Phosphorylation of CEACAM1 Molecule by Calmodulin Kinase IID in a Three-dimensional Model of Mammary Gland Lumen Formation*

Received for publication, July 10, 2013, and in revised form, November 26, 2013 Published, JBC Papers in Press, December 3, 2013, DOI 10.1074/jbc.M113.496992

Tung Nguyen, Charming-Jui Chen, and John E. Shively†

From the Department of Immunology, Beckman Research Institute of City of Hope, Duarte, California 91010

Carinoembryonic antigen-related cell adhesion molecule-1 (CEACAM1), a transmembrane protein, expressed on normal breast epithelial cells is down-regulated in breast cancer. Phosphorylation of Thr-457 on the short cytoplasmic domain isoform (CEACAM1-SF) that is predominant in normal epithelial cells is required for lumen formation in a three-dimensional model that involves apoptosis of the central acinar cells. Calmodulin kinase IID (CaMKIID) was selected as a candidate for the kinase required for Thr-457 phosphorylation from a gene chip analysis comparing genes up-regulated in MCF7 cells expressing wild type CEACAM1-SF compared with the T457A-mutated gene (Chen, C. J., Kirshner, J., Sherman, M. A., Hu, W., Nguyen, T., and Shively, J. E. (2007) J. Biol. Chem. 282, 5749–5760). Up-regulation of CaMKIID during lumen formation was confirmed by analysis of mRNA and protein levels. CaMKIID was able to phosphorylate a synthetic peptide corresponding to the cytoplasmic domain of CEACAM1-SF and was covalently bound to biotinylated and T457C-modified peptide in the presence of a kinase trap previously described by Shokat and co-workers (Maly, D. J., Allen, J. A., and Shokat, K. M. (2004) J. Am. Chem. Soc. 126, 9160–9161). When cell lysates from wild type-transfected MCF7 cells undergoing lumen formation were incubated with the peptide and kinase trap, a cross-linked band corresponding to CaMKIID was observed. When these cells were treated with an RNAi that inhibits CaMKIID expression, lumen formation was blocked by over 90%. We conclude that CaMKIID specifically phosphorylates Thr-457 on CEACAM1-SF, which in turn regulates the process of lumen formation via apoptosis of the central acinar cells.

Mammary morphogenesis, a process that includes formation of acini that secrete milk into a central lumen, can be mimicked by growth of normal mammary epithelial cells in a three-dimensional culture in which Matrigel is used as a source of extracellular matrix (1–3). This model system allows the identification of key molecules and processes that allow individual cells to migrate and form acini that undergo lumen formation by an apoptotic process (4), followed by expression and secretion of the components of milk. In addition to providing insights into this important biological process, when the basic step of lumen formation does not occur properly, the model resembles the early stage of breast cancer known as ductal carcinoma in situ. We, and others, have utilized this model system to identify the molecules involved in the process of lumen formation. For example, Brugge and co-workers (5) have shown that expression of a Her2 reporter in the normal mammary epithelial cell line MCF10A fills the interior of the lumen with rapidly dividing cells, suggesting that a key signal of terminal differentiation is either absent or over-ridden. Further work by this group has tentatively identified tumor necrosis factor-related apoptosis-inducing ligand and BIM as key molecules in initiating the process of apoptosis that creates the lumen (2, 6). Our own studies have focused on the role of carinoembryonic antigen-related cell adhesion molecule-1 (CEACAM1), a type 1 transmembrane protein, that is expressed on normal breast epithelial cells and is down-regulated in breast cancer (7, 8). We have identified calpain-9 as a key effector of apoptosis in our model system (9).

Because CEACAM1 is expressed on epithelial cells throughout the body and epithelial cells form the lining of lumina in most tissues, it is possible that it plays a general role in lumen formation. In man, CEACAM1 is expressed as multiple alternatively spliced mRNAs, giving rise to type 1 membrane proteins with 3–4 Ig-like ectodomains and long (72 amino acids) or short (12 amino acids) cytoplasmic domains (10). All isoforms have a common N-terminal domain that regulates its cell-cell interaction.

Significance: Lumen formation, a hallmark of epithelial cells, is lost upon malignant transformation.

Background: CEACAM1, a cell-cell adhesion molecule that induces lumen formation, requires phosphorylation on Thr-457 for its function.

Results: Biochemical and RNAi approaches identified that CaMKIID was responsible for phosphorylation of Thr-457 and lumen formation.

Conclusion: CaMKIID, up-regulated during lumen formation, is associated with CEACAM1-mediated apoptosis, a key feature of lumen formation.

Grant CA84202.

To whom correspondence should be addressed: Dept. of Immunology, Beckman Research Institute of City of Hope, 1450 East Duarte Rd., Duarte, CA 91010. E-mail: jshively@coh.org.

© 2014 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.
adhesion properties (11, 12). In the case of the breast where the short cytoplasmic domain (CEACAM1-SF) predominates (7, 13, 14), signal transduction is conveyed (in part) by a very short stretch of amino acids that interact with the actin cytoskeleton via the key residue Phe-454 (15). Experimental proof involves transfection of MCF7 cells, which neither express CEACAM1-SF nor form a lumen in three-dimensional culture, with an F454A null mutant of CEACAM1-SF that fails to form a lumen compared with the wild type gene (15). Further mutational analysis of the short cytoplasmic domain identified Thr-457 as another key residue that is phosphorylated during lumenn formation and whose function can be abrogated if mutated to alanine along with downstream Ser-459 (15). In that study, it was speculated that Ser-459 is a back-up phosphorylation site that becomes operational in the T457A mutant, because the phosphorylation mimic T457D permits lumen formation.

The possibility that the short cytoplasmic domain of CEACAM1 can be phosphorylated was previously addressed by Obrink and co-workers (16) who showed that protein kinase C (PKC) can phosphorylate a Thr in the rodent equivalent position of Thr-457 in the human protein. However, the rodent and human sequences are slightly different, and the human sequence lacks a critical basic amino acid that is usually required for phosphorylation of Thr or Ser by PKCs. Furthermore, a database search (NetPhosK server) of the human sequence returns no kinases with a high score, leaving us with no clear leads as to the critical kinase that phosphorylates the human sequence. Because it was possible that the kinase was induced during lumen formation as part of a larger program, we turned to a comparative gene chip analysis. MCF7 cells were transfected with either wild type CEACAM1-SF or the T457A,S459A null mutant, and the mRNA levels were compared on cells grown in the three-dimensional model for 4 days when lumen formation is most active (9). In this analysis we identified several key proteins involved in the apoptotic process, namely calpain-9 and PKC-δ, and found that calmodulin kinase IID (CaMKIID) was elevated by 2.67-fold (log base 2) in the wild type versus the mutant transfectants. Although functional analysis of CaMKIID was not performed in that study, we were intrigued by the expression of this rather unique isoform of CaMKII and hypothesize that it may have a specific function in lumen formation. In this respect, we have previously shown that lumen formation in the three-dimensional model system involves apoptosis of the central acinar cells (4), and CaMIKD expression induces apoptosis of cardiac myocytes (17).

CaMKIIs are an abundant class of Ser and Thr kinases activated by Ca2+ /calmodulin (Ca2+/CaM). CaMKIIs are encoded by four different genes (A, B, D, and G) expressed in the majority of cells (18). CaMKIIA and -B, abundant proteins in the brain, are associated with synaptic processing, learning, and memory (19). Expression of CaMIKD was implicated in apoptosis of myocardiocytes during ischemia (17), regulation of vascular smooth muscle polarization and migration (20), and down-regulation in breast cancer tumor cells (21). In addition, CAMKII in the absence of CaM can bind G-actin and bundle F-actin (22). Intrigued by the functional relevance of CaMKIID to apoptosis and cancer, its up-regulation in our comparative gene chip analysis, and the fact that CEACAM1 binds CaM (23, 24) and G-actin (15), we decided to explore the possibility that CaMKIID may be responsible for the phosphorylation of Thr-457 in CEACAM1-SF and may play an essential role in lumen formation.

We found that recombinant CaMKIID was indeed capable of phosphorylating a synthetic peptide comprising the short cytoplasmic domain of CEACAM1, whereas PKCs and other kinases tested had little or no activity toward this substrate. CaMKIID was able to phosphorylate a biotinylated and T457C-modified SF peptide in the presence of a kinase trap previously described by Shokat and co-workers (25). The up-regulation of CaMIKD during lumen formation was confirmed, as well as the ability of RNAi to inhibit both its up-regulation and lumen formation. We conclude that CaMIKD plays an essential role in lumen formation in this model system and that Thr-457 in the short cytoplasmic domain isoform of CEACAM1 is likely a critical target of this kinase.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal antibodies anti-CaMKIID and anti-PKCδ were from Abnova (Taipei, Taiwan); polyclonal anti-PKCδ was from Santa Cruz Biotechnology (Santa Cruz, CA); anti-β-actin was from Abcam (Cambridge, MA); anti-biotin was from Thermo Scientific (Lafayette, CO). Anti-CEACAM1 antibody T84.1 was previously described (24). Anti-Thr(P)-286 CaMKIID was from Cell Signaling Technology (Beverly, MA). Infrared-labeled IRDye secondary antibodies were from LI-COR Biotechnology (Lincoln, NE). Stealth RNAi siRNA oligonucleotide RNAi-negative control medium GC duplex, Lipo-fectamine RNAiMAX transfecting reagent, and Opti-MEM reduced serum medium were from Invitrogen: 1) 5′-UCUGU-GAGCCAGGCCCCUACUGCUU-3′; 2) 5′-ACAUUGGAUCAUG- GAUGGAAUGGCAAAG-3′; 3) 5′-GGAUCAUAGAGAAAACUA- AGAAAGAGAA-3′. pcDNA3/CEACAM1–4S-eGFP plasmid and CEACAM1-SF synthetic peptides and GST–CEACAM1 cytoplasmic domain fusion proteins were prepared as described previously (24). Calmodulin antagonist W7 (N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide), CaMKII–specific inhibitor KN-93, and inactive analog KN-92 were from Calbiochem. Recombinant CKI, CKII, and CaMKII were from New England Biolabs (Ipswich, MA). Recombinant CaMKIIb, -B2, and -D and PKC mixtures were from Invitrogen. The recombinant kinase trap-linker was synthesized according to Maly et al. (25). A CaMKII peptide substrate corresponding to residues 1–10 of glycogen synthase was from Santa Cruz Biochemicals (catalog sc-3119).

Cell Lines—The human mammary adenocarcinoma cell line MCF7 was obtained from ATCC (HTB-22). MCF7 cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin/amphotericin B, 1% sodium pyruvate, 2% sodium bicarbonate, and 1% nes-sential amino acids.

Transfection—The cloning of CEACAM1-SF into the pHβ-actin expression vector, as well as construction and expression of the double null mutant of the cytoplasmic domain of CEACAM1-SF, (T457A,S459A), was described previously (24).

Matrigel Culture and Inhibition Studies—The three-dimen-sional Matrigel (BD Biosciences) sandwich assay has been pre-
CaMKII and CEACAM1 in Lumen Formation

Viously described (15). Briefly, 10-ml culture dishes were coated with 1 ml of Matrigel and incubated at 37 °C for 30 min until the Matrigel solidified, and cells (1 × 10^5) in 10 ml of mammary epithelial basal medium plus pituitary gland extract (Lonza Group, Ltd.) were added to each well. After 3 h of incubation, the floating cells were removed, and the bound cells were overlaid with 1 ml of 50% Matrigel in mammary epithelial basal medium plus bovine pituitary extract. At day 4, the acini were recovered by adding 10 ml of dissolving solution (BD Biosciences) into each dish and incubated at 4 °C for 3 h by gentle rotation. The cells were harvested and RNA isolated for RT-PCR analysis. For inhibition of lumen formation with KN93 (or KN92 control) or W7, 12-well plates were used. Each well was coated with 250 μl of 100% Matrigel, and cells (1 × 10^5) were seeded into each well and coated with 50% Matrigel as above. Inhibitors (KN-93 or KN92 or W7 or DMSO control, 30 μl in DMSO) were added to media (3 ml) and changed every other day. After 6 days of incubation, lumen formation was scored under an inverted light microscope. Statistical analysis was performed using Fisher’s exact test.

For RNAi inhibition, cells were transfected with Stealth RNAi siRNA oligos using the Lipofectamine RNAiMAX-transfecting reagent (Invitrogen). Briefly, cells were split, seeded at 50% confluence in T25 flasks overnight, and washed twice with PBS prior to the treatment. Stealth RNAi and siRNA oligos (100 nm) and transfection agent (1:100 dilution) were separately diluted in Opti-MEM I reduced serum medium, mixed, and then incubated together for 20 min before addition to the cells. After 6 h, without removing the transfection solution, cells were added back to minimal essential medium with 10% fetal bovine serum overnight, harvested, and transferred to Matrigel for the lumen formation assay.

RT-PCR and Quantitative PCR—Total RNA was extracted from cells using the TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instruction. From 1 μg of each total RNA, 20 μl of cDNA was synthesized with an OMM iSCRIPT RT kit (Qiagen, Valencia, CA). PCR was performed with the iCycler Thermal Cycler (Bio-Rad) for 35 cycles at 55 °C annealing temperature. The FideliTaq™ PCR master mix (United States Biochemical, Cleveland, OH) was used in each experiment to ensure tube to tube consistency in PCRs. Primers (sequence available upon request) were designed for amplification of specific genes. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control amplifier set was used to assess RNA integrity. Reaction products (15 μl) were visualized after electrophoresis in 1.4% agarose gel containing SYBR Safe DNA gel stain (Invitrogen) +.

To quantify the mRNA expression level of CaMKII/B/D/G primers, were designed to carry out quantitative PCR using the CFX96 real time PCR detection system (Bio-Rad). Briefly, we amplified 1 μl of cDNA from the reverse transcription reaction with 20 pmol of each primer in a total volume of 25 μl using the iQ™ supermixes (Bio-Rad) and the following conditions: initial denaturation step at 94 °C for 3 min, followed by 40 cycles of 94 °C for 10 s and 55 °C for 30 s. The fluorescence was measured at the end of the annealing step at 55 °C. Subsequently, a melting curve was recorded between 55 and 95 °C every 0.5 °C with a hold every 1 s. Levels of mRNA were compared after correction by use of concurrent GAPDH message amplification. Samples were done in triplicate, and the values shown were normalized to their own GAPDH readings.

Immunoblotting and Immunoprecipitation—Cells were lysed with lysis buffer (10 mm Tris-HCl, pH 7.4, 100 mm NaCl, 1 mm EDTA, 1 mm EGTA, 50 mm NaF, 1 mm PMSF, 1 mm Na3VO4, 0.05% sodium deoxycholate, 10% glycerol, 1% Triton X-100, and proteinase inhibitor mixture (Roche Applied Science) on ice for 30 min, and protein concentration was determined using the Bio-Rad protein assay. Fifty micrograms of protein from each sample were resolved by SDS-gel electrophoresis and immunoblotted with appropriate primary and infrared-labeled IRDye secondary antibodies. Immunoprecipitation of CEACAM1 was performed with anti-CEACAM1 mAb 26H7 (a kind gift of R. Blumberg) using Pierce protein A/G Plus-agarose (Thermo Scientific, Rockford, IL) per the manufacturer’s protocol. Detection was carried out using the Odyssey® infrared imaging system (LI-COR Biotechnology, Lincoln, NE) according to the manufacturer’s instruction.

In Vitro Phosphorylation and Mass Spectrometry—Two micrograms of GST fusion proteins, including CEACAM1-long cytoplasmic domain (GST-LF) and CEACAM1-short cytoplasmic domain (GST-SF), were subjected to in vitro phosphorylation by dioleoyl-rac-glycerol/phosphatidylserine-activated purified recombinant PKC at 5 units/ml (Sigma) with 50 μM [γ-32P]ATP (PerkinElmer Life Sciences), followed by addition of 125 μM cold ATP, separated on NuPAGE Novex 4–12% BisTris gel (Invitrogen), and autoradiographed to locate the 32P-phosphorylated protein bands. A duplicate gel was stained with GelCode Blue Stain Reagent (Thermo Fisher Scientific) to visualize total protein loaded.

Calmodulin-activated CaMKII/B/D, phosphatidyserine-activated PKC mix/B2/D (Invitrogen), and casein kinase 1/2 (New England Biolabs Inc.) were used to carry out in vitro phosphorylation of CEACAM1-SF synthetic peptides. Briefly, 50 μμμ of synthetic peptides were mixed with activated kinases in the presence of 100–200 μM ATP at 30 °C for 2 h. Phosphorylated peptides were acidified to pH 4 with acetic acid, captured on nickel-nitritiotriacetate acid silica resin (Qiagen, Inc., Valencia, CA), collected on ZipTips (Millipore), eluted onto stainless steel sample plates, and co-crystallized with 2,5-dihydroxybenzoic acid as the matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) matrix. Single-stage mass spectrometric analyses were performed with a Protos 2000 MALDI-quadrupole (MALDI-Q)-time-of-flight instrument (PerkinElmer Sciex, Framingham, MA), and multistage mass spectrometric fragmentation spectra were obtained with a prototype MALDI-Q-ion trap as described previously (26). Spectra were analyzed by m/z Moverz software (ProteoMetrics, LLC, New York). Additional mass spectral analyses were performed on a Thermo Electron LTQ-FT-MS hybrid linear ion trap mass spectrometer.

Kinase Trap Assay—The kinase trap was based on the principle of having an ATP analog with a dialdehyde to cross-link the active site lysine of a kinase and a cysteine of a peptide substrate in which cysteine replaced the target Ser or Thr residue (25). The CEACAM1-SF peptide was synthesized with biotin at the N terminus and Cys replaced Thr-457 (biotin-
His-Phe-Gly-Lys-Cys-Gly-Ser-Ser-Gly-Pro-Leu-Gln-COOH). Glycogen synthase peptide (cPLSRTLSSVSS10), a known CaMKII substrate, was used as a positive control. In the presence or absence of 100 μM cross-linker dialdehyde, peptides at 20 μM were incubated with activated purified kinases or 4-day Matrigel-cells lysates in kinase assay buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM MgCl2, 100 μM 2-mercaptoethanol) for 1 h at room temperature and quenched with SDS sample loading buffer afterward. The sample mixtures were resolved by NuPAGE Novex 4–12% BisTris gel and then either silver-stained with SilverQuest™ staining kit (Invitrogen) or transferred and Western-blotted with appropriate antibodies. In-gel kinase trap assays were carried out under the same principle, except that the activated purified kinases or 4-day Matrigel-grown cell lysates were first resolved natively by NuPAGE Novex 4–20% Tris glycine with Tris glycine running and native sample buffer. The gels were then denatured (50 mM Tris-HCl, pH 8.0, 20 mM DTT, 6 M guanidine hydrochloride), renatured (50 mM Tris-HCl, pH 8.0, 5 mM DTT, 0.04% Tween 20, 100 mM NaCl, 5 mM MgCl2), incubated in kinase assay buffer (20 mM HEPES, pH 7.7, 10 mM MgCl2, 2 mM DTT, 0.1 mM EGTA), washed with Tris glycine transfer buffer with 20% MeOH, pH 10.5, transferred, and Western-blotted with appropriate antibodies. Detection was carried out using the Odyssey® infrared imaging system (LI-COR Biotechnology, Lincoln, NE) according to the manufacturer’s instruction.

Kinase Enrichment Assay—The kinase enrichment kit (Thermo Fisher Scientific) was used to selectively label and enrich for kinase using a nucleotide derivative desthiobiotin-tagged ATP per the manufacturer’s instructions. Briefly, lysates from MCF7 cells that had been stably transfected with CEACAM1-4S and grown in Matrigel for 0–4 days were buffer-exchanged with reaction buffer, labeled with 5 or 20 μM desthiobiotin-ATP, captured on high capacity streptavidin-agarose resin, washed, and eluted with SDS reducing sample buffer by boiling. Eluted proteins were analyzed by SDS-PAGE and Western blot analysis.

Confocal Microscopy and Halo-tagged Samples—For co-localization of CEACAM1 and CaMKII, pCDNA3/CEACAM1-4S-eGFP and pFN21A HaloTag CMV Flexi-CaMK2D (Promega Corp., Madison, WI) MCF7 stable transfectants were grown in 12-well plates in Matrigel. For detection and visualization, N-terminal Halo-tagged CaMK2D was stained with HaloTag TMR fluorescent ligand per manufacturer’s protocol. Plates were observed on a Zeiss LSM 510 inverted confocal microscope.

RESULTS

Phosphorylation of GST-CEACAM1 Cytoplasmic Domain Fusion Proteins—In previous work, we showed that GST-CEACAM1 cytoplasmic domain fusion proteins could be used to study the interaction of these domains with actin (15). We reasoned that they may also act as substrates for protein kinase assays. As shown in Fig. 1, a mixture of PKCs was able to phosphorylate the GST-CEACAM1-LF fusion protein but not the GST-CEACAM1-SF. This result contrasts with the finding that PKCs were able to phosphorylate the rodent version of CEACAM1-SF (16). This result is not unexpected because the human version does not have the appropriate consensus sequence for PKC phosphorylation. Because we were not interested in the phosphorylation of CEACAM1-LF, which has multiple serines in its 72-amino acid domain and is a minor expressed isoform in the breast, we did not perform further studies on the LF isoform. In addition, we were concerned that when testing additional kinases, we would encounter problems by phosphorylation of GST itself, potentially confounding our study. Therefore, further work was performed on synthetic peptides.

Phosphorylation of CEACAM1-SF Synthetic Peptides—Because the exact start of the cytoplasmic domain of CEACAM1-SF is in doubt and may depend on its association with membrane lipids (27), the peptide was synthesized with N-acyl-mercaptoundecanoic acid. The inclusion of N-acyl-mercaptoundecanoic acid was precipitated on its ability to insert this peptide into liposomes or to form micelles, thus mimicking the lipid microenvironment (15). The peptide was then mixed with kinase and appropriate cofactors, including ATP, calcium, and/or lipid as required, and the product was analyzed by MALDI-TOF MS over time. In most cases, the major peak observed corresponded to the intact peptide with and without a sodium adduct (as expected from the use of sodium phosphate buffers). The peak height of phosphorylated products was compared with unphosphorylated species to calculate the maximum phosphorylation. Because the peptide contains two serine residues in addition to Thr-457, several monophosphorylated peptides were synthesized as substrates to determine whether various kinases could diphosphorylate the peptide or whether a monophosphorylated version was preferred. The results shown in Table 1 demonstrate that among the kinases tested CaMKII had the highest activity toward the wild type peptide, although CKI had the highest activity toward monophosphorylated Thr-457. CaMKII also had some activity toward the wild type peptide, although none of the kinases were able to triphosphorylate the diphosphorylated substrate. Notably, CaMKII and a mixture of isoforms (CaMKII) had activity, but 2.5-fold less than CaMKIIID (Table 1), suggesting the possibility that CaMKIIIB or holoenzymes...
with a mixture of isoforms may play a minor role in CEACAM1-SF phosphorylation. A representative series of mass spectra from the analysis of CaMKIID with several of the peptide substrates are shown in Fig. 2. In Fig. 2 (lower panel), the protonated molecular ion (MH⁺) and sodium adduct (MNa⁺) both gain 80 atomic mass units after treatment with CAMKIID as expected for the phosphorylated products with an approximate yield of 50% (Table 1). In Fig. 2 (middle and upper panels), the monophosphorylated peptides form minimal amounts of diphosphorylated products (indicated by arrows). The loss of phosphoric acid from the starting material, which occurs in the mass spectrometer, is typical of phosphopeptides and did not affect the analysis. After completing these studies, we were requested during the review to determine whether CaMKIID was able to phosphorylate the T457A mutant version of the peptide. Indeed, this peptide was phosphorylated to the extent of 25% on Ser-459, as confirmed by MS/MS analysis on an LTQ-FT hybrid linear ion trap mass spectrometer (data not shown). This result confirms our previous study that showed it is necessary to mutate both Thr-457 and Ser-459 to completely block lumen formation of CEACAM1-SF-transfected MCF7 cells (15). Although these data suggest that CaMKIID is the leading candidate for phosphorylation of the wild type peptide, confirmatory studies are required.

Kinase Trap Assays for CEACAM1-SF Peptides—Shokat and co-workers (25) have developed a mechanism-based kinase trap for the identification of novel kinases. This intriguing method involves synthesis of the peptide substrate with a cysteine residue in place of the target Ser or Thr and an N-terminal biotin to allow recovery of the “trapped” peptide for further analysis. The trapping is performed by co-incubation of the peptide and kinase with a reagent that incorporates an ATP analog that binds the kinase with a dialdehyde linker that reacts with the thiol of the peptide and the amino group of an essential lysine found in the active site of all kinases. To test the ability of the approach to identify which kinase phosphorylates CEACAM1-SF in vivo, we synthesized the cross-linker reagent and the biotinylated CEACAM1-SF peptide with the substitution T457C (Biotin-His-Phe-Gly-Lys-Cys-Gly-Ser-Ser-Gly-Pro-Leu-Gln-COOH) and incubated them with several recombinant kinases, including CaMKIID. A second peptide corresponding to a known CaMKII substrate (glycogen synthase 1–10) was used as a positive control. Fig. 3A demonstrates that a mixture of purified baculovirus recombinant rat truncated CaMKIIs can trap either a known peptide substrate or the CEACAM1 SF peptide when analyzed by SDS-gel electrophoresis. Similar studies with known substrates for casein kinase I (CKI) also demonstrated that the “trap” worked efficiently (data not shown). We incubated lysates from vector or wild type CEACAM1-transfected MCF7 cells grown in Matrigel for 4 days with the CEACAM1-SF peptide plus or minus the ATP-trap reagent. After incubation, proteins were separated by SDS-gel electrophoresis and Western-blotted with anti-biotin antibody. The minus ATP-trap control results shown in Fig. 3B demonstrate that most of the peptide migrates to the bottom of the gel with two weak bands at 30 and 48 kDa for the vector control. These two bands coincide with the known molecular masses of CKI and several of the isoforms of CaMKIID. In contrast to the vector control, the wild type control shows an increase in the intensity of the 48-kDa band, a result that may indicate an SDS-resistant association of the CEACAM1-SF peptide with CaMKIID, based on the molecular size. In the experiment where the incubations included the ATP-trap, similar bands were observed but were more intense, indicating that the ATP-trap increased peptide association, as expected for the formation of a covalent bond between the kinase and the peptide. When comparing the vector versus the wild type–transformed cell lysates, we observed the appearance of a new band at 59 kDa, an expected size for isoforms of CaMKIID. Because there are a large number of kinases present in the cell and CaMKII has 10 subunits that may include different isozymes (28), we cannot prove by this analysis alone that a specific kinase has been positively identified. However, we can conclude that the results are consistent with the appearance of bands at 48 and 59 kDa, corresponding to the correct molecular sizes for CaMKIID and -B.

To make a positive identification of the bands tentatively identified for CaMKIID and CKI, vector and wild type CEACAM1-SF-transfected MCF7 lysates were incubated with peptide and cross-linker; proteins were separated by SDS-gel electrophoresis and immunoblotted with antibodies to CaMKIID or CKI (Fig. 3C). In the case of CaMKIID, the kinase was trapped to a larger extent in WT-transfected versus vector controls. Although the immunoblot is less conclusive for CKI, perhaps due to the use of a less specific polyclonal antibody, a similar result was observed.

Although the kinase trap approach was originally developed for cell lysates preincubated with peptide and ATP-trap followed by separation on SDS gels, it suffers from the fact that many kinases may be inactivated during cell lysis or may nonspecifically react with the peptide substrate. We reasoned that the kinase trap could be combined with other approaches in which the kinases were maintained in a more active form and pre-separated from other proteins. One such approach is the “in-gel” kinase assay that involves running a cell lysate into a native gel, followed by a denaturation-renaturation step to refold proteins (29). We performed the in-gel kinase approach followed by incubation of the renatured gel with the ATP-trap reagent and peptide substrate. The gel was then soaked in SDS and transferred to nitrocellulose for Western blot analysis with an anti-biotin antibody. The results shown in Fig. 4A shows two

### TABLE 1

**Phosphorylation of CEACAM1-SF synthetic peptides**

| Kinase | WT | Thr(P)-457 | Ser(P)-459 | Thr(P)-457–Ser(P)-459 |
|--------|----|------------|------------|----------------------|
| CKI    | 0  | 0          | 0          | 0                    |
| CKII   | 20 | 0          | 0          | 0                    |
| CaMKIID| 20| 0          | 0          | 0                    |
| CaMKIIB| 20| 0          | 0          | 0                    |
| CaMKII | 20| 0          | 0          | 0                    |
| PKC mix| 10| 10         | 0          | 0                    |
| PKCB2  | 10| 0          | 0          | 0                    |
| PKCD   | 0 | 0          | 0          | 0                    |
strong bands found in lysates from cells transfected with wild type (WT) CEACAM1-SF but not the vector control. Because the vector control-transfected cells failed to form lumena in Matrigel, the results identify two potential kinases that were activated by CEACAM1-SF in MCF7 cells grown in three-dimensional culture. Although the migration of the two cross-linked bands correspond to the migration of authentic CaMKIID and CKI, the low resolving power of native gels prevents us from making unequivocal identifications. Nonetheless, the specificity of the trap for CEACAM1-SF-transfected cells was demonstrated, further strengthening the case for selecting CaMKIID as a possible candidate for the in-cell phosphorylation of CEACAM1-SF during lumen formation.

A further analysis was performed using the kinase enrichment kit from Thermo Scientific. In this approach, the cell lysate was incubated with a desthiobiotin derivative of ATP, which in the presence of an activated kinase reacts with lysine at its active site forming a covalent bond between desthiobiotin and lysine. The kinase desthiobiotin adduct is purified on streptavidin beads, separated by SDS-gel electrophoresis, and Western-blotted with anti-kinase antibodies. Although this approach is not substrate-specific (i.e. no peptide substrate was added), it can demonstrate the presence of an active kinase in the analyzed lysate. As shown in Fig. 4B, we identified a time-dependent activation of PKC-δ, a kinase we previously associated with lumen formation in these cells (9), and an additional band for CaMKIID. Furthermore, both bands were more strongly detected in lysates from CEACAM1-SF-transfected cells than in vector controls. Taken together the three approaches indicate that CaMKIID merits further consideration as a kinase associated with both CEACAM1-SF expression and lumen formation.

Expression of CaMKIID in Cells Undergoing Lumen Formation—As stated earlier, CaMKIID was selected as a possible candidate for the phosphorylation of CEACAM1-SF based on its increased expression in a comparative gene chip assay. Because these assays often exaggerate the fold changes in mRNA levels and do not necessarily reflect changes in protein levels, the mRNA and protein levels of CaMKII genes were analyzed by RT-PCR and Western blot analysis. As seen in Fig. 5A, none of the cells tested expressed the CaMKIIA mRNA, although all the cells tested (vector control and wild type CEACAM1-SF) expressed the CaMKIIB and -D mRNAs. Quantitative PCR analysis for CaMKIID revealed an expression maximum for this gene product at day 4 (Fig. 5B), whereas the other isoforms showed no trend (data not shown). When a similar time course analysis was performed by immunoblotting for CaMKIID, a major band was observed that increased with time, suggesting that this gene product was specifically induced at the protein level compared with vector control (Fig. 6A). Because multiple splice forms for the CaMKIID gene are known (28) and the Western blot detects 2–3 gene products, only one of which is overexpressed during lumen formation, we conclude that a distinct splice form of CaMKIID may be involved. However, sequence analysis of the cDNAs made from the mRNA isolated from CEACAM1-SF-transfected MCF7 cells revealed that all nine CaMKIID splice isoforms were found (data not shown).

In order for CaMKIID to phosphorylate CEACAM1-SF, the two proteins must co-localize, however briefly, at some stage of lumen formation. To determine the stage, CEACAM1-GFP

FIGURE 2. Mass spectrometric analysis of CEACAM1-SF peptides phosphorylated with CaMKIID. Lower panel, wild type peptide has peaks corresponding to the MH⁺ and MNa⁺ ions plus product peaks corresponding to addition of HPO₃. Middle panel, Thr-457-monophosphorylated SF peptide has peaks corresponding to MH⁺ and MNa⁺ and loss of H₃PO₄, all of which correspond to substrate. Evidence for a diphosphorylated peptide (arrow) is almost completely lacking. Upper panel, Ser-459-monophosphorylated SF peptide has peaks corresponding to MH⁺, MNa⁺, and loss of H₃PO₄, all of which correspond to substrate. Evidence for a diphosphorylated peptide (arrow) is almost completely lacking.
fusion protein-transfected MCF cells (15) were co-transfected with a Halo-CaMKIID fusion protein that can be detected with HaloTag TMR fluorescent ligand (see under “Experimental Procedures” for details). In this analysis we found the two proteins co-localized at the two-cell stage of cell aggregation in three-dimensional culture (data not shown). Further analysis of the time course of lumen formation was made difficult by the tendency of the overexpressed CaMKIID to inhibit acinus formation.

Knockdown of CaMKIID by RNAi Blocks Lumen Formation—To test the functional role of CaMKIID in lumen formation, several RNAi oligos that target CaMKIID were transfected into MCF7 cells expressing CEACAM1-SF. As shown in Fig. 6B, all three oligos tested reduced CaMKIID expression by >90%, although two controls (low GC RNAi and Lipofectamine only) had minimal effects. When the RNAi-treated cells were tested for lumen formation in three-dimensional culture (data not shown). Further analysis of the time course of lumen formation was made difficult by the tendency of the overexpressed CaMKIID to inhibit acinus formation.

Knockdown of CaMKIID by RNAi Blocks Lumen Formation—To test the functional role of CaMKIID in lumen formation, several RNAi oligos that target CaMKIID were transfected into MCF7 cells expressing CEACAM1-SF. As shown in Fig. 6B, all three oligos tested reduced CaMKIID expression by >90%, although two controls (low GC RNAi and Lipofectamine only) had minimal effects. When the RNAi-treated cells were tested for lumen formation in three-dimensional culture, oligo 2 reduced lumen formation to 15% compared with 93% lumen formation for no treatment, 81% for the transfection reagent control, and 67% for the RNAi transfection control (Table 2). Notably, oligo 1 was no better than the RNAi control, although oligo 3 had intermediate lumen formation (39%), suggesting a range of efficiency for these oligos in three-dimensional culture. A representative microscopic view of the resulting acini for a control versus oligo 2-treated cells is shown in Fig. 7. These results confirm the hypothesis that CaMKIID plays an essential role in lumen formation in the three-dimensional model system.

Inhibition of CaMKII with KN93 or W7 Blocks Lumen Formation—To test the effect of pharmacological inhibitors of CaMKII on the ability of CEACAM1-SF-transfected MCF7 cells to form lumena in three-dimensional culture, the cells were treated with either KN93, a specific CaMKII inhibitor, DMSO vehicle, or KN92, an inactive analog of KN93. Cells treated with vehicle or two different doses of KN92 formed lumena (>80%), although lumena were blocked in a dose-dependent manner with KN93 (Table 2). Although KN93 is not specific for CaMKIID, these data confirm the importance of CaMKII in lumen formation. Further studies were also performed with W7, a generic CaM antagonist that prevents the binding of CaM to its target proteins. Similar to KN93, this inhibitor caused a dose-related decrease in lumen formation for CEACAM1-SF-transfected MCF7 cells grown in a 4-day culture (Table 2).
Phosphorylation of CaMKIID and CEACAM1-SF during Lumen Formation—Upon binding CaM, CaMKII kinases become activated by autophosphorylation of Thr-286 (28). Thus, it was of interest to determine whether this residue was phosphorylated in CEACAM1-SF-transfected MCF7 cells under lumen formation in three-dimensional culture. Lysates from WT, but not from vector control or T457A,S459A double mutated CEACAM1-SF (MCF7-SF) were incubated with a desthiobiotin-ATP reagent (see “Experimental Procedures”), purified on streptavidin beads, eluted proteins separated by SDS-gel electrophoresis, and immunoblotted with anti-PKC-δ or anti-CaMKIID. The positions of each kinase are indicated with arrows.

DISCUSSION

Early on, we were struck by the fact that CEACAM1-SF was expressed lumenally in normal breast and down-regulated in breast cancer, especially in the invasive front lacking lumen formation (8). This observation took on phenotypic meaning in results (Table 2). It should be noted that we have no phosphoryspecific antibodies for CEACAM1-SF and that due to its extensive glycosylation (over 50%) and the need to use polyclonal antibodies for phospho-Thr, the immunoblot bands are quite diffuse. Nonetheless, they agree well with our previous studies on phospho-Thr levels that show increased phospho-Thr on CEACAM1-SF in MCF7 cells undergoing lumen formation (15). Immunoblot analysis with phospho-Ser antibodies was inconclusive, suggesting that phosphorylation of Ser-459 is a minor site.

immunoblot analysis: untreated (MCF7 cells grown in three-dimensional culture for 4 days in the presence or

Because this is exactly what we showed with MCF7 cells, including the documentation that lumen forma-

FIGURE 6. Expression of CaMKIID protein and its inhibition with RNAi in

TABLE 2

Inhibition of lumen formation by RNAi or calmodulin kinase IID inhibitors

MCF7 cells transfected with CEACAM1–4S were treated as described, and 200 colonies were counted and scored for lumen formation. p values were calculated relative to no treatment or DMSO control.

the normal breast cell line MCF10F, a variant of the more pop-

ular MFC10A line, which expressed CEACAM1–SF and formed a lumen in three-dimensional culture, while silencing of CEACAM1 with antisense abrogated this lumen formation (8).

The logical reverse experiment was to take a cell line that did not express CEACAM1 nor form a lumen in three-dimensional culture and reinitiate this phenotype by forced expression of the CEACAM1 gene. Because this is exactly what we showed with MCF7 cells, including the documentation that lumen forma-
including CaMKIID, have been recently shown to bind to G-actin in the absence of Ca\(^{2+}\) and to bundle actin when activated by Ca\(^{2+}\)/CaM (22). Given that new information, one can envision a stepwise series of events that start with Ca\(^{2+}\)/CaM release and proceeds all the way to actin binding, polymerization, and bundling at the site of CEACAM1 activation (Fig. 10). Given the multimeric structure of CaMKIIs and their ability to bind G-actin, they are perfectly positioned to deposit large amounts of G-actin to a membrane enriched in a G-actin-binding protein such as CEACAM1-SF. However, this step must be controlled in some way to prevent a constitutive unloading of cargo. CEACAM1-SF fulfills this requirement by only binding G-actin in the presence of Ca\(^{2+}\) by the unmasking of critical residue Phe-457 (27). Because unmasking Phe-457 also leads to Ca\(^{2+}\)/CaM binding, the situation is perfectly poised to simultaneously activate CaMKII by transfer of Ca\(^{2+}\)/CaM from CEACAM1-SF to CaMKIID. Ca\(^{2+}\)/CaM-activated CaMKIID would then phosphorylate its substrate CEACAM1-SF and transfer one G-actin to CEACAM1-SF. Because there are 14 subunits on one holoenzyme, the process could continue in an iterative manner (Fig. 10). Finally, CaMKIID could bundle actin as it polymerizes along the CEACAM1-SF enriched membrane. It should be stressed that this is only a model and may need to be modified as more data on both CEACAM1-SF and CaMKIID become available. At present, we are studying the binding of Ca\(^{2+}\)/CaM to CEACAM10SF peptides by NMR. Future studies will focus on the putative exchange of G-actin for Ca\(^{2+}\)/CaM on CaMKIID. So far, the evidence suggests that association of CEACAM1-SF with the cytoskeleton is a key step that coordi-
nates signaling from the cytoskeleton components to the apoptotic pathway. In that respect, we have already identified the involvement of additional key components, including PKC-δ, and the unusual calpain isoform calpain-9 is required for apoptosis (9). Taken together, these data suggest the existence of an extremely complex and fine-tuned mechanism for lumen formation, not surprising given the need to respond to a large number of environmental cues, including cell-cell and cell-extracellular matrix interactions and hormones produced by the pituitary gland.

Finally, the kinase requirement for CEACAM1-SF has undergone a significant evolutionary change from mouse to man. This finding further challenges us to determine why such a change was necessary and at what point in evolution did the change occur? Answers to these questions not only await further investigation but, in addition, may reveal additional biochemical differences at the level of the mammary gland between the two species.

REFERENCES

1. Bissell, M. J., Radisky, D. C., Rizki, A., Weaver, V. M., and Petersen, O. W. (2002) The organizing principle: microenvironmental influences in the normal and malignant breast. 

2. Mailleux, A. A., Overholtzer, M., Schmelze, T., Bouillet, P., Strasser, A., and Brugge, J. S. (2007) BIM regulates apoptosis during mammary ductal morphogenesis, and its absence reveals alternative cell death mechanisms. 

3. Mailleux, A. A., Overholtzer, M., and Brugge, J. S. (2008) Lumen formation during mammary epithelial morphogenesis: insights from in vitro and in vivo models. 

4. Kirshner, J., Chen, C. J., Liu, P., Huang, J., and Shively, J. E. (2003) CEACAM1–4S, a cell-cell adhesion molecule, mediates apoptosis and reverts mammary carcinoma cells to a normal morphogenic phenotype in a 3D culture. 

5. Debnath, J., Mills, K. R., Collins, N. L., Regnato, M. J., Muthuswamy, S. K., and Brugge, J. S. (2002) The role of apoptosis in creating and maintaining luminal space within normal and oncogene-expressing mammary acini. 

6. Mills, K. R., Regnato, M., Debnath, J., Queenan, B., and Brugge, J. S. (2004) Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is required for induction of apoptosis during lumen formation in vitro. 

7. Huang, J., Simpson, J. F., Glackin, C., Riether, L., Wagener, C., and Shively, J. E. (1998) Expression of biliary glycoprotein (CD66a) in normal and malignant breast epithelial cells. 

8. Huang, J., Hardy, J. D., Sun, Y., and Shively, J. E. (1999) Essential role of biliary glycoprotein (CD66a) in morphogenesis of the human mammary epithelial cell line MCF10F. 

9. Chen, C. J., Nguyen, T., and Shively, J. E. (2010) Role of calpain-9 and PKC-δ in the apoptotic mechanism of lumen formation in CEACAM1 transfected breast epithelial cells. 

10. Barnett, T. R., Drake, L., and Pickle, W., 2nd (1993) Human biliary glycoprotein gene: characterization of a family of novel alternatively spliced RNAs and their expressed proteins. 

11. Turvide, C., Rojas, M., Stanners, C. P., and Beauchemin, N. (1991) A mouse carcinoembryonic antigen gene family member is a calcium-dependent cell adhesion molecule. 

12. Gray-Owen, S. D., and Blumberg, R. S. (2006) CEACAM1: contact-dependent control of immunity. 

13. Gaur, S., Shively, J. E., Yen, Y., and Gaur, R. K. (2008) Altered splicing of CEACAM1 in breast cancer: identification of regulatory sequences that control splicing of CEACAM1 into long or short cytoplasmic domain isoforms. 

14. Dery, K. J., Gaur, S., Gencheva, M., Yen, Y., Shively, J. E., and Gaur, R. K. (2011) Mechanistic control of carcinoembryonic antigen-related cell adhesion molecule-1 (CEACAM1) splice isoforms by the heterogeneous nuclear ribonuclear proteins hnRNP I, hnRNP A1, and hnRNP M. 

15. Chen, C. J., Kirshner, J., Sherman, M. A., Hu, W., Nguyen, T., and Shively, J. E. (2007) Mutation analysis of the short cytoplasmic domain of the cell-cell adhesion molecule CEACAM1 identifies residues that orchestrate actin binding and lumen formation. 

16. Edlund, M., Wikström, K., Toomik, R., Ek, P., and Obrink, B. (1998) Characterization of protein kinase C-mediated phosphorylation of the short cytoplasmic domain isoform of C-CAM. 

17. Zhu, W., Woo, A. Y., Yang, D., Cheng, H., Crow, M. T., and Xiao, R. P. (2007) Activation of CaMKIIδC is a common intermediate of diverse death stimuli-induced heart muscle cell apoptosis. 

18. Braun, A. P., and Schulman, H. (1995) The multifunctional calcium/calmodulin-dependent protein kinase: from form to function. 

19. Edlund, M., Wikström, K., Toomik, R., Ek, P., and Obrink, B. (1998) Characterization of protein kinase C-mediated phosphorylation of the short cytoplasmic domain isoform of C-CAM. 

20. Mercure, M. Z., Ginnan, R., and Singer, H. A. (2008) Calcium/calmodulin-dependent protein kinase II–regulated development of vascular smooth muscle cell polarization and migration. 

21. Tombs, R. M., Mikkelsen, R. B., Jarvis, W. D., and Grant, S. (1999) Down-regulation of δ CaM kinase II in human tumor cells. 

22. Hoffman, L., Farley, M. M., and Waxham, M. N. (2013) Calcium-calmodulin-dependent protein kinase II isoforms differentially impact the dynamics and structure of the actin cytoskeleton. 

23. Krutchinsky, A. N., Kalkum, M., and Chait, B. T. (2001) Automatic identification of proteins with a MALDI-quadrupole ion trap mass spectrometer. 

24. Lu, R., Niesen, M. J., Hu, W., Vadehi, N., and Shively, J. E. (2011) Interaction of actin with carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) receptor in liposomes is Ca2+ - and phospholipid-dependent. 
28. Hudmon, A., and Schulman, H. (2002) Structure-function of the multifunctional Ca\(^{2+}\)/calmodulin-dependent protein kinase II. *Biochem. J.* 364, 593–611

29. Kameshita, I., and Fujisawa, H. (1989) A sensitive method for detection of calmodulin-dependent protein kinase II activity in sodium dodecyl sulfate-polyacrylamide gel. *Anal. Biochem.* 183, 139–143

30. Salas, M. A., Valverde, C. A., Sánchez, G., Said, M., Rodríguez, J. S., Portiansky, E. L., Kaetzel, M. A., Dedman, J. R., Donoso, P., Kranias, E. G., and Mattiazzi, A. (2010) The signalling pathway of CaMKII-mediated apoptosis and necrosis in the ischemia/reperfusion injury. *J. Mol. Cell. Cardiol.* 48, 1298–1306

31. Patel, P. C., Lee, H. S., Ming, A. Y., Rath, A., Deber, C. M., Yip, C. M., Rocheleau, J. V., and Gray-Owen, S. D. (2013) Inside-out signaling promotes dynamic changes in the carcinoembryonic antigen-related cellular adhesion molecule 1 (CEACAM1) oligomeric state to control its cell adhesion properties. *J. Biol. Chem.* 288, 29654–29669