration was determined by using Bio-Rad reagent. IEF was conducted in slab gels composed of 6.5% (wt/vol) acrylamide, 0.2% (wt/vol) N,N’-methylene-bis-acrylamide, 0.1% (vol/vol) glycerol, 8 M urea, and ampholites (pI 2.5–5 and 3–10). Samples were resolved by using a Pharma- cia Biotech Multiphor II apparatus. Gels were precropped for 20 min at 700 V, and samples were resolved for 20 min at 500 V, 90 min at 2,000 V, and finally 10 min at 2,500 V. Proteins were fixed in 15% TCA for 10 min, then dried at 125°C for 15 h at 25 mM Tris-192 mM glycine containing 20% methanol. Immunoblotting was conducted with anti-human CRHSP-28 antibodies (1 μg/ml) and detected by enhanced chemiluminescence using a horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (1:5,000). Intensity of CRHSP-28 phosphosioforms on the immunoblots was quantified by densitometric analysis using a PDI model DNA35 scanner interfaced with the Protein and DNA Imageware System (PDI, Huntington Station, NY). Two-dimensional gel electrophoresis was performed as described (31).

Subcellular fractionation. Cells were scraped with a rubber policeman and sonicated in ice-cold lysis buffer containing (in mM) 50 Tris-HCl, 25 NaF, 10 tetrasodium pyrophosphate, 0.2 benzamidine, 0.5 phenylmethylsulfonyl fluoride, and protease inhibitor cocktail, pH 7.4. After centrifugation at 100,000 g for 1 h, the supernatant was used as soluble fraction and the pellet was again sonicated in lysis buffer containing 0.2% Triton X-100 and centrifuged to obtain solubilized membrane proteins.

Phosphoamino acid analysis. Cells were incubated in phosphate-free RPMI media containing 0.3 mM/L [γ-32P]orthophosphoric acid for 6 h. After being labeled, cells were treated with 100 μM CCh for 2 min, washed with ice-cold phosphate-buffered saline, and scraped in lysis buffer containing 150 mM NaCl and 0.2% Triton X-100. Samples were centrifuged at 21,000 g for 30 min, then pre-cleared with protein A beads for 1 h at 4°C. Anti-CRHSP-28 antibody (2 μg) was added to 800 μg of total cell protein overnight at 4°C and precipitated by a 1-h incubation with protein A beads. Immunoprecipitated proteins were washed five times in lysis buffer, with the final wash conducted in buffer lacking NaCl. Immunoprecipitates were denatured in SDS sample buffer. After electrophoresis, proteins were transferred to polyvinylidene difluoride membrane and autoradiographed. The area of the membrane corresponding to CRHSP-28 was excised and hydrolyzed by incubation in 6 M HCl at 110°C for 90–150 min. Samples were lyophilized, suspended in water, and spotted on a phosphocellulose plate. Following addition of 1 μg each of phosphoserine, -threonine, and -tyrosine, thin-layer electrophoresis was run at 20 mA for 45 min in a pH 2.5 running buffer consisting of (vol/vol) 5.9% acetic acid, 0.8% formic acid (88%), 0.3% pyridine, and 0.3 mM EDTA. Standards were visualized by ninhydrin staining, whereas 32P-labeled amino acids were detected by autoradiography.
**In vitro kinase assays.** Phosphorylation of recombinant CRHSP-28 by CKII and the catalytic subunit of PKA was conducted in a buffer containing (in mM) 50 Tris-HCl, pH 7.5, 150 NaCl, 10 MgCl₂, 5 dithiothreitol (DTT), and 0.01 μCi [γ-32P]ATP. GSK3 and CKI assays were conducted in the same buffer containing 2 mM DTT. Reactions were conducted in the presence of 100 ng CRHSP-28 and 1 μCi [γ-32P]ATP (3,000 Ci/mmol) in a final volume of 25 μL. After incubation for 2 h at 30°C, samples were separated by SDS-PAGE, and the radioactivity incorporated into CRHSP-28 was determined by liquid scintillation counting.

**Phosphopeptide mapping.** In vitro phosphorylated recombinant CRHSP-28 or 32P-labeled CRHSP-28 immunoprecipitated from CCh-treated T84 cells was resolved by SDS-PAGE and autoradiographed. The 28-kDa band was excised, incubated in 50% 25 mM ammonium bicarbonate (pH 8.0)-50% acetonitrile for 10 min, and then dried. The sample was hydrated in 25 mM ammonium bicarbonate containing 0.1 mg/ml chymotrypsin at 37°C for 12 h. Recovered peptides were concentrated and resolved by IEF.

**Immunohistochemistry.** T84 cells were plated at 12 × 10⁶ cells/cm² on Transwell permeable, clear polyester membranes (4-μm pores). After 24 h, media were changed and cells were allowed to equilibrate for a least 15 min at 37°C before treatments. After stimulation, cells were washed three times with ice-cold phosphate-buffered saline and then fixed in 4% paraformaldehyde for 20 min at room temperature followed by dehydration in ethanol. Sections were blocked in 10% donkey serum containing 0.2% Triton X-100 and incubated with an Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (1:1,000). Actin filaments were stained with rhodamine-phalloidin (165 nM). Immunofluorescence microcopy was conducted by using a Bio-Rad model 1024 confocal microscope with a mixed krypton/argon gas laser. Captured images were converted to TIFF files by using Bio-Rad software and were processed for publication by using Photoshop software.

**RESULTS**

**CRHSP-28 expression in T84 cells.** To determine if CRHSP-28 is expressed in T84 cells, lysates were immunoblotted with anti-human CRHSP-28 antibodies. The CRHSP-28 antibodies specifically reacted with a single 28-kDa protein in T84 cell lysates that was identical in size to signals observed in lysates prepared from rat pancreas and lacrimal gland (Fig. 1A). Immunoblotting after SDS-PAGE further confirmed that the antibodies reacted with a single protein with a molecular mass and acidic pI corresponding to that previously reported for CRHSP-28 in acinar cells (15) (Fig. 1B). Although CRHSP-28 contains no obvious primary or secondary structure to support its association with membranes, the protein has been shown to partition into microsomal fractions of pancreas (26, 27). In accordance with these findings, CRHSP-28 immunoreactivity was equally associated with soluble and particulate fractions of T84 cells (Fig. 1C).

**CRHSP-28 undergoes regulated serine phosphorylation.** In acinar cells, CRHSP-28 is phosphorylated on serine residues following stimulation with the physiological secretagogue cholecystokinin (15). To determine if CRHSP-28 undergoes regulated phosphorylation in T84 cells, the protein was immunoprecipitated from confluent monolayers that had been metabolically labeled with [32P]orthophosphate (Fig. 2A). Autoradiography of the immunoprecipitated protein demonstrated a prominent 28-kDa signal that was absent in samples containing protein A beads alone. Stimulation of cells with the muscarinic receptor agonist CCh in-
CRHSP-28 phosphorylation following muscarinic receptor activation. The IEF pattern of CRHSP-28 phosphoisoforms allowed us to examine the regulated phosphorylation of the protein in T84 cells by immunoblotting. A time course for the effects of CCh on CRHSP-28 phosphorylation is illustrated in Fig. 4. CRHSP-28 phosphoisoforms were quantified by densitometry and expressed as a percent of total CRHSP-28 protein in all conditions.

Fig. 4. CRHSP-28 is transiently phosphorylated following muscarinic receptor activation. T84 cells were treated with 100 μM CCh for the indicated times, and CRHSP-28 phosphorylation was analyzed by immunoblotting following isoelectric focusing (IEF). A, top: immunoblot of CRHSP-28 in T84 lysates following SDS-PAGE, demonstrating that CRHSP-28 protein levels remain constant during CCh treatment. A, bottom: single representative experiment illustrating the rapid and transient phosphorylation of CRHSP-28 in response to CCh. B: CRHSP-28 isoforms were quantified by densitometry and expressed as a percent of total CRHSP-28 present in each sample. Data are means ± SE of 3 independent experiments, each performed in duplicate.
three phosphoisoforms. Under basal conditions, the majority of CRHSP-28 was in the nonphosphorylated α-form with a comparably smaller proportion in the β-form. A pronounced increase in CRHSP-28 phosphorylation was detected within 2 min of CCh stimulation, as indicated by the acidic shift of the protein from the α- to the β- and γ-forms. The amount of CRHSP-28 in the γ-form increased from undetectable levels to ~40% of total, whereas the α-form decreased correspondingly from 68% to 20% of total protein. CRHSP-28 phosphorylation was transient, with a maximal increase seen at 2 min that then declined over 30 min and returned to near basal conditions by 60 min in the continued presence of agonist. The amount of CRHSP-28 in the β-form remained constant throughout the time course, representing ~40% of total. Immunoblotting of lysates following SDS-PAGE showed that CRHSP-28 expression was not altered by CCh treatment (Fig. 4A, top). This was also true in cells when total protein translation was inhibited by preincubation with a high concentration of cycloheximide (10 μg/ml) before the 60-min challenge with CCh (data not shown).

The sensitivity of CRHSP-28 phosphorylation to CCh treatment was determined after 2 min of stimulation (Fig. 5). CCh-stimulated CRHSP-28 phosphorylation occurred at concentrations as low as 10 μM and was maximal at 300 μM. Concentrations <10 μM partially shifted CRHSP-28 from the α-form to the β-form; however, the γ-form of the protein was unchanged. At CCh concentrations >100 μM, there was a pronounced increase in the γ-form. Based on the disappearance of the α-form, the EC₅₀ for the CCh effect was ~25 μM.

![Fig. 5. Concentration-dependent effects of CCh on CRHSP-28 phosphorylation.](image)

Notably, the intensity of the β-form in control cells ranged from 10 to 30% between experiments, indicating that the protein is variably phosphorylated under basal conditions. These findings are compatible with the basal levels of CRHSP-28 phosphorylation seen by ³²P labeling (see Fig. 2).

In addition to muscarinic receptor agonists, treatment of cells with 100 μM histamine for 2 min induced a marked phosphorylation of CRHSP-28 that was comparable with the effects of CCh treatment. However, CRHSP-28 phosphorylation was not altered by growth factor stimulation (15 nM IGF-I or EGF or 10% fetal bovine serum) of serum-starved quiescent cultures (data not shown).

CRHSP-28 is phosphorylated by a Ca²⁺-dependent mechanism. To define the signal transduction pathway responsible for mediating CRHSP-28 phosphorylation in T84 cells, confluent monolayers were treated with agents that bypass receptor occupation to activate cellular messengers. Treatment of cells with CPT-cAMP to activate cAMP-dependent protein kinase, TPA to activate protein kinase C isoforms, or 8-Br-cGMP to activate cGMP-specific protein kinases had no effect on CRHSP-28 phosphorylation (Fig. 6A). Furthermore,
Calmodulin inhibitors do not inhibit CRHSP-28 phosphorylation. In agreement with the Ca\textsuperscript{2+}-dependent phosphorylation of CRHSP-28, it has been reported (21) that the protein is a substrate for the multifunctional CaMKII in vitro. To determine if CRHSP-28 is similarly regulated in intact cells, T84 monolayers were treated with KN93, a potent and selective inhibitor of CaMKII (17). Pretreatment of T84 cells for up to 20 h with concentrations of KN93 as high as 30 \(\mu\)M had no effect on CRHSP-28 phosphorylation in response to CCh (results not shown). The KN93 used was active, because 10 \(\mu\)M of the compound was found to inhibit >90% of purified CaMKII activity when tested in vitro (data not shown). Previous studies in pancreatic β-cells indicate that the permeability of KN93 may be increased by using the hemolytic agent α-toxin (2), which produces small pores in the plasma membrane and allows diffusion of molecules <2 kDa into the intracellular compartment. Thus to increase the permeability of KN93, T84 cells were permeabilized with α-toxin and then stimulated by an elevation of intracellular Ca\textsuperscript{2+} (Fig. 7A). Although increasing cellular Ca\textsuperscript{2+} strongly stimulated CRHSP-28 phosphorylation in α-toxin-treated cells, inclusion of KN93 had no effect on phosphorylation of the protein under control or Ca\textsuperscript{2+}-stimulated conditions.

KN93 acts to inhibit the association of calmodulin with CaMKII (25). To further investigate a potential role for an additional calmodulin-activated kinase in mediating CRHSP-28 phosphorylation, the broad-specific cell-permeable calmodulin antagonist W7 was used. Pretreatment of cells with 300 \(\mu\)M W7 (IC\textsubscript{50} for calmodulin, 25 \(\mu\)M) for 30 min partially increased the basal phosphorylation of CRHSP-28 but had no effects on CCh-stimulated activity. Similar results were obtained by using the calmodulin antagonist trifluoperazine (data not shown).

In vitro phosphorylation of CRHSP-28. Prediction analysis (http://www.cbs.dtu.dk/services/NetPhos/) indicates that CRHSP-28 contains potential sites for serine phosphorylation by CKII (Ser-26, -75, and -136), CKI (Ser-115 and -119), PKA or protein kinase G (Ser-100), and GSK3 (Ser-100, -111, and -115). Phosphorylation by GSK3 requires the COOH-terminal serine to be phosphorylated. The CKI site at Ser-119 is

neither cAMP nor cGMP analogs altered CRHSP-28 phosphorylation in response to CCh. In contrast, agents that increase cellular Ca\textsuperscript{2+} markedly stimulated CRHSP-28 phosphorylation, including the Ca\textsuperscript{2+}-ionophore ionomycin and the endoplasmic reticulum Ca\textsuperscript{2+}-ATPase inhibitor thapsigargin, which causes the release of Ca\textsuperscript{2+} from intracellular stores (Fig. 6, B and C). CRHSP-28 phosphorylation in response to CCh was also fully inhibited by buffering the intracellular Ca\textsuperscript{2+} concentration with BAPTA-AM (Fig. 6D). Pretreatment of cells with BAPTA-AM completely blocked CRHSP-28 phosphorylation irrespective of the presence of Ca\textsuperscript{2+} in the extracellular media. Together, these data establish that CRHSP-28 phosphorylation is fully dependent on elevated cellular Ca\textsuperscript{2+} and independent of cAMP-, cGMP-, and TPA-activated kinases.

Fig. 7. The Ca\textsuperscript{2+}/calmodulin-dependent kinase II (CaMKII) antagonist KN93 does not inhibit CCh-stimulated CRHSP-28 phosphorylation. A: T84 cells were permeabilized with α-toxin and incubated under conditions of basal Ca\textsuperscript{2+} (50 nM) for 15 min in the presence or absence of 30 \(\mu\)M KN93. Cells were then incubated in media containing basal or elevated Ca\textsuperscript{2+} (10 \(\mu\)M) with or without KN93 for 2 min before being harvested in IEF buffer. IEF and immunoblotting were used to detect CRHSP-28 phosphorylation.

Fig. 8. Characterization of CRHSP-28 kinase in T84 cells. Recombinant CRHSP-28 was incubated for 2 h at 30°C with casein kinase I (CKI), casein kinase II (CaMKII), Ca\textsuperscript{2+}/calmodulin-dependent protein kinase I (CaMKI), CaMKII, protein kinase A (PKA), or glycogen synthase kinase 3 (GSK3) in the presence of [\(\gamma\text{-32P}\)]ATP. A: proteins were resolved by SDS-PAGE, and phosphorylated CRHSP-28 was quantified by liquid scintillation counting. Data are means ± SE of at least 3 determinations. B: phosphorylated CRHSP-28 was transferred to polyvinylidene difluoride membrane and subjected to phosphoamino acid analysis. C: recombinant CRHSP-28 was phosphorylated in vitro by CKII or CaMKII or was immunoprecipitated from CCh-stimulated \(32\text{P}\)-labeled T84 cells and resolved by SDS-PAGE. Phosphorylated protein was digested with chymotrypsin, and the resulting peptides were separated by IEF. Phosphopeptide maps were identified with a phosphoimager.
four residues COOH-terminal to Ser-115, suggesting that the protein may be acted on by both enzymes. The sequence surrounding Ser-100 also shows specificity for phosphorylation by CaMKII (20). No obvious consensus sites for CRHSP-28 phosphorylation by CaMKII are present. Studies were conducted to evaluate the ability of these enzymes to phosphorylate recombinant CRHSP-28 in vitro (Fig. 8A). Of the kinases tested, CRHSP-28 was most highly phosphorylated by CKII, incorporating ~0.14 mol of phosphate per mole of protein. In contrast, CRHSP-28 incorporated ~10-fold less phosphate when incubated with CaMKII. Little or no phosphorylation of CRHSP-28 was detected following incubation with PKA, CaMKI, CKI, GSK3, or sequential incubation with CKI and GSK3. As seen for phosphorylation of CRHSP-28 in intact cells, CKII- and CaMKII-mediated phosphorylation of the protein occurred exclusively on serine residues (Fig. 8B).

To begin to identify which kinase mediates CRHSP-28 phosphorylation in vivo, phosphopeptide mapping was conducted on in vitro-phosphorylated CRHSP-28 and endogenous CRHSP-28 immunoprecipitated from 32P-labeled T84 cells (Fig. 8C). Chymotryptic digestion of CRHSP-28 following CKII or CaMKII phosphorylation produced distinct and non-overlapping phosphopeptide profiles. The phosphopeptide generated following CKII phosphorylation focused at a more alkaline pH than that seen for CaMKII. The phosphopeptide map generated from in vitro-phosphorylated CRHSP-28 by CaMKII was identical to that for endogenous CRHSP-28 from T84 cells that had been stimulated with CCh. Thus, although CRHSP-28 is more highly phosphorylated by CKII in vitro, these findings indicate that CKII does not phosphorylate CRHSP-28 in intact cells. Rather, these data are consistent with CRHSP-28 phosphorylation being mediated by CaMKII or a CaMKII-like enzyme.

CRHSP-28 is localized to the apical cytoplasm of polarized T84 cells. Confocal immunofluorescence microscopy was used to analyze the expression of CRHSP-28 in T84 cells. When grown on glass slides, CRHSP-28 was present in a punctate pattern throughout the cytoplasm and was particularly abundant on vesicular structures in perinuclear regions of cells (Fig. 9A). Analysis of T84 monolayers that had been cultured on permeable membrane supports to promote the development of a polarized phenotype revealed that CRHSP-28 was abundant in the perinuclear and apical regions of cells under basal conditions (Fig. 9B). Stimulation of cells with 100 μM CCh for 2 min promoted the accumulation of CRHSP-28 within the apical cytoplasm immediately below the plasma membrane. The apical accumulation of CRHSP-28 was most evident immediately on CCh stimulation and then returned to a prestimulatory appearance within 15 to 30 min (data not shown).

Effects of cytoskeletal-disrupting agents on CRHSP-28 phosphorylation and CaMKII localization in T84 cells. Subcellular fractionation and immunofluorescence microscopy in pancreatic acinar cells suggests that CRHSP-28 enters an apical endosomal compartment following secretagogue stimulation (26). Because membrane trafficking in epithelial cells is mediated by microtubule and actin-cytoskeleton proteins, the effects of pharmacological agents that target disruption of these structures on CRHSP-28 phosphorylation was analyzed (Fig. 10). Pretreatment of cells with the microtubule-stabilizing agent nocodazole at 33 μM, a concentration previously shown to fully depolymerize microtubules in T84 cells (28), slightly elevated basal

Fig. 9. CRHSP-28 is localized to a perinuclear and apical compartment of polarized T84 cells. T84 cells were cultured on glass slides (A–C) or raised supports (D–G) to induce a polarized morphology having apical (AP) and basal (BL) regions. Sections were fixed in 4% paraformaldehyde and examined by confocal microscopy. CRHSP-28 antibodies (2 μg/ml) were identified by using an FITC-conjugated secondary antibody (1:400). Nuclei were stained with To-Pro T-3 nucleic acid stain (8 μM). A: punctate staining of CRHSP-28 in perinuclear regions of cells grown on glass coverslips. B: same section as in A, showing nuclear staining. C: overlay of A and B showing CRHSP-28 in green and nuclei in red. D: CRHSP-28 staining is present throughout the cytoplasm of control cells grown on raised supports. E: CRHSP-28 translocates to subapical regions of cells following stimulation with 100 μM CCh for 2 min. F and G: dual staining of CRHSP-28 (green) and nuclei (red) in the same sections shown in D and E.
levels of CRHSP-28 phosphorylation but had no significant effect on CCh-stimulated phosphorylation of the protein. Conversely, disruption of actin filaments by using 20 μM cytochalasin D, as previously described (24), caused an ~50% reduction in CCh-stimulated CRHSP-28 phosphorylation. Cells treated with cytochalasin D for 1 h remained viable as determined by trypan blue exclusion.

Consistent with previous studies of mucosal epithelium (19), immunofluorescence localization of CaMKII in T84 cells indicated that the enzyme was highly localized to a subapical region that strongly overlapped with the prominent actin filament web (Fig. 11). In cell sections where cytochalasin D treatment fully disrupted actin filaments, CaMKII was effectively displaced from the subapical region and was diffusely localized throughout the cytoplasm. Although cytochalasin D treatment inhibited CRHSP-28 phosphorylation, the compound had no effects on CRHSP-28 localization in CCh-treated cells where the actin cytoskeleton was clearly disrupted. Analysis of multiple sections from cytochalasin D-treated cells by rhodamine-phalloidin staining indicated that actin filaments were clearly disrupted in only ~50% of the cells. Less-than-complete disruption of the actin cytoskeleton was evident even following prolonged incubations with cytochalasin D. This partial disruption of actin filaments likely reflects a slow rate of filament turnover, because cytochalasin D acts to inhibit the polymerization of actin monomers rather than to depolymerize existing structures.

**DISCUSSION**

In the current study, use of pharmacological agents including Ca²⁺ ionophore, thapsigargin, and BAPTA-AM as well as elevated Ca²⁺ concentrations in α-toxin-permeabilized cells clearly established the Ca²⁺ dependence of CRHSP-28 phosphorylation in T84 cells. Parente et al. (21) previously demonstrated that CSPPP28/CRHSP-28 is phosphorylated by CaMKII in vitro. The current data confirm and expand these findings by using phosphopeptide mapping to establish that a CaMKII-like enzyme in intact cells acts on CRHSP-28.

Unexpectedly, the calmodulin antagonists KN93, W7, and trifluoperazine did not inhibit CRHSP-28 phosphorylation in response to CCh. The explanation for the lack of effects of these agents is uncertain. One possibility is that CaMKII is partially autoactivated under basal conditions in T84 cells. Autoactivation of CaMKII entails the trapping of calmodulin on the enzyme (3). Calmodulin trapping occurs as a result of a phosphorylation-induced increase in the affinity of calmodulin for the enzyme, making this association independent of elevated Ca²⁺. Because calmodulin trapping is transient in the presence of elevated Ca²⁺, high levels of calmodulin may exist locally around the enzyme.
zyme to support its activation in the presence of inhibitors. Moreover, the high concentration of CaMKII present at the cell apex may create a localized environment that further limits access of the inhibitors to the enzyme. Other possibilities include that CRHSP-28 may be closely associated with CaMKII and/or calmodulin within the cell, which may act to overcome competitive inhibition by calmodulin antagonists. Finally, it cannot be ruled out that CRHSP-28 phosphorylation is regulated by a calmodulin-independent enzyme that creates an identical phosphopeptide map to that seen for CaMKII.

The serine phosphorylation of CRHSP-28 by CKII was 10-fold more efficient than that seen for CaMKII. These findings are consistent with the multiple CKII consensus sites present in the protein. CRHSP-28 does not contain an Arg-X-X-Ser sequence typical of CaMKII substrates (11). It does contain a lysine residue positioned three amino acids NH2-terminal to Ser-100; whether lysine can substitute as a basic amino acid for CaMKII recognition is uncertain. Although phosphopeptide mapping clearly suggests that CRHSP-28 is regulated by CaMKII rather than CKII, the stoichiometry of phosphorylation by these enzymes was less than optimal, both incorporating <1 mol of phosphate per mole of protein. These findings may reflect that CRHSP-28 forms homooligomers in vitro (9, 23) that potentially restrict access of the kinase to the protein.

The transient phosphorylation of CRHSP-28 following CCh stimulation suggests that the protein may translocate within the cytoplasm to alter its association with kinase and phosphatase enzymes. Although CRHSP-28 subcellular fractionation was not altered by CCh treatment (data not shown), the dual association of the protein with soluble and particulate fractions of cell lysates is consistent with a dynamic localization of CRHSP-28 within the cell. Also supporting CRHSP-28 movement within the cytoplasm was the apparent CCh-induced accumulation of the protein along the apical plasma membrane where CaMKII was shown to be highly concentrated. The finding that cytochalasin D treatment both disrupted the localization of CaMKII and significantly inhibited CRHSP-28 phosphorylation supports the hypothesis that apical translocation is necessary to bring CRHSP-28 in close proximity with the kinase.

Recent studies implicating CRHSP-28 and its associated proteins (26, 30) in membrane-trafficking events in polarized epithelia support a phosphorylation-dependent regulatory function for the protein. On the basis of the Ca2+-sensitive interaction of CRHSP-28 with thezymogen granule membrane protein annexin VI, as well as the peripheral association of CRHSP-28 with endosome-enriched membrane fractions of acinar cells, we recently proposed (26) that CRHSP-28 is recruited to the cell apex during exocytosis and enters an early endosomal compartment during membrane retrieval. One possibility is that phosphorylation promotes the dissociation of CRHSP-28 from a multiprotein complex on vesicle membranes, likely involving annexin VI (26) and/or MAL2 (30). Release of CRHSP-28 to the cytosol would facilitate its dephosphorylation by a constitutively active phosphatase. Clearly, the Ca2+-dependence of CRHSP-28 phosphorylation is in line with the Ca2+-dependent effects of the protein on digestive enzyme secretion (27) and annexin VI binding in acinar cells (26). Identification of the precise serine phosphorylation sites on CRHSP-28 will be invaluable in elucidating the important regulatory role of this protein in epithelial membrane trafficking.

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DISCLOSURES

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