Calmodulin Binds and Stabilizes the Regulatory Enzyme, CTP:Phosphocholine Cytidylyltransferase*

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CTP:phosphocholine cytidylyltransferase (CCTα) is a proteolytically sensitive enzyme essential for production of phosphatidylcholine, the major phospholipid of animal cell membranes. The molecular signals that govern CCTα protein stability are unknown. An NH2-terminal PEST sequence within CCTα did not serve as a degradation signal for the proteasine, calpain. Calmodulin (CaM) stabilized CCTα from calpain proteolysis. Adenoviral gene transfer of CaM in cells protected CCTα, whereas CaM small interfering RNA accentuated CCTα degradation by calpains. CaM bound CCTα as revealed by fluorescence resonance energy transfer and two-hybrid analysis. Mapping and site-directed mutagenesis of CCTα uncovered a motif (LQERVDKVK) harboring a vital recognition site, Gln243, whereby CaM directly binds to the enzyme. Mutagenesis of CCTα Gln243 not only resulted in loss of CaM binding but also led to complete calpain resistance in vitro and in vivo. Thus, calpains and CaM both access CCTα through two major sites: PEST (proline-glutamate-serine-threonine) sequences, in part, by docking to two major motifs within its subunits: PEST (proline-glutamate-serine-threonine) sequences or CaM binding motifs. Each isoform consists of two distinct subunits, a larger 80-kDa calmodulin subunit and a smaller 30-kDa regulatory subunit, forming a heterodimeric structure. The large and small subunits consist of four (I–IV) and two (I–II) domains, respectively. EF-hand motifs within each subunit allow for heterodomain interactions and calcium binding. Calpain cleaves its substrates, in part, by docking to two major motifs within its subunits: PEST (proline-glutamate-serine-threonine) sequences and calmodulin (CaM) binding domains. PEST sequences within IkBα and the ATP-binding cassette transporter 1 serve as proteolytic signatures for calpain degradation (11, 12). Likewise, CaM binding domains within a calcium-ATPase pump and inducible nitric-oxide synthase impact their sensitivity to calpain hydrolysis (13, 14). A “calmodulin-like” domain also exists within the catalytic subunit of calpain that facilitates interaction with some PEST sequences or CaM binding motifs (15, 16). Indeed, data base analysis (available on the World Wide Web) identified a consensus PEST sequence within the CCTα NH2-terminal domain. Thus, this PEST sequence or other structural motifs that recognize CaM might brand CCTα for its elimination within cells.

Despite being a calpain substrate, CCTα is a relatively stable enzyme. CCTα is highly abundant in cells, is cytosolic in pneumocytes, and has an extended half-life (17). Typically, larger, hydrophobic, cytosolic proteins and regulatory enzymes exhibit faster turnover rates (18). The half-life of CCTα (~8 h) also exceeds that of other metabolic enzymes, including hydroxymethylglutaryl-CoA reductase and phosphoenolpyruvate carboxykinase.

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The abbreviations used are: PtdCho, phosphatidylcholine; CCTα, CTP:phosphocholine cytidylyltransferase; CaM, calmodulin; MLE, murine lung epithelial; LDL, low density lipoprotein; ERK, extracellular signal-regulated kinase; siRNA, small interfering RNA; GST, glutathione S-transferase; TNT, transcription and translation; Ox-LDL, oxidized LDL; YFP, yellow fluorescent protein; FRET, fluorescence resonance energy transfer; CFP, cyan fluorescent protein.
vate carboxykinase (19). These observations strongly suggest existence of covalent modifications, stabilizing ligands, or binding partners that enhance the life span of CCTα in vivo.

CaM (16.7 kDa) is a highly conserved calcium-sensing protein that binds and modulates stability of some cytoskeletal and ion transport proteins (20–22). CaM binds proteins in its calcium-bound (holo-CaM) or calcium-free form (apo-CaM). CaM binding proteins are thus classified into Ca2+-dependent, Ca2+-independent, and Ca2+-inhibited proteins (20). Many CaM binding proteins harbor recognition motifs characterized by a basic amphipathic helix, moderate to high helical hydrophobic moment, and a net positive charge (20). Other motifs described include an IQ motif ((I/L)QXXRXX), a calcium-binding EF hand motif, and a net positive charge (20). Other motifs described include an IQ motif ((I/L)QXXRXX), a hydrophobic moment, and a net positive charge (20). Other motifs described include an IQ motif ((I/L)QXXRXX), a calcium-binding EF hand motif, and a net positive charge (20).

In the present study, we investigated the hypothesis that specific molecular sequence signatures confer stability to CCTα. We show for the first time that CCTα is a CaM-binding enzyme and that CaM protects CCTα from calpain degradation. A conceptually unique finding of this study is that a highly conserved residue (Gln243) (rather than an NH2-terminal PEST sequence) serves as an essential molecular recognition site for competition between CaM and calpain for access to CCTα. Mutagenesis of Gln243 within CCTα totally blocked CaM binding but also the ability of calpain to degrade CCTα in vitro and in vivo. The intermolecular competition between a proteinase and a stabilizing protein for access to a single recognition site within CCTα represents a novel mechanism regulating an enzyme’s availability.

**EXPERIMENTAL PROCEDURES**

Materials—The sources of murine lung epithelial (MLE) cells, LDL, CCTα, extracellular signal-regulated kinase (ERK) antibodies, TrueBlot IgG, FuGENE6 transfection kits, and transcription and translation (TNT) coupled reticulocyte lysate were described previously (23). Rabbit polyclonal antibodies to M- and µ-calpain were from ABR-Affinity BioReagents (Golden, CO). Rabbit monoclonal calmodulin antibody was purchased from Upstate (Billerica, MA). Rabbit polyclonal calmodulin kinase II antibody was purchased from Epitomics (Burlingame, CA). Purified µ-calpain, recombinant CaM, and the calpain substrate peptide, LLVY, were purchased from Calbiochem. The pCR-TOPO4 cloning kit, Escherichia coli One Shot competent cells, pENTR Directional TOPO cloning kits, and the Gateway mammalian expression system were purchased from Invitrogen. The QuikChange site-directed mutagenesis kit and the X-blue competent cells were purchased from Stratagene (La Jolla, CA). The gel extraction kit and QIAprep Spin Miniprep Kit were from Qiagen (Valencia, CA). Nucleofector transfection kits were from Amaxa (Gaithersburg, MD). Calmodulin-Sepharose 4B beads were purchased from Amershambiosciences. Immobilized glutathione-agarose beads were purchased from Pierce. BD TALON purification and buffer kits were purchased from BD Biosciences. Calmodulin siRNAs were purchased from Dharmacon (Chicago, IL). A mammalian calmodulin cDNA, pEx1-CaM, was kindly provided by Dr. Madeline Shea (University of Iowa, Iowa City, IA).

Calmodulin siRNAs were purchased from Dharmacon (Chicago, IL). A mammalian calmodulin cDNA, pEx1-CaM, was kindly provided by Dr. Madeline Shea (University of Iowa, Iowa City, IA) (24). All DNA sequencing was performed by the University of Iowa DNA core facility.

**Construction of CCTα PEST Mutants**—A CCTα variant harboring point mutations in the PEST domain (CCTPESTsdm) was constructed where Thr25 and Ser32 were mutated to Ala using the QuikChange site-directed mutagenesis kit. A full-length CCTα template (pCMV-CCTα) plasmid DNA was used as a template. The primers used to mutate Thr25 were 5′-GCCCTAATGGGACGAGCAGGAGAGATGGG-3′ (forward) and 5′-CCATCTTCTCTCTGCTCATTAGGGCC-3′ (reverse). The primers used to mutate Ser32 were 5′-GAAGATGGAAT-CTCGTCCAAAAGTCGACGGG-3′ (forward) and 5′-GGCGTGACATTGAGGAGATTCCATCTCC-3′ (reverse). PCR conditions were as follows: initial denaturation at 95 °C for 2 min and then denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, elongation at 68 °C for 6 min, 18 cycles for three steps.

An internal deletion mutant lacking the PEST sequence (CCTPESTsoe) was constructed using splicing by overlapping extension PCR. Full-length CCTα cloned into TOPO4 (TOPO-CCTαfl plasmid) was used as a template. Four primers, CCTPEST1 to -4, were designed (CCTPEST1, 5′-CAGCATGGATGCGAGATTCAAGC3′; CCTPEST2, 5′-ACTGACATGGCTGACATTTCTCTCTTCGTGAGATTGACTTTA-3′; CCTPEST3, 5′-CAAATGCAATTACCAGAGAAGGGAGAAGATTGCAGGTGACT-3′; CCTPEST4, 5′-TCAGTCTCTTCATCTCCCTCTGCTG-3′). In the first step, primers CCTPEST1 and CCTPEST2 were used to amplify an NH2-terminal fragment of CCTα. In the second step, primers CCTPEST3 and CCTPEST4 were used to amplify a COOH-terminal CCTα fragment. Each fragment flanked the PEST. In the last step, the two gel-purified fragments from the steps above were used as a template in the final PCR using primers CCTPEST1 and CCTPEST4 to amplify a desired 1050-bp product lacking the PEST. This fragment was purified and cloned into pCR4-TOPO. The PCR conditions were as follows: 95 °C for 30 s and 18 cycles at 95 °C for 30 s, 55 °C for 30 s, and 68 °C for 5 min. After DNA sequence confirmation, TOPO-CCTPESTsoe was used as the template using the primers 5′-AAGCTTATGGATGCGAGATTCAAGC3′ (forward) and 5′-TCCTCCCTCTTCATCTGGATGACT-3′ (reverse) to amplify the 1050-bp CCTα fragment. The forward primer contains a recognition site for HindIII, the reverse primer contains a recognition site for XhoI. The PCR product was cloned into pCR4-TOPO, followed by digestion with the same enzymes prior to directional cloning into pcDNA3.1/V5-his.

**Construction of Glutathione S-Transferase (GST)-tagged CCTα Domain and Carboxyl-terminal Mutants**—A series of internal CCTα domain deletion mutants were constructed using TOPO-CCTαmem and TOPO-CCTαcat, TOPO-CCTαpest that were first generated as described previously or by using splicing by overlapping extension PCR. These constructs were used as a template in PCR to generate GST-CCTαmem and GST-CCTαcat, two constructs devoid of the membrane binding domain or catalytic domain, respectively. The forward primer, 5′-CACCAGATGAGTCCAGTCACAGT-3′, and reverse primer, 5′-GTCCCCTCTCATCTCGCCTGA-3′, were used in the PCR conditions as follows: 95 °C for 30 s and 25 cycles at
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95 °C for 30 s, 55 °C for 30 s, and 68 °C for 1 min. The PCR products were gel-purified and cloned into pENTR-TOPO.

A series of carboxyl-terminal deletion mutants were constructed as follows. pCMV5-CCT was used as a template for PCR using the forward primer 5’-CACATGGAGCACAGAGTTTCG-3’ and reverse primer 5’-TTGCAAGTGGTATTTCTTTTC-3’ for CCT315. 5’-ACTTGATGGCCTGGGACTAT-3’ for CCT260, 5’-CACCCTTGCACAATCTTTTGA3’ for CCT267, 5’-TGGAAGTGGATTTTCTCTTG-3’ for CCT243, 5’-GACAAGTGGGTAGATGTG-3’ for CCT210, 5’-TGATCTTTTCCCTACATTTTCA-3’ for CCT260, and 5’-CCTTACCTTTG-3’ for CCT250. An NH2-terminal CCTα deletion mutant (CCTN40) was constructed using the forward primer 5’-CACCTTGCCAGGAGTGGCT-3’ and reverse primer 5’-GTCCCTCTTCATCCTGCTGA-3’. The PCR conditions were as follows: 95 °C for 30 s and 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 68 °C for 1 min. All PCR products were gel-purified and cloned into pENTR-TOPO. Finally, for cloning into GST fusion constructs, 150 ng of pENTR-TOPO plasmids and 150 ng of pDEST27 GST destination vector were incubated with LR Clonase enzyme mix at 25 °C for 1 h per the manufacturer’s instructions. The reactions were terminated by adding 1 µl of proteinase K solution and heated at 37 °C for 10 min. The plasmids were transformed into E. coli TOP10 competent cells, followed by plasmid preparation.

A CCTα variant harboring a point mutation (CCTQ243A) where Gln243 was mutated to Ala was generated using the QuikChange site-directed mutagenesis kit. GST-CCT51 plasmid was used as a template for PCR using forward primer 5’-GAAAAAGAATACACCTTGGCAGGATTTGATAAGG-3’ and reverse primer 5’-CCTTATCAACTCGTTCTG-3’. The thermal cycling program was as follows: initial denaturation at 95 °C for 2 min and then denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 68 °C for 10 min, 18 cycles for three steps. The desired PCR product was gel-purified and fused to GST as above. A CCTα carboxyl-terminal deletion mutant harboring a similar point mutation (CCT267Q243A) was generated by PCR using GST-CCT267 as the template using methods described above. All of the above PCR products in pENTR-TOPO were verified by DNA sequencing.

In Vitro TNT—CCTα cDNA constructs cloned into pCR4-TOPO4 (2 µg of plasmid/reaction) were added directly to the rabbit reticulocyte lysate, incubated in 50 µl of reaction solution containing 2 µg of plasmids, 25 µl of rabbit reticulocyte lysate, 2.5 µl of RNase inhibitor, 1.2 µl of 1 mM amino acids (minus methionine), 2 µl of 17 RNA polymerase, and 5 µl of [35S]methionine (40 µCi/reaction). The reaction mixture was incubated at 30 °C for 30 min as described (23).

Calpain Proteolysis Assay—A 25-µl reaction volume containing 10 µl of TNT reaction products, 10 µl of calpain buffer (20 mM Tris, pH 7.5, 2 mM dithiothreitol, 1% Tween 20, and 0.015% Triton X-100), and 0.25–1 µg of purified µ-calpain was incubated at 37 °C for 0–1 h after adding CaCl2 to a final concentration of 200 µM. The reaction was terminated by adding 2× SDS protein loading buffer and heating to 95 °C for 5 min. Effects of calpain hydrolysis of CCTα were further tested in separate studies by inclusion of varying concentrations of CCTα, CaM, or the calpain substrate, LLVY, in the reaction mixture. In these studies, calpain was present at a fixed concentration of 0.6 pmol/reaction. The digestion products were resolved by SDS-PAGE, and gels were processed for autoradiography or immunoblotting. In other experiments, purified recombinant GST-CCTα and GST-CCTα mutants were used as substrates for calpain (0.005–0.05 µg) digestion.

Cell Culture—MLE cells were cultured in Dulbecco’s minimum essential medium containing 0% fetal bovine serum for up to 48 h with or without Ox-LDL (100 µg/ml). Cell lysates were prepared by brief sonication in 150 mM NaCl, 50 mM Tris, 1.0 mM EDTA, 2 mM dithiothreitol, 0.025% sodium azide, and 1 mM phenylmethylsulfonyl fluoride (Buffer A) at 4 °C prior to analysis. Cytosolic and microsomal preparations were isolated as described (25).

Lipoprotein Oxidation—Lipoproteins were dialyzed in phosphate-buffered saline at 4 °C for 24 h followed by oxidation in 5 µM CuSO4/phosphate-buffered saline for 24 h at 37 °C. Confirmation of lipoprotein oxidation was by the malonaldehyde assay and by detection of apoprotein B-100 degradation as described (7).

CCT Activity—Enzyme activity was determined by measuring the rate of incorporation of [methyl-14C]phosphocholine into CDP-choline using a charcoal extraction method (7). Assays were conducted with and without exogenous PtdCho/oleic acid lipid activator in the reaction mixtures.

PtdCho Analysis—Cells were pulsed-labeled with 1 µCi of [methyl-3H]choline chloride during the final 3 h of incubation, lipids were extracted and resolved, and activity within PtdCho was analyzed as described (7).

Immunoblot Analysis—Equal amounts of total protein (5–20 µg) in sample buffer were resolved using 10% SDS-PAGE and transferred to nitrocellulose, and immunoreactive CCTα or calmodulin was detected as described (23). The dilution factor for primary and secondary antibodies was 1:2000. CCTα was purified to homogeneity as described (26).

Co-immunoprecipitation—200 µg of total protein from MLE cell lysates were prefiltered with 20 µl of TrueBlue anti-lg beads for 1 h at 4 °C. 5 µg of CCTα, ERK, CaM, rabbit IgG, or calmodulin kinase II antibodies were added for a 2-h incubation at 4 °C. 20 µl of TrueBlue anti-lg beads were added for an additional 2-h incubation. Beads were spun down and washed five times using 50 mM HEPES, 150 mM NaCl, 0.5 mM EGTA, 50 mM NaF, 10 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 20 µM leupeptin, and 1% (v/v) Triton X-100 (radioimmune precipitation) buffer as described (23). Beads were heated at 100 °C for 5 min with 80 µl of protein sample buffer prior to SDS-PAGE and immunoblotting.

Expression and Knockdown of Recombinant Proteins—CCTα PEST mutants were expressed in cells using the Amaxa nucleofector system per the manufacturers’ instructions. Cellular expression of green fluorescent tagged plasmids using this device was achieved at >90% in MLE cells. Transfection of GST-CCTα fusion constructs was also conducted for 24 h in Dulbecco’s minimum essential medium/F-12 medium containing 0% fetal bovine serum using 18 µl of FuGene6 reagent and 6–10 µg/dish of the desired plasmid. After 24 h, the cells were
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Fluorescence Resonance Energy Transfer (FRET) Analysis—PCR-based strategies were used to create compatible restriction enzyme sites that would allow construction of chimeric cDNAs for FRET. A 1050-bp CCTα fragment was amplified in PCR using the primers 5’- ACAAGATCTATGGTACGACAG- 
AGTTCTAG-3’ (forward) and 5’- ACTGTCGACTTCATCTGGAT- 
TCATCTGTA-3’ (reverse) using pCMV5-CCTα as a template. The forward primer contains a recognition site for BglII, and the reverse primer contains a recognition site for SalI. The PCR product was gel-purified and digested with the same enzymes prior to cloning into pAmCyan-C1 (Clontech), generating CFP-CCTα.

An Adv-CaM vector (below) was used as template in PCR using the primers 5’- ACTGATCTATGGTACGACAG- 
ACGG-3’ (forward) and 5’- ACTGTCGACTTCATCTGGAT- 
TCATCTGTA-3’ (reverse) to amplify a 450-bp CaM fragment. The forward primer contains a recognition site for BglII, and the reverse primer contains a recognition site for SalI. This PCR product was gel-purified and digested with BglIII and SalI prior to cloning into a linearized pZsYellow1-C1 vector (Clontech), generating YFP-CaM.

For analysis of CCTα and CaM interaction by FRET, cells were first plated at 0.12 × 10⁶ cells/well in a two-chamber cover glass system. Cells were co-transfected with YFP-CaM and CFP-CCTα (2 μg of plasmid/chamber) with Fugene 6 (6 μl). The analysis was detected at the single cell level using a combination laser-scanning microscope system (LSM510/Confocor2; Zeiss, Jena, Germany). To achieve excitation, the 458-nm line of an argon ion laser was focused through the Zeiss ×60 oil differential interference contrast objective lens onto the cell. Emissions of YFP (the FRET acceptor) and CFP were collected through 535–595-nm and 470–500-nm barrier filters, respectively. Photobleaching was performed with 50 iterations and 100% intensity of a 514-nm laser. FRET quantitation of fluorescence images was generated using Zeiss Rel3.2 image software. The average fluorescence intensities per pixel were calculated following background subtraction.

Construction of an Adenoviral CaM Vector—A pEx1-CaM plasmid was used as a PCR template using the primers 5’- CTG- 
GAGATGGCTGACCAACTGACTGA-3’ (forward) and 5’-GGATACTTTTGTGTCATCTGTA-3’ (reverse) to amplify a 450-bp CaM fragment. The forward primer has an engineered Xhol site, and the reverse primer has an engineered BamHI site. PCR conditions were as follows: 95 °C for 30 s and 35 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. The PCR products were cloned into pCR4-TOPO followed by digestion with Xhol and BamHI. The pacAD5 cytomegalovirus IRESEGFP pA vector was also digested with Xhol and BamHI. These digestion products were fractionated by gel electrophoresis, and the desired 450-bp and 7.5-kb fragments were then purified and ligated using T4 DNA ligase at 25 °C for 1 h. The Gene Transfer Vector Core (University of Iowa) used the newly constructed adenovirus-CaM shuttle plasmid to generate a first generation adenovirus-CaM expression vector (27). Adenovirus vectors expressing the CaM transgene driven by the cytomegalovirus promoter (Adv-CaM) or an empty control vector containing the cytomegalovirus promoter but no trans-

...harvested in Buffer A followed by brief sonication. In some studies, advancement 24 h after transfection, 100 μg/ml Ox-LDL was added for an additional 24 h. For overexpression of CaM, 1 × 10⁶ cells were infected with Adv-CaM or an empty vector (Adv-Con) at MOI = 40 for 6 h prior to an additional 24-h incubation with Ox-LDL. For CaM knockdown, 1 × 10⁶ cells were electroporated with 1 nmol of siRNA. Cells were either transfected with siRNA (sense sequence, 5’-UGACAAACCUUGGAGA- 
GAAUU-UU-3’; antisense sequence, 5’- PUUCUCCCAAGGU- 
UUGUCAUU-3’) against CaM or with a control siRNA. 72 h after transfection, cells were exposed to Ox-LDL for an additional 24 h prior to harvest. In rescue studies, cells were either transfected with CaM siRNA or control siRNA; 48 h after transfection, cells were infected with Adv-CaM for another 24 h. Finally, Ox-LDL was added to the medium for an additional 24 h prior to harvest.

CCTα Degradation—CCTα degradation was determined by nucleofecting CaM siRNA or control siRNA in MLE cells as above, and 72 h later, cells were preincubated for 1 h in methionine-deficient medium and then pulsed with [35S]methionine (60 μCi/ml) for 4 h at 37 °C as described (25). Cells were rinsed, chased in medium replete with methionine and cysteine for 0–8 h, and processed for CCTα immunoprecipitation, SDS-PAGE, and autoradiography as described (7).

GST Pull-down Assays—After plasmid transfection, cellular lysates were prepared as described above, followed by incubation with 20 μl of immobilized glutathione-agarose beads at 4 °C for 1 h. After incubation, the beads were spun down and rinsed three times using buffer containing 250 mM NaCl and 0.2% Nonidet P-40. Beads were heated at 100 °C for 5 min with 80 μl of protein sample buffer for subsequent immunoblotting. For purification of recombinant GST-CCTα, 100 μl of immobilized glutathione-agarose beads were incubated with cell lysates (prepared from two 100-mm dishes) at 4 °C for 4 h. After incubation, beads were washed as described above. Recombinant GST-CCTα proteins were eluted using a 5 mM glutathione Buffer A solution, followed by concentration using YM-30 spin columns.

Calmodulin-Sepharose Binding Assay—20 μl of CaM-Sepharose beads were incubated with cell lysates (30 μg) or purified rat liver CCTα (1–2 μg) with or without calcium at 4 °C for 2 h. After incubation, beads were spun down and washed three times using buffer containing 300 mM NaCl and 0.1% Nonidet P-40. Beads were heated at 100 °C for 5 min with 40 μl of protein sample buffer. Released products were resolved using SDS-PAGE prior to immunoblotting.

Mammalian Two-hybrid Binding Assay—CCTα was PCR-amplified using GST-CCTα as a template and cloned into the pM vector (Clontech) that expresses the CCTα-Gal4BD fusion protein. CaM was also PCR-amplified using the Adv-CaM plasmid and cloned into a pVP16 Gal4AD vector that expresses a CaM-Gal4AD fusion protein. After sequence confirmation, CCTα-Gal4BD, CaM-Gal4AD, and pGS5CAT reporter vector were co-electroporated into cells per the manufacturers’ instructions. 48 h after transfection, cells were lysed and assayed for β-galactosidase activities. pM-53 and pVP16-T plasmids served as positive controls, pM3-VP16 and pVP16-CP plasmids served as negative controls.

...and then purified and ligated using T4 DNA ligase at 25 °C for 1 h. The Gene Transfer Vector Core (University of Iowa) used the newly constructed adenovirus-CaM shuttle plasmid to generate a first generation adenovirus-CaM expression vector (27). Adenovirus vectors expressing the CaM transgene driven by the cytomegalovirus promoter (Adv-CaM) or an empty control vector containing the cytomegalovirus promoter but no trans-

...plasmids served as positive controls. pM3-VP16 and pVP16-CP plasmids served as negative controls.
gene (Adv-Con) were used in experiments. Adenoviral vectors were replication-deficient (deletion of the E1 gene) and free of wild type contamination as determined by plaque assay and by PCR for E1 sequences. The particle titers of adenoviral stocks were \(10^{12}\) particles/ml that were used in studies.

**Statistical Analysis**—Statistical analysis was performed using one-way analysis of variance with a Bonferroni adjustment or Student's unpaired \(t\) test. Data are expressed as mean ± S.E.

**RESULTS**

**CCT\(\alpha\) PEST Mutants Are Not Resistant to Calpain**—A strong PEST sequence was identified in CCT\(\alpha\) using the PEST-FIND algorithm (PEST find Analysis webtool) (Fig. 1A). The calculated PEST score was \(8.56\) (PEST scores greater than \(0\) are considered highly significant). There are four less conserved CCT\(\alpha\) PEST sequences, with scores ranging from \(-1.37\) to \(-18.37\). Mutagenesis was performed to disrupt this signature motif and potentially reduce the ability of calpain to degrade CCT\(\alpha\). Mutagenesis of highly conserved Thr\(^{33}\) and Ser\(^{32}\) to Ala resulted in loss of the PEST sequence as identified by this algorithm. First, three constructs, CCT\(\alpha\) full-length (CCT\(\alpha\)_FL) and CCT\(\alpha\) mutants with disrupted PEST motifs, were synthesized in vitro and incubated with calpain, and reaction products were resolved and visualized by autoradiography.

Calpain produced a dose-dependent decrease in the levels of the 42-kDa CCT\(\alpha\)_FL product (Fig. 1B, upper left). CCT\(\alpha\)_FL was degraded within 15 min of calpain exposure (Fig. 1B, lower left) (6). Calpain (0.5 \(\mu\)g) effectively hydrolyzed CCT\(\alpha\)_SDM and CCT\(\alpha\)_SOE within 60 min (Fig. 1B, upper right), and at higher calpain concentrations (1 \(\mu\)g) levels of PEST mutants were undetectable within 30 min (Fig. 1B, lower right). Thus, disruption of the PEST sequence in CCT\(\alpha\) does not protect against calpain in vitro. To assess the physiologic role of the CCT\(\alpha\) PEST motif, functional CCT\(\alpha\)_FL or CCT\(\alpha\)_PEST plasmids were transiently expressed in MLE cells prior to exposure to Ox-LDLs that activate calpains (7).

**FIGURE 1.** A PEST sequence is not required for calpain-mediated cleavage of CCT\(\alpha\). A, the black box identifies a highly conserved PEST sequence within CCT\(\alpha\). The dashed-underlined sequences are weak PEST sequences. The amino acids indicated by black arrows were mutated to alanine to disrupt the PEST sequence. B, full-length CCT\(\alpha\) (CCT\(\alpha\)_FL) or CCT\(\alpha\) variants with PEST mutations (CCT\(\alpha\)_SDM) or an internal PEST deletion mutant (CCT\(\alpha\)_SOE) were translated in vitro using a rabbit reticulocyte system. Newly synthesized products were incubated with calpain at various concentrations (top) or times (bottom) in a proteolysis assay. After hydrolysis, products were run on SDS-PAGE, and gels were dried prior to autoradiography. C and D, MLE cells were nontransfected (NT) or nucleofected with GST-CCT\(\alpha\)_FL or GST-CCT\(\alpha\)_PEST. After 24 h, Ox-LDL (100 \(\mu\)g/ml) was added for an additional 24 h, and cells were harvested and analyzed for CCT activity (C) or CCT\(\alpha\) immunoblot analysis (D). Results are mean ± S.E. from \(n=3\) experiments.

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ments. Further, consistent with other calpain substrates, CCTα degradation products are not easily identified, since these fragments are probably cleared rapidly in cells by endopeptidases or the proteasome thereby often evading detection (11, 28). The sizes of products of the 20 and 26 S proteasome are only 500 Da (5–6 residues) and would run near the dye front of the PAGE, requiring more sensitive approaches for their detection. Oxidized lipids also impaired CCT activity in cells transfected with either CCTαFL or CCTPEST (Fig. 1C). In these experiments, high level expression of CCTαFL or CCTPEST plasmids by nucleofection was observed; reductions of the levels of both overexpressed and endogenous CCTα proteins were observed after Ox-LDL treatment (Fig. 1D). These data indicate that the CCTα N-terminal PEST sequence within does not confer resistance to actions of calpains.

**CCTα Is a CaM-binding Protein**—An alternative recognition signal for calpain in substrates is a CaM-binding domain (13, 14). We first investigated whether CCTα interacts with CaM. Thus, CaM or ERK were immunoprecipitated, followed by immunoblotting with anti-CCTα antibodies. CCTα was detected in association with immunoprecipitated CaM from cell lysates (Fig. 2A, top); consistent with our prior studies, CCTα also bound to ERK, whereas this association was not detected using negative controls (rabbit IgG or beads alone). Conversely, immunoprecipitation of CCTα or calmodulin kinase II followed by immunoblotting with CaM antibody revealed that CaM was detected in association with CCTα and calmodulin kinase II (positive control) but not with preimmune serum (negative control) (Fig. 2A, bottom). Next, cells were transfected with GST-CCTα fusion proteins, and GST pull-down products were eluted and processed for CCTα and CaM immunoblotting (Fig. 2B). CaM was detected in association with overexpressed, purified GST-CCTα, whereas this association was not demonstrated in untransfected cells or by using agarose beads. Further, cell lysates were run over CaM-agarose beads, the beads were extensively rinsed using buffer containing 0.1% Nonidet P-40, and products were eluted. The elution products were then resolved by SDS-PAGE prior to CaM immunoblotting. Different calcium concentrations ranging from 0 to 2000 μM were used in the binding buffer. Indeed, CaM interacts with CCTα in a calcium-independent manner (Fig. 2C). Preliminary studies also showed that varying calcium concentrations after the proteins were bound using the CaM-agarose assay did not dissociate CaM from CCTα (data not shown). To confirm a more direct interaction between CCTα and CaM, purified CCTα was run over CaM-agarose beads, and the beads were processed as above for CCTα binding (Fig. 2D). CaM was detected in association with incubation of 1–2 μg of purified CCTα.

To assess in vivo binding between CaM and CCTα, we used mammalian two-hybrid assays and FRET (Fig. 3). Cells were co-transfected with CCTα-Gal4BD and CaM-Gal4AD plasmids as fusion proteins together with a plasmid construct encoding a β-galactosidase reporter gene (pG5CAT). CCTα-Gal4BD and CaM-Gal4AD transfected separately did not increase reporter activity (Fig. 3A, inset). Co-transfection of the CCTα-Gal4BD and CaM-Gal4AD plasmids together stimulated reporter activity comparable with the positive control plasmid indicative of CaM-CCTα binding. Finally, we also employed FRET analysis using an acceptor photobleaching technique (29) (Fig. 3B). In FRET, energy is transferred from a donor fluorophore molecule to an acceptor fluorophore molecule when proteins are in close (nanometer range) proximity. Thus, FRET is a powerful tool providing more direct visual evidence of protein-protein interaction in vivo. If FRET is observed using the acceptor photobleaching method, the donor emission (CFP) signal increases after a nearby acceptor fluorophore (YFP) is inactivated by irreversible photobleaching. Cellular transfection with YFP-CaM chimera led to diffuse cellular fluorescence of YFP-CaM in line with CaM localization in both

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**FIGURE 2.** **CCTα is a CaM-binding enzyme.** A, co-immunoprecipitation. MLE cells were lysed and incubated with TrueBlot beads alone or with rabbit IgG, CaM, or ERK1 polyclonal antibodies (top). Cells were also lysed and incubated with beads alone or with rabbit IgG, CCTα, or calmodulin kinase II (CaMKII) polyclonal antibodies (bottom). Immunoprecipitants were resolved by SDS-PAGE prior to CCTα immunoblotting. Non-transfected (WT) cell lysates or agarose beads alone processed similarly served as a negative control. B and D, CaM-Sepharose binding assay. Cell lysates (30 μg) C were incubated with CaM-Sepharose beads at different Ca2+ concentrations. After extensive rinsing, elution products were resolved by SDS-PAGE prior to CaM immunoblotting. Cell lysates were incubated with agglutination-agarose beads and rinsed, and products were resolved by SDS-PAGE prior to CaM immunoblotting. Non-transfected (NT) cell lysates or agarose beads alone processed similarly served as a negative control. C and D, CaM-Sepharose binding assay. Cell lysates (30 μg) C were incubated with CaM-Sepharose beads at different Ca2+ concentrations. After extensive rinsing, elution products were resolved by SDS-PAGE prior to CaM immunoblotting. Cell lysates were incubated with agglutination-agarose beads and rinsed, and products were resolved by SDS-PAGE prior to CaM immunoblotting.
Calmodulin Stabilizes CCTα

A

B

FIGURE 3. CaM binds CCTα in vivo. A, a mammalian two-hybrid assay. Cells were co-transfected using electroporation with CCTα-Gal4BD (CCTα) and CaM-Gal4AD (CaM) plasmids as fusion proteins separately (inset) or in combination with a plasmid construct encoding a β-galactosidase reporter gene (pG5CAT) per the manufacturers’ instructions. Cells were lysed and assayed for β-galactosidase activities. pM-S3/pVP16-T and pM3-VP16/pVP16-CP plasmids served as positive and negative controls, respectively. B, FRET analysis. Cells were transfected with YFP-CaM, and CFP-CCTα and CaM-CCTα interaction at the single cell level was imaged using laser-scanning microscopy before and after photobleaching. Shown in the upper sets of panels is single cell imaging showing that after acceptor photobleaching, fluorescence intensity of YFP decreased, and CFP increased, confirming protein interaction between CaM and CCTα. Bottom, the same FRET was confirmed quantitatively by graphing of fluorescence intensities.

cytosol and nucleus as described previously (30) (data not shown). Consistent with the ability of CaM to recruit binding partners to the nucleus (31), co-transfection of these fluorescent plasmids led to detection of a robust CFP-CCTα signal within the nucleus, making CCTα accessible to CaM (Fig. 3B, top). More importantly, the emission fluorescence values of both the donor CFP-CCTα and acceptor YFP-CaM before and after acceptor photobleaching in the region of interest are shown (Fig. 3B, upper arrow and lower plots). These data show that upon bleaching, there was decreased acceptor fluorescence (YPF) coupled with an increase in donor emission fluorescence (CFP), because the acceptor cannot take in energy after its photobleaching. As a whole, these data complement the physical interaction data in Fig. 2, demonstrating that CCTα binds CaM in vivo and in vitro.

Mapping the CaM Binding Site within CCTα—CaM has a propensity to bind amphipathic α-helices (20). There are two major helices within the CCTα membrane binding domain (Fig. 4A) (32). We hypothesized that these helices might harbor a potential CaM binding domain. We employed a reductionist approach by expressing GST-tagged CCTα constructs lacking functional domains (Fig. 4B). Following cellular plasmid transfection, lysates were resolved by SDS-PAGE followed by immunoblotting using GST antibodies to confirm expression of these mutants (Fig. 4C); cellular lysates were also run over glutathione-agarose beads, and after stringent washing, GST pull-down products were eluted and resolved by SDS-PAGE prior to CCTα and CaM immunoblotting. As shown in Fig. 4C, each of these constructs was sufficiently expressed and exhibited appropriate mobilities as fusion proteins on immunoblots. CCTα immunoblotting, as expected, revealed a missing band after expression of GST-CCTα as the antiserum is directed against the catalytic domain (Fig. 4D, top). Full-length CCTα and GST-CCTα mutants devoid of the catalytic core (CCTαCAT), PEST sequence (residues 16–32) (CCTαPEST), NH2-terminal sequence (residues 1–40) (CCTαN40), and carboxyl terminus (residues 315–367) (CCTα315) all bound CaM (Fig. 4D, bottom). However, deletion of the CCTα membrane binding domain (residues 236–315) (CCTαMEM) totally disrupted CaM-CCTα association. Thus, CaM binds CCTα within the membrane domain.

We next tested several GST-CCTα mutants that were progressively truncated within the membrane-binding domain (at the carboxyl terminus) to further localize a CaM binding motif (Fig. 5A, upper map). C288 retains helix 1 and helix 2, C267 contains helix 1, and both mutants bound CaM (Fig. 5A, bottom). However, binding of CaM was not observed with mutants C243 and C210. Thus, CaM binds CCTα in a span of residues from 243 and 267 in helix 1. Additional mapping studies (Fig. 5B) revealed that C260 and C250 also bound CaM, thus localizing CaM interactions with CCTα to a motif, LQERVDKVK. This sequence displays some similarity to CaM IQ-binding motifs with regard to conservation at Gln243 (20). To evaluate the significance of this highly conserved site, we constructed a series of mutants that were progressively truncated within the membrane domain (residues 243–315) (CCTαMEM) totally disrupted CaM-CCTα association. Thus, CaM binds CCTα within the membrane domain.

CaM Modulates CCTα Stability and Function—The above data suggest that CaM binds CCTα within a distinct recognition motif. Since CaM stabilizes proteins, we next executed gain-of-function and loss-of-function analysis by manipulating its expression in vivo. First, purified CCTα was used in the calpain digestion assay in the absence or presence of exogenous CCTα, CaM, or the calpain peptide substrate, LLVY. Each reaction contained 0.6 pmol of calpain. After hydrolysis, reaction products were processed for levels of immunoreactive CCTα and quantified by densitometry. As shown in Fig. 6A, 0.6 pmol of calpain effectively cleaved 80% of purified CCTα (0.7 pmol) in the absence of CaM; only at a 9-fold excess of recombinant CCTα (6.3 pmol) was calpain hydrolysis of the enzyme significantly inhibited (Fig. 6A, left). Importantly, calpain-mediated CCTα hydrolysis was significantly reduced by increasing either the CaM/CCTα molar ratio or the amounts of a calpain substrate in the reaction mixture (Fig. 6A, middle and right, respectively). In these studies, CCTα was present at 0.7 pmol/reaction (Fig. 6A, middle and right). Of note, even at very low molar
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To confirm that endogenous CaM regulates CCTα protein stability, we performed CaM knockdown using siRNA, followed by measurements of CCTα half-life by pulse-chase (Fig. 7, A and B). Consistent with prior studies, in the presence of scrambled siRNA, CCTα exhibited a half-life of ~8 h (25). CCTα turnover was significantly accelerated in cells pretreated with CaM siRNA, since enzyme levels were almost undetectable by 2 h (Fig. 7B). These effects of CaM siRNA were associated with significant reductions in PtdCho synthesis (Fig. 7, C and D). As a whole, these observations indicate that endogenous CaM stabilizes CCTα in vivo.

A Q243A Mutant Confers Resistance to Calpain—Calpain efficiently cleaves its substrates, in part, by docking to CaM binding motifs within target substrates (33). Since Q243A
Calmodulin Stabilizes CCT<sub>α</sub>

![Diagram](image)

**FIGURE 5.** CaM binds within the CCT<sub>α</sub> membrane binding domain. A, top, map illustrating the functional domains of individual CCT<sub>α</sub> mutants lacking portions of helix 1 or helix 2 domains or both. Bottom, overexpressed GST-CCT fusion proteins were incubated with glutathione-agarose beads and rinsed extensively, and elusion products were resolved by SDS-PAGE prior to CCT<sub>α</sub> immunoblotting (above) or CaM immunoblotting (below). Non-transfected cell lysates (NT, far right lane) and A431 cell lysate (far left lane) served as a negative and positive control, respectively. B, top, map illustrating individual CCT<sub>α</sub> mutants constructed that harbor truncations within helix 1. All constructs tested harbor at least 243 upstream residues of CCT<sub>α</sub>. Bottom, overexpressed GST-CCT fusion proteins were processed by GST pull-down analysis and CCT<sub>α</sub> immunodetection of CaM in association with GST-CCT<sub>α</sub>. C, top, CCTFLQ243A (a full-length CCT<sub>α</sub> with a Q243A mutation (FLQ243A)) and C267Q243A (a truncated CCT<sub>α</sub> with 267 residues and a Q243A mutation (C267Q243A)) mutants were constructed to test functionality of CaM binding within CCT<sub>α</sub>. Bottom, overexpressed GST-CCT fusion proteins were processed by GST pull-down analysis as above and CCT<sub>α</sub> immunodetection of CaM (above) or CaM immunoblotting (below). Data in each panel represent at least n = 3 experiments.

To the best of our knowledge, these studies demonstrate a novel regulatory model whereby competition between a proteinase and CaM for access to a single, highly conserved residue (Gln<sup>243</sup>) within a CaM binding motif profoundly affects levels of an enzyme. The specific site (Gln<sup>243</sup>) in CCT<sub>α</sub> appears to serve as a structurally unique recognition signal for both calpains and CaM that may vie for occupancy within CCT<sub>α</sub>. The studies are also the first to show that mutagenesis of a single residue within a CaM dock site blocks the ability of a proteinase to hydrolyze its substrate. This site, rather than a consensus PEST sequence, is integrally linked to CCT<sub>α</sub> proteolysis by calpains. Last, a new finding here is that CCT<sub>α</sub> is a CaM-binding protein and that CaM antagonizes CCT<sub>α</sub> degradation by calpains in vitro and in vivo. Evidence supporting CaM interactions with CCT<sub>α</sub> includes (i) co-immunoprecipitation of CCT<sub>α</sub> with CaM from cellular lysates, (ii) immunodetection of CaM in association with GST-CCT<sub>α</sub> fusion proteins in GST pull-downs, (iii) detection of CCT<sub>α</sub> in CaM-Sepharose binding assays, (iv) mammalian two-hybrid studies, and (v) FRET analysis. Manipulation of CaM expression by adenoviral overexpression or CaM siRNA in lung epithelia differentially altered stability of CCT<sub>α</sub> in the native state and in response to destabilizing effects of oxidized lipoproteins. Thus, CaM appears to be a physiologically relevant binding partner for CCT<sub>α</sub> that may serve to protect the enzyme from proteolytic cleavage.

Although the ratio of immunoreactive CaM versus CCT<sub>α</sub> levels in lung epithelia appears relatively low, this does not preclude CaM as a bona fide binding partner that protects CCT<sub>α</sub> under native conditions from calpains. The relative amounts of CaM, calpains, and CCT<sub>α</sub> in cells depend, in part, upon the
The avidity of antibodies for their detection. CCT α stability will also be governed by the binding affinities of calpains versus CaM for CCT α/H9251. Thus, high affinity binding of CaM to CCT α/H9251 might be sufficient to overcome lower stoichiometries of CaM relative to calpain.

**FIGURE 6.** Calmodulin protects CCTα from proteolysis. A, left, varying amounts of purified CCTα were incubated with purified μ-calpain (0.6 pmol/reaction for 30 min). Middle, varying amounts of CaM were incubated with purified CCTα (0.7 pmol) and recombinant μ-calpain (0.6 pmol/reaction). The ratios of CaM/CCTα are represented on the abscissa. Right, varying amounts of the calpain substrate, LLVY, were incubated with purified CCTα (0.7 pmol) and recombinant μ-calpain (0.6 pmol/reaction). In each panel, the digestion products were resolved by SDS-PAGE prior to CCT α immunoblotting. The percentage hydrolysis of the 42-kDa CCT α was then quantified densitometrically.

**FIGURE 7.** Endogenous CaM stabilizes CCTα. A and B, CCTα turnover was determined by nucleofecting CaM siRNA (●) or control siRNA (○) as in Fig. 6D, and 72 h later, cells were preincubated for 1 h in methionine-deficient medium and then pulsed with [35S]methionine (60 μCi/ml) for 4 h. Cells were rinsed, chased in medium replete with methionine and cysteine for 0–8 h, and processed for CCTα immunoprecipitation, SDS-PAGE, and autoradiography. CCTα bands on the autoradiogram were quantitated; enzyme half-life is presented in A, and a typical autoradiogram is shown in B. The data in A and B represent n = 3 separate experiments. C and D, CaM siRNA reduces PtdCho synthesis. Cells were exposed to control or CaM siRNA as above and pulsed with [methyl-3H]choline to determine radiolaabeled incorporation of choline into PtdCho (C). We confirmed effects of CaM siRNA by measuring immunoreactive CCTα, CaM, and β-actin levels in D. *p < 0.05 versus control.
where CCT activity recovers after CaM actions are blocked (34). These temporal differences of CaM siRNA effects on CCT activity versus CCT half-life are also consistent with a loss of CaM siRNA efficacy by 96 h, since many siRNA duplexes exhibit time-dependent and labile effects (35). For example, recent studies indicate that maximal efficacy of nonvector synthetic RNA interference is highly dependent upon the half-life of the targeted protein; thus, shorter half-life proteins like CaM (t_{1/2} = 10–12 h) (36) will usually exhibit maximal knockdown between 12 and 48 h, whereas longer half-life proteins (>24 h) may exhibit maximal reductions after several days (35). Thus, loss of CaM siRNA effects after half-life measurements were concluded by ~78 h will be associated with partial restoration of CCT activity by 4 days, as observed in our studies. Second, in pulse-chase studies, the stability of only newly synthesized CCTα is determined and not preformed enzyme. The unmeasured preformed or preexisting pool of enzyme may be highly phosphorylated at specific sites or membrane-associated, providing greater stability (37). Because CCTα is regulated at multiple post-translational levels, our half-life measurements in the setting of CaM siRNA treatment do not take into account co-regulation by such mechanisms. Third, even these more modest inhibitory effects of CaM siRNA on CCT activity were physiologically significant, because they were sufficient to reduce PtdCho synthesis (Fig. 8).

Calpains, in part, target PEST motifs within substrates to facilitate degradation (12, 15). The program PESTFIND identified a strong PEST motif (residues 16–32) within CCTα, evidenced by a hydrophilic stretch of amino acids containing two prolines, several acidic residues, and one serine flanked by an NH2-terminal lysine. Mutagenesis of threonine 25 and serine 32 to alanine within CCTα removed this motif as a high value PEST target. Indeed, both the CCTα T25A/S32A double mutant and an internal deletion mutant devoid of the PEST motif retained sensitivity to calpain degradation in vitro and after cellular expression. As with c-Fos and Ca2+/ATPase, the CCTα PEST motif may not serve as a proteolytic signal (16, 38) with the caveat that the PEST domain is sufficiently exposed in vivo. Unlike cAMP-dependent kinase (39), the PEST motif is probably unmasked in our system because ~40% of CCTα was membrane-associated, a feature that activates CCTα, exposing its NH2-terminal domain (40).

A CaM binding motif within CCTα might serve as an alternative recognition signal for calpains. Data base analysis (available on the World Wide Web) of the CCTα sequence initially predicted a putative CaM binding domain within the distal catalytic domain-membrane binding domain interface (residues 205–240) on the basis of hydrophobicity, an average hydrophobic moment, and propensity for α-helix formation. Mapping studies using GST-CCTα carboxyl-terminal truncated mutants, however, localized a CaM binding motif to residues 242–250 exclusively within the membrane-binding domain (Fig. 9). This motif has some features resembling other CaM binding domains. This region resides within α-helix-1, consistent with the predilection of CaM binding domains to localize in amphipathic helices (20). Second, the presence of a calpain cut site juxtaposed upstream of this domain is in line with calpain cleavage of substrates at or near CaM binding domains (21).
to block degradation of CCT motif (Q243A) not only negates CaM binding but was sufficient amino acid substitution of glutamine within the CaM binding motifs (20).

2 and a basic residue at position 11, features characteristic of IQ motifs in proteins that bind CaM in a calcium-independent manner (20). The CaM binding domain within CCTα harbors a highly conserved glutamine at position 2 and a basic residue at position 11, features characteristic of IQ motifs (20).

A remarkable observation from our studies is that a single amino acid substitution of glutamine within the CaM binding motif (Q243A) not only negates CaM binding but was sufficient to totally block degradation of CCTα by calpain (Fig. 8A). These effects were recapitulated in cells exposed to oxidized lipids, where the expressed Q243A CCTα mutant construct was resilient to calpains evidenced by the stability of the overexpressed protein and preservation of enzyme activity. We did not examine the functionality of other residues, since Glu is highly conserved within the CaM motif. Presumably, polarity and/or electrostatic interactions between Gln (Q243) or other residues in the motif, and calpain might enhance accessibility of the protease to its adjacent CCTα cleavage site or help sequester calcium (Fig. 9). Of note, Gln within the motif may also have several favorable electrostatic interactions with the backbone of CaM at Leu111, Gly112, and Glu113 or via binding to domain IV of the large calpain subunit at basic loops (Protein Data Bank code 1AJI). Our results differ from studies of the calcium ATPase and inducible nitric-oxide synthase, where deletion of an entire canonical CaM binding region either attenuated or was insufficient to modify calpain activity (14, 16). Conversely, removal of CaM binding domains within caldesmon and calponin do not alter substrate recognition by calpain (41). Unlike our results, Padanyi et al. (13) demonstrated that point mutagenesis at Trp1093 in the calcium ATPase pump increased the accessibility of a calpain hydrolysis site within a CaM binding domain. Thus, our data suggest a somewhat unique molecular model whereby availability of CCTα will be influenced by stoichiometry and binding affinities of CaM versus calpain utilizing Gln as a critical recognition site. Interestingly, a point mutation at a highly conserved Gln (Q3180P) was recently identified within an IQ motif of the gene encoding abnormal spindle-like microcephaly-associated protein; this mutation is linked to an inherited disorder characterized by neurodevelopmental arrest of brain growth, raising the possibility that interactions between abnormal spindle-like microcephaly-associated protein, calpains, and CaM might play a role in disease pathogenesis (42).

Our study demonstrates that CaM stabilizes a protein in cells, an issue not addressed in prior work (14, 16, 21, 22). Of note, CaM inhibition reduces PtdCho synthesis and impairs lung growth, but these studies relied on the use of nonselective approaches (34, 43–45). Adenoviral gene transfer of CaM into lung epithelia was achieved with high efficiency (∼95%) and specificity, allowing for gain-of-function analysis. Complementary loss-of-function experiments were facilitated by cellular electroporation of CaM siRNA constructs that also proved to be efficient (Figs. 6 and 7). Ad5–CaM overexpression effectively blocked Ox-LDL-induced CCTα breakdown and rescued acceleration of enzyme turnover after CaM knockdown, indicating that siRNA effects were selective for CaM. These stabilizing effects of CaM on CCTα protein were the dominant effect, since separate in vitro experiments showed that recombinant CaM only produced modest (∼2-fold) activation of purified CCT (data not shown). These studies coupled with our physical protein interaction data underscore a potentially important biochemical and physiologic role for CaM in regulating PtdCho biosynthesis. Future work using structural analysis of CaM–CCTα complexes will provide newer insight into the conformational environment for these interactions. Testing of these mechanistic associations in vivo also awaits the generation of suitable transgenic or knock-in animal model systems that conditionally express relevant molecular sites within CCTα or CaM in epithelia.

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