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

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CaM kinase II (CaMKII) is a calcium/calmodulin-dependent protein kinase involved in regulating a variety of cellular functions. In this study, we investigated the role of CaMKII in the phosphorylation of CRHSP-28, a member of the tumor protein D52 (TPD52) family, which is highly expressed in exocrine glands and regulates digestive enzyme secretion from pancreas, salivary, lacrimal, and gastric glands. We found CRHSP-28 highly expressed in T84 cells, consistent with an important regulatory role in apical membrane trafficking. Stimulation of T84 cells with carbachol (CCh) induced rapid, concentration-dependent phosphorylation of CRHSP-28 on two serine residues. Isoelectric focusing and immunoblotting were used to characterize cellular mechanisms governing CRHSP-28 phosphorylation. Phosphorylation depended on elevated cytosolic Ca2+, being maximally induced by ionomycin and thapsigargin and fully inhibited by BAPTA-AM. In vitro phosphorylation of recombinant CRHSP-28 was 10-fold greater by casein kinase II (CKII) than Ca2+/calmodulin-dependent protein kinase II (CaMKII). However, phosphopeptide mapping studies demonstrated that CaMKII induced an identical phosphopeptide profile to endogenous CRHSP-28 immunoprecipitated from T84 cells. Although calmodulin antagonists had no effect on CCh-stimulated phosphorylation, disruption of actin filaments by cytochalasin D inhibited phosphorylation by 50%. Confocal microscopy indicated that CRHSP-28 is expressed in perinuclear regions of cells and accumulates immediately below the apical membrane of polarized monolayers following CCh stimulation. CaMKII was also localized to the subapical cytoplasmic web and was clearly displaced following actin filament disruption. These data suggest that CRHSP-28 phosphorylation is regulated by a CaMKII-like enzyme and likely involves a translocation of the protein within the apical cytoplasm of 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 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 amphotericin were from Amersham Pharmacia Biotech (Piscataway, NJ); α-toxin, 12-O-tetradecanoylphorbol 13-acetate (TPA), 8-(4-chlorophenylthio)-cAMP (cPT-cAMP), carbachol (CCh), ionomycin, d-phospho-L-serine, d-phospho-L-threonine, d-phospho-L-tyrosine, EDTA, ninyhdrin, phenylmethylsulfon 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 were purchased from Pierce (Rockford, IL); peroxidase-conjugated donkey antimyoglobin (1) from Biochemicals (Lakewood, NJ). The human adenoma cell line T84 was obtained from the American Type Culture Collection (Rockville, MD) and was passaged by using trypsin-EDTA. Media were supplemented with 10% fetal bovine serum and containing N2 medium containing 0.5 M 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 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 amphoteries (pI 2.5–5 and 3–10). Samples were resolved by using a Pharmacia Biotech Multiphor II apparatus. Gels were prefocused 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 then transferred to nitrocellulose 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 phosphoisoforms 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 phenylmethylsulfon fluoride, and protease inhibitor cocktail, pH 7.4. After centrifugation at 100,000 g for 90 min, 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 [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 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 mgATP. GSK3 and CKI assays were conducted in the same buffer containing 2 mM DTT. Phosphorylation by Ca²⁺/calmodulin-dependent protein kinase I (CaMKI) and CaMKII was conducted in a buffer containing (in mM) 50 HEPES, pH 7.0, 10 MgCl₂, 1 CaCl₂, 0.01 mgATP, and 30 μg/ml calmodulin. 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 (1:400), and nuclei were visualized by using 8 μM To-Pro T-3 nucleic acid stain. CaMKII was analyzed by using the polyclonal antibody G301 (1:100) and was stained with an Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (1:1,000). Actin filaments were stained with rhodamine-phalloidin (165 nM). Immunofluorescence microscopy 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). Separation of T84 cell proteins by two-dimensional 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

produced an approximate twofold increase in phosphate incorporation into the 28-kDa protein. Phosphoamino acid analysis indicated that CRHSP-28 was phosphorylated exclusively on serine residues (Fig. 2B). No radioactivity was incorporated into phosphothreonine or phosphotyrosine.

Characterization of CRHSP-28 phosphoisoforms by IEF. IEF was used to further characterize the phosphorylation state of CRHSP-28 in T84 cells (Fig. 3). Identification of CRHSP-28 isoforms by immunoblotting (labeled IB) after IEF exhibited that the protein focused primarily in two bands under basal conditions (labeled α and β). On stimulation with CCh (100 μM, 2 min), CRHSP-28 shifted to a third band having a more acidic isoelectric point (labeled γ), corresponding to an increased phosphorylation state of the protein. For comparison, phosphorylated isoforms of CRHSP-28 were identified on the IEF gels following immunoprecipitation (labeled IP) from 32P-labeled cells. Radiolabeled CRHSP-28 focused only in the two acidic β- and γ-isoforms but was absent in the α-isoform, as would be expected with an increased negative charge of the protein following phosphate incorporation. Collectively, these data indicate that CRHSP-28 separates into three distinct phosphoisoforms when separated by IEF, a single nonphosphorylated α-form and two more acidic phosphorylated β- and γ-forms.

Fig. 3. Detection of CRHSP-28 phosphoisoforms by isoelectric focusing (IEF). T84 cells were treated as control or with 100 μM CCh for 2 min, and lysates (30 μg/lane) were separated by IEF and identified by immunoblotting (IB) with anti-CRHSP-28 antibodies (1 μg/ml). Immunoblotting reveals an acidic shift in CRHSP-28 from predominantly the α-isoform in control to the β- and γ-isoforms following CCh stimulation. CRHSP-28 that was immunoprecipitated (IP) from 32P-labeled T84 cells was resolved in parallel and detected by autoradiography. A lysate incubated with protein A beads (Bd) alone served as control. Note the radiolabeling of the β- and γ-isoforms of CRHSP-28 and the absence of radiolabel in the α-isoform.

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. A: T84 cells were treated with indicated concentrations of CCh for 2 min, and CRHSP-28 phosphorylation was determined by IEF and immunoblotting. B: CRHSP-28 phosphoisoforms 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. Gel shows a single representative experiment.

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,
CRHSP-28 phosphorylation in response to CCh. In contrast, agents that increase cellular Ca\(^{2+}\) markedly stimulated CRHSP-28 phosphorylation, including the Ca\(^{2+}\)-ionophore ionomycin and the endoplasmic reticulum Ca\(^{2+}\)-ATPase inhibitor thapsigargin, which causes the release of Ca\(^{2+}\) from intracellular stores (Fig. 6, B and C). CRHSP-28 phosphorylation in response to CCh was also fully inhibited by buffering the intracellular Ca\(^{2+}\) concentration with BAPTA-AM (Fig. 6D). Pretreatment of cells with BAPTA-AM completely blocked CRHSP-28 phosphorylation irrespective of the presence of Ca\(^{2+}\) in the extracellular media. Together, these data establish that CRHSP-28 phosphorylation is fully dependent on elevated cellular Ca\(^{2+}\) and independent of cAMP-, cGMP-, and TPA-activated kinases.

Calmodulin inhibitors do not inhibit CRHSP-28 phosphorylation. In agreement with the Ca\(^{2+}\)-dependent of cAMP-, cGMP-, and TPA-activated kinases. CRHSP-28 phosphorylation in response to CCh was control or Ca\(^{2+}\)-dependent phosphorylation of cells with BAPTA-AM completely blocked CRHSP-28 phosphorylation irrespective of the presence of Ca\(^{2+}\) in the extracellular media. Together, these data establish that CRHSP-28 phosphorylation is fully dependent on elevated cellular Ca\(^{2+}\) and independent of cAMP-, cGMP-, and TPA-activated kinases.

Calmodulin inhibitors do not inhibit CRHSP-28 phosphorylation. In agreement with the Ca\(^{2+}\)-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 \(\beta\)-cells indicate that the permeability of KN93 may be increased by using the hemolytic agent \(\alpha\)-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 \(\alpha\)-toxin and then stimulated by an elevation of intracellular Ca\(^{2+}\) (Fig. 7A). Although increasing cellular Ca\(^{2+}\) strongly stimulated CRHSP-28 phosphorylation in \(\alpha\)-toxin-treated cells, inclusion of KN93 had no effect on phosphorylation of the protein under control or Ca\(^{2+}\)-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-specificity cell-permeable calmodulin antagonist W7 was used. Pretreatment of cells with 300 \(\mu\)M W7 (IC\(_{50}\) 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 was quantified by liquid scintillation counting. Data are means \pm SE of at least 3 determinations. A: proteins were resolved by SDS-PAGE, and phosphorylated CRHSP-28 was quantified by densitometry. B: phosphorylated CRHSP-28 was transferred to polyvinylidene difluoride membrane and subjected to phosphoimager analysis. Recombinant CRHSP-28 was phosphorylated in vitro by CKII or CaMKII or was immunoprecipitated from CCh-stimulated \(32^P\)-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.

![Fig. 7. The Ca\(^{2+}\)/calmodulin-dependent kinase II (CaMKII) antagonist KN93 does not inhibit CCh-stimulated CRHSP-28 phosphorylation. A: T84 cells were permeabilized with \(\alpha\)-toxin and incubated under conditions of basal Ca\(^{2+}\) (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\(^{2+}\) (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.](http://www.cbs.dtu.dk/services/NetPhos/)
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 incubation with PKA, CaMKI, CKI, GSK3, or sequential incubation with CKI and GSK3. As seen for incubation with 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-destabilizing agent nocodazole at 33 μM, a concentration previously shown to fully depolymerize microtubules in T84 cells (28), slightly elevated basal

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**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 basolateral (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 en...

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**Fig. 10.** Actin filament disruption inhibits CRHSP-28 phosphorylation. Cells were pretreated for 1 h with 20 μM cytochalasin D or 3 h with 33 μM nocodazole and then stimulated with 100 μM CCh for 2 min. CRHSP-28 phosphorylation was determined by IEF and immunoblotting. Phosphorylation was quantified by densitometry and expressed as the percent of total CRHSP-28 present in the blot. Data are means ± SE of 3 independent experiments, each performed in duplicate.

**Fig. 11.** CaMKII localization to the subapical cytoplasm is dependent on an intact actin cytoskeleton. Cells were treated as control (A, B) or pretreated with 20 μM cytochalasin D (C–F) for 1 h; all cells were then stimulated with 100 μM CCh for 2 min. A and C: T84 cell sections were stained with the CaMKII antibody G301 (1:100) followed by an Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (1:1,000). B, D, and F: actin filaments were stained with rhodamine-phalloidin (165 nM). E: CRHSP-28 immunostaining was detected by using an FITC-conjugated secondary antibody (1:400). Note that actin filament disruption displaces CaMKII from the subapical compartment, resulting in a diffuse staining of the enzyme within the cytoplasm, whereas CRHSP-28 is unaltered and remains localized in subapical regions. AP, apical membrane; BL, basolateral membrane.
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-Ser sequence typical of CaMKII substrates (11). It does contain a lysine residue positioned three amino acids NH2-terminal to Ser100; 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 a calmodulin-independent enzyme that further limits access of the inhibitors to the protein, the stoichiometry of phosphorylation by these enzymes 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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