Carcinoembryonic Antigen Cell Adhesion Molecule 1 Directly Associates with Cytoskeleton Proteins Actin and Tropomyosin*

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CEA cell adhesion molecule 1 (CEACAM1), a type 1 transmembrane and homotypic cell adhesion protein belonging to the carcinoembryonic antigen (CEA) gene family and expressed on epithelial cells, is alternatively spliced to produce four major isoforms with three or four Ig-like ectodomains and either long (CEACAM1-L) or short (CEACAM1-S) cytoplasmic domains. When murine MC38 (methylcholanthrene-induced adenocarcinoma 38) cells were transfected with human CEACAM1-L and stimulated with sodium pervanadate, actin was found to co-localize with CEACAM1-L at cell-cell boundaries but not in untreated cells. When CEACAM1-L was immunoprecipitated from pervanadate-treated MC38/CEACAM1-L cells and the associated proteins were analyzed by two-dimensional gel analysis and mass spectrometry, actin and tropomyosin, among other proteins, were identified. Whereas a glutathione S-transferase (GST) fusion protein containing the l-isoform (GST-Cyto-L) bound poorly to F-actin in a co-sedimentation assay, the S-isoform fusion protein (GST-Cyto-S) co-sedimented with F-actin, especially when incubated with G-actin during polymerization (K_D = 7.0 μM). Both GST-Cyto-S and GST-Cyto-L fusion proteins bind G-actin and tropomyosin by surface plasmon resonance studies with binding constants of 0.7 × 10^-8 and 1.0 × 10^-7 M for GST-Cyto-L to G-actin and tropomyosin, respectively, and 3.1 × 10^-8 and 1.3 × 10^-7 M for GST-Cyto-S to G-actin and tropomyosin, respectively. Calmodulin or EDTA inhibited binding of the GST-Cyto-L fusion protein to G-actin, whereas calmodulin and G-actin, but not EDTA, stimulated binding to tropomyosin. A biotinylated 14-amino acid peptide derived from the juxtamembrane portion of the cytoplasmic domain of CEACAM1-L associated with both G-actin and tropomyosin with K_D values of 1.3 × 10^-5 and 1.8 × 10^-5 M, respectively. These studies demonstrate the direct interaction of CEACAM1 isoforms with G-actin and tropomyosin and the direct interaction of CEACAM1-S with F-actin.

CEACAM11 (biliary glycoprotein, CD66a) is a member of the carcinoembryonic antigen (CEA) family, which in turn belongs to the Ig superfamily (1–4). Alternative splicing of the transcripts of a single gene results in expression of at least four CEACAM1 isoforms (5, 6), all of which contain a transmembrane region, followed by a 74-amino acid long (CEACAM1-L) or a 14-amino acid short (CEACAM1-S) cytoplasmic domain. CEACAM1 is a highly glycosylated type 1 transmembrane protein expressed on the surface of epithelial, endothelial, and granulocytic cells (7). The human as well as the rat and mouse isoforms of CEACAM1 have been characterized as homotypic cell adhesion molecules (8, 9). Murine CEACAM1 also functions as a receptor for murine hepatitis virus (10), whereas human CEACAM1 can bind bacterial membrane proteins from Escherichia coli, Salmonella typhimurium, or Neisseria gonorrhoeae (11, 12). CEACAM1 expression is down-regulated in human colon (13) and prostate (14) cancer and in 30% of breast cancers (15). Transfection of rat CEACAM1-L into a human tumorigenic prostate cell line rendered the cells non-tumorigenic in nude mice (16), suggesting a tumor inhibitory function of CEACAM1. Transfection of human CEACAM1-L into the breast cancer cell line MCF7 leads to massive apoptosis when the cells are cultured in three-dimensional matrigel (17). The effect of the ratio of cytoplasmic domain isoforms on tumorigenicity reveals that an excess of the murine CEACAM1-L over the CEACAM1-S isoform is dominant and renders murine colon adenocarcinoma CT51 cells non-tumorigenic (18).

To understand the biological function of the two isoforms, studies have focused on the identification of cellular proteins interacting with the cytoplasmic domain of CEACAM1-L. CEACAM1-L has been shown to be phosphorylated in vitro on its tyrosine as well as its serine and threonine residues (19), in neutrophils after antibody binding (20), or in hepatocytes after activation of the insulin receptor (21, 22). The reported association of human CEACAM1-L or its murine and rat homologues with Src family kinases (23, 24) as well as the protein-tyrosine phosphatases SHP-1 (25) and SHP-2 (26) suggests a signal transduction role for CEACAM1-L. Along with interactions with kinases and phosphatases, rat CEACAM1 isoforms have been shown to bind calmodulin (27). Calmodulin is a cytoplasmic regulator of enzymatic and cytoskeletal functions in cells, and its direct association to the long and the short cytoplasmic domain of human, rat, or mouse CEACAM1 synthetic peptides has been reported (28). Most recently, binding of the cytoplasmic domain of rat CEACAM1-L to actin has been reported (29, 30). Sadekova et al. (30) have shown that when Swiss 3T3 cells are microinjected with murine CEACAM1-L, Rho GTPase activation is required for localization to cell-cell boundaries and co-localization with actin filaments.

To gain more direct evidence of the interaction of liquid chromatography; LC, liquid chromatography; RU, relative units; BSA, bovine serum albumin.

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CEACAM1-L with the cytoskeleton, we transfected murine MC38 cells with human CEACAM1-L and showed that actin and tropomyosin, among other proteins, were immunoprecipitated with CEACAM1 from MC38/CEACAM1-L cells stimulated with sodium pervanadate (10 mM H2O2 and 0.1 mM Na3VO4) for 15 min. The nucleotide sequences of Cyto-L and Cyto-S correspond to RI sites and subcloned into pGEX-Bam incorporating 5'-captoethanol) and subsequently stored at −80 °C for 1 h at 4 °C. Samples were analyzed by one-dimensional SDS-polyacrylamide gel electrophoresis on 8–16% Tris glycine gradient gels (NOVEX) as described previously (35).

**Table I**

| Peptide sequence                      |
|--------------------------------------|
| Cyto-L-(419–432)                      |
| Cyto-L-scrambled                      |
| Cyto-L-Thr(P)-425                     |
| Cyto-S-Thr(P)-425, Ser(P)-429         |
| Cyto-S-(419–432)                      |
| Cyto-S-T425E,S429E                    |
| Cyto-S-T425A,S429A                    |
| Cyto-L-(419–432)                      |

**Experimental Procedures**

**Transfection and Cell Culture—**Murine MC38 cells (methylcholanthrene-induced adenocarcinoma) were transfected with full-length human CEACAM1-L cDNA (6) inserted into the pH β-actin vector (31), transfected with Lipofectin (Life Technologies, Inc.), and selected in 0.1–1.0 mg/ml G418 for 8 weeks. The cells were negative prior to transfection and uniformly positive for CEACAM1-L after transfection and selection in G418. Transfected cells were grown to confluency in Dulbecco’s modified Eagle’s high glucose medium with 10% calf serum before each experiment. Cells (1 × 107 cells/ml) were treated with sodium pervanadate (10 mM H2O2 and 0.1 mM Na3VO4) for 15 min at 37 °C in 5% CO2 in Dulbecco’s modified Eagle’s high glucose medium with 10% fetal calf serum. Cells were harvested by resuspension in Dulbecco’s modified Eagle’s high glucose medium without fetal calf serum and centrifuged.

**Antibodies, Recombinant Proteins, and Synthetic Peptides—**The murine anti-CD66 mAb T84.1, which recognizes an epitope in the extracellular NH2-terminal domain of human CEACAM1, was used for immunoprecipitation and immunoblotting (32). The murine anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology, Inc.) was used for immunoblotting. Hors eradish peroxidase-labeled goat anti-mouse antibody (Pierce) was used for immunoprecipitation and immunoblotting (32).

**Results**

**Immunoprecipitation and Gel Electrophoresis—**Immunoprecipitation of CEACAM1-L from CEACAM1-L-transfected MC38 cells was performed using the anti-CD66 mAb T84.1 and protein G-agarose (Roche Molecular Biochemicals) as described in the manufacturer’s manual. Harvested cells were frozen overnight at −80 °C and extracted with 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40 (v/v), 0.5% sodium deoxycholate (v/v)) by vortexing for 30 s at 4 °C. Preclearing of the cell lysates was performed by incubation for 2 h with 4 μg of mAb T84.1 (isotype matched anti-CEA 1 antibody), addition of 20 μl of protein G-agarose, and continued incubation for 2 h at 4 °C. Following the preclearing, 4 μg of mAb T84.1 was added to the lysates for 2 h, followed by addition of 20 μl of protein G-agarose and incubation overnight at 4 °C. Precipitated proteins were recovered by incubation of the slurry for 1 h at 4 °C in solubilization buffer (9 μl urea, 4% Nonidet P-40, 2% amylphosphates, pH 2–11, 2% β-mercaptoethanol) and subsequently stored at −20 °C. Samples were analyzed by one-dimensional SDS-polyacrylamide gel electrophoresis on 8–16% Tris glycine gradient gels (NOVEX) as described previously (35).

Two-dimensional gel electrophoresis was performed as described by O’Farrell (36) using the Bio-Rad Protein Ixy system. Samples were analyzed in the first dimension on a pH 2–11 gradient (Sigma amorphous), followed by a separation on a 10% SDS-polyacrylamide gel in the second dimension. Polyacrylamide gels were silver-stained according to Shevchenko et al. (37). For immunoblotting, proteins were transferred onto a nitrocellulose membrane (Bio-Rad) using the Bio-Rad semidyem blotting system and subsequently probed with the monoclonal anti-CEACAM1 antibody (T84.1) followed by goat anti-mouse Texas Red-conjugated antibody (Molecular Probes). Imaging was performed on a Leica TCS-SP spectral confocal microscope.

**Mass Spectrometry—**Silver-stained protein spots were excised from two-dimensional gels and in-gel digested with trypsin (Promega) as described by Shevchenko et al. (37). All solutions were prepared fresh prior to the digestion using HPLC-grade chemicals (Sigma) and 0.2 μm dichloromethane, dried, and cleaved in the usual manner. O-Benzyl-protected phosphoserine and phosphothreonine Fmoc amino acids were purchased from Novabiochem and coupled to the peptide/solid support (0.1 mmol) according to the manufacturer’s instructions. Peptides were purified to >95% purity by reversed-phase HPLC on a Poros RP2 or Oligo R3 column and then confirmed by electrospray ionization mass spectrometry on a Finnigan LCQ ion trap mass spectrometer. "Conflon Microscopy—"Immunofluorescent staining of CEACAM1-L-transfected MC38 cells with antibodies and subsequent confocal microscopy experiments were performed according to Griffiths (34). Cells (1 × 106) were grown for 2 or 24 h at 37 °C on polylysine-coated glass coverslips, treated or untreated with pervanadate (0.1 μl, 15 min), and fixed with 2% paraformaldehyde (v/v) in phosphate-buffered saline. Fixed cells were permeabilized with 0.01% saponin (w/v), 0.25% gelatin (v/v), and 0.1% Nonidet P-40 (v/v) in phosphate-buffered saline and then stained with Oregon Green-conjugated phalloidin (Molecular Probes) and anti-CEACAM1 antibody (T84.1) followed by goat anti-mouse Texas Red-conjugated antibody (Molecular Probes). Imaging was performed on a Leica TCS-SP spectral confocal microscope.
of filtered MilliQ water. To ensure successful tryptic digestion, two additional washing steps involving dehydration of the gel pieces with 100% acetonitrile (MeCN) and rehydration using 100 mM NH₄HCO₃ were added before and after the reduction/alkylation step. Extracted peptides were concentrated in a SpeedVac and resuspended in 10 μl of 0.1% trifluoroacetic acid. To minimize introduction of contaminating proteins, all in-gel digestion steps were carried out in a laminar flow hood. LC/MS/MS analyses were performed using an Apple Macintosh.

The standard gradient was from 2 to 92% Buffer B over 120 min using low TFA buffers (Buffer A, 0.02% trifluoroacetic acid; Buffer B, 90% MeCN, 0.014% trifluoroacetic acid) at 50 p.s.i. Sample injection was performed at 1500 p.s.i. for 2 min followed by 10 min of washing at high pressure with Buffer A for desalting and removal of contaminating components. Prior to the injection of the preformed gradient, the system pressure was reduced to 50 p.s.i. over 1 min. The 150-μm inner diameter × 350-μm outer diameter on-line microspray needles were pulled using a laser-based micropipette puller (Sutter Instrument Co.) to a terminal inner diameter of ~5 μm. The tip was packed at 4000 p.s.i. using a Zorbax, 5 μm C₁₈ packing as described previously by Davis et al. (39). The packed tip was connected to a 75-μm inner diameter × 350-μm outer diameter transfer line using a PEEK capillary Tee (Valco) and graphite ferrules. A 0.3-mm gold wire was introduced through the off-axis inlet to apply the electrospray potential. All mass spectral analyses were performed using a Finnigan LCQ ITMS equipped with a custom microspray interface. The LCQ was operated under Automatic Gain Control and enabled dynamic exclusion in the Navigator view. The automatic gain control targets are as follows: full MS, 5e⁶; MS/MS, 2e⁶; MS/MS, 2e⁶; and MS/MS, 2e⁶. The default maximum injection time was 500 ms with a single microscan count.

Co-sedimentation Assays—Co-sedimentation assays were performed with the actin protein binding kit (Cytoskeleton) essentially according to the manufacturer’s instructions. Briefly, G-actin (2.7 μM) was polymerized in the presence or absence of the GST-Cyto fusion proteins (2–18 μM) for 60 min at 28 °C and pelleted at 150,000 × g for 1 h in a Beckman L8-M ultracentrifuge using an SW50.1 rotor. The pellets and supernatant fractions were run on 12% polyacrylamide SDS gels and stained with Coo massie Blue. Bands were scanned on a Bio-Rad G-10 calibrated densitometer, integrated, and expressed as GST-Cyto-S fusion protein/actin ratios. Experiments were repeated three times, and the results were plotted as GST-Cyto-S fusion protein/actin versus [GST-Cyto-S fusion protein] (Hill plots). The Kᵦ was determined from the [GST-Cyto-S fusion protein] at half-maximal binding. Controls included α-actinin (positive binding), BSA (negative binding), and the GST-only protein (negative binding).

SPR Analysis—Biomolecular interaction analyses were carried out in HBS Buffer (150 mM NaCl, 0.05% (v/v) Surfactant P20, 10 mM HEPES, pH 7.4) using the BIAcore® 2000 (BIAcore, Inc.). Depending on the experiment, CaCl₂ was added to concentrations ranging from 10 mM EDTA to 100 mM HCl added to the HBS. G-actin or tropomyosin were immobilized on a CM5-rg sensorchip (BIAcore) using the Amine Coupling Kit (BIAcore). The surface of the sensorchip was activated with 30 μl of 100 mM N-ethyl-N’-(dimethylaminopropyl)carbodiimide hydrochloride, 400 μM N-hydroxysuccinimide using a flow rate of 5 μl/min. For immobilization of the protein, 1 μg of G-actin or 5 μg of tropomyosin in 100 μl of 10 mM sodium acetate, pH 4.0, were applied (flow rate, 5 μl/min). Subsequently, the sensorchip was deactivated with 30 μl of 1 M ethanolamine hydrochloride, pH 8.5 (flow rate, 5 μl/min), and conditioned with 10 μl of 100 mM HCl (flow rate, 5 μl/min). Binding studies and regeneration of the chip surface between injections were carried out at a flow rate of 20 μl/min unless otherwise noted. Samples were diluted in HBS Buffer immediately prior to injection. Between sample injections the surface was regenerated with 15 μl of 6 M guanidine HCl followed by three 30-s injections of HBS.

RESULTS

Identification of CEACAM1-L-associated Proteins—Previous studies (30) demonstrated that murine CEACAM1-L interacts with the cytoskeleton in a Rho kinase-dependent manner. This study suggested that the interaction was indirect, requiring CEACAM1-L modification or interaction through associated proteins. Possible modifications for CEACAM1-L such as phosphorylation on one or both of its tyrosines located in the cytoplasmic domain have been shown previously to affect its biological properties (18, 26, 30). We explored these possibilities further using murine MC38 cells transfected with human CEACAM1-L. This murine colon cancer cell line, in contrast to most human colon cancer cell lines, does not express CEACAM1, and when transfected with the human CEACAM1-L cDNA and grown for 24 h on polylysine-coated glass slides, the cell line expressed high levels of CEACAM1-L that localize to cell-cell junctions with little evidence of co-localization with actin (red only). When these cells were treated with sodium pervanadate, a treatment which increases tyrosine phosphorylation by inhibition of tyrosine phosphatases (40, 41), CEACAM1-L remained at cell-cell boundaries and co-localized with actin (yellow, Fig. 1D). These results show that pervanadate treatment causes CEACAM1-L to associate with the actin cytoskeleton in a phosphorylation-dependent manner. In contradiction to our results, Sadekov et al. (30) showed extensive co-localization of murine CEACAM1-L with actin in murine CT-51 carcinoma cells; however, their cells exhibited a rounded morphology with extensive cortical actin. Indeed, when our transfected cells were grown for only 2 h on glass slides, they exhibited a rounded morphology, cortical actin, and co-localization of actin with CEACAM1-L at the cell-cell junctions (Fig. 1A and B). Because this does not mimic the morphology of these cells when grown on plastic, we believe the results shown in Fig. 1C and D, for cells grown on slides for 24 h are more relevant.

Pervanadate has been shown previously to stimulate tyrosine phosphorylation of murine CEACAM1-L (26). To provide more direct evidence of CEACAM1-L tyrosine phosphorylation and association of CEACAM1-L with the cytoskeleton, CEACAM1-L was immunoprecipitated from MC38/CEACAM1-L cell lysates,
before and after pervanadate treatment, using the mAb T84.1. This antibody binds to the extracellular amino-terminal domain of CEACAM1 as well as other members of the human CEA family. However, it does not cross-react with murine CEACAM1 and is therefore specific for human CEACAM1-L in our model system. To minimize contamination of the immunoprecipitates with proteins binding nonspecifically to either antibody or protein G-agarose, the cell lysates were precleared for 3 h with the CEA-specific mAb T84.66 and protein G-agarose prior to the immunoprecipitation with mAb T84.1. The anti-CEA mAb T84.66 is an isotype-matched, control antibody that does not react with CEACAM1-L.

Successful immunoprecipitation of CEACAM1-L was verified by immunoblotting immunoprecipitates with anti-CEACAM1 antibody T84.1 (Fig. 2A). The 15-min treatment of the cells with sodium pervanadate led to about a 2-fold difference in the amount of immunoprecipitated CEACAM1-L. This difference may be due to the decreased solubility of the immunoprecipitates in solubilization buffer after pervanadate treatment. Due to this difference we may have underestimated the amount of co-immunoprecipitated proteins by a factor of 2 in subsequent experiments. Immunoblotting was also used for detection of tyrosine phosphorylation using the anti-phosphotyrosine mAb 4G10 (Fig. 2B). Precipitates from untreated cells showed no detectable tyrosine phosphorylation of CEACAM1-L, either due to the lack of tyrosine phosphorylation or the activity of tyrosine phosphatases in the lysates of pervanadate untreated cells. However, upon treatment of the cells with sodium pervanadate, tyrosine phosphorylation of CEACAM1-L was readily detectable, either due to in vivo inhibition of phosphatases, activation of tyrosine kinases, or both.

Analysis of silver-stained SDS gels of immunoprecipitated samples revealed the co-precipitation of two major proteins at 200 and 45 kDa with CEACAM1-L (Fig. 2C) which correlate directly with the increased tyrosine phosphorylation of CEACAM1-L due to pervanadate treatment. The identification of these proteins as myosin and actin is based on further studies shown below. Whereas de novo co-precipitation of proteins occurred mainly for the 200- and 45-kDa proteins, other proteins present in immunoprecipitates from untreated cells co-precipitated in higher amounts after treatment of the cells with sodium pervanadate (Fig. 2C). These results indicate either a general phosphorylation event caused increased protein association with CEACAM1-L or a specific tyrosine phosphorylation of CEACAM1-L caused enhanced association of proteins to its phosphorylated cytoplasmic domain. These data do not distinguish the two possibilities. The silver-stained gels do not reveal a band corresponding to immunoprecipitated CEACAM1-L, which due to its high level of glycosylation stains poorly with silver.

To obtain better resolution and identification of the immunoprecipitated proteins, immunoprecipitates from cells before and after treatment with sodium pervanadate were further analyzed by two-dimensional gel electrophoresis. Two-dimensional gel analysis of immunoprecipitates from untreated cells revealed ~15 co-precipitated proteins, 5 of which were highly abundant (spots 1–5 in Fig. 3A), and the remaining were barely detectable even using silver staining. Upon treatment of the cells with sodium pervanadate, the intensity of the co-precipitated proteins (Fig. 3B) increased significantly (up to 5-fold). A close analysis of the two gels suggests that no new spots appeared, but many of the spots increased in intensity. Prominent among these were spots 1, 2, 4, and 5, later identified as β- and γ-actin, keratin, and myosin, all components of the cytoskeleton. In addition to these five prominent proteins visible in Fig. 3A, increased co-precipitation of proteins 6–8 was observed (Fig. 3B). Two of these proteins were later identified as tropomyosin (spot 6) and vimentin (spot 7). From these immunoprecipitation studies, we conclude pervanadate treatment leads to (a) increased tyrosine phosphorylation of CEACAM1-L, and (b) increased binding of co-precipitating proteins. These effects may be direct (due to phosphorylation of CEACAM1-L) or mediated by other events caused by pervanadate (indirect). However, because the results are specifically due to pervanadate treatment, we proceeded to identify the CEACAM1-L-associated proteins.

Identification of CEACAM1-binding Proteins by LC/MS/MS—Seven of the most abundant spots were excised from the silver-stained two-dimensional gel obtained from immunoprecipitates from the sodium pervanadate-treated cells (Fig. 3B). The isolated protein spots were subjected to in-gel digestion
These proteins were identified with high confidence (Xcorr > 2 in SEQUEST) and excellent sequence coverage (11–44%). The isoforms of actin were identified based on their position on the two-dimensional gel. The more basic isoforms of actin are expressed in most cells, whereas expression of the more acidic α-actin isoform is restricted to contractile muscle cells (43). Based on mass spectrometry alone we were not able to identify peptides unique to either of the analyzed actin isoforms. Sufficient sequence information was obtained from spot 6 to positively identify it as tropomyosin isoform 2 (TM2). The identification of five of the analyzed proteins as members of the large and highly conserved family of cytoskeletal proteins demonstrates their interaction with CEACAM1-L, especially after treatment of the cells with sodium pervanadate. Two of the identified proteins were cytotkeratin (spot 4) and the IgG light chain (data not shown). The finding of cytotkeratin has to be interpreted with caution because this is a ubiquitous contaminant in analyses of this type (due to human skin particles in the air and on surfaces). The IgG light chain is from the immunoprecipitating antibody and is co-eluted from the protein G beads.

In these studies, the co-precipitation of myosin and tropomyosin with actin is not unexpected, given their usual association in motor-actomyosin fibers and the shape changes induced in these cells by pervanadate. Scanning densitometry of the two-dimensional gels reveals a 5-fold increase in actin, a 10-fold increase in myosin, and a 2-fold increase in TM2 after pervanadate treatment. Although these data are suggestive, they do not prove a direct association of the phosphorylated form of CEACAM1-L with these members of the cytoskeleton. As mentioned earlier, we believe that we have underestimated the amount of co-precipitated proteins, because lesser amounts of CEACAM1-L were immunoprecipitated in the pervanadate-treated versus untreated cells (Fig. 2A). To gain further insights into the potential associations of these proteins with CEACAM1-L, we next attempted to show a direct association of the cytoplasmic domain of CEACAM1-L with actin. We chose actin first because of its prominent role in thin filaments. In later experiments we also investigated the role of tropomyosin, because its interaction with actin is well described and its interaction with CEACAM1-L previously undescribed.

Co-sedimentation Assays for GST-Cyto Fusion Proteins with the Cytoskeletal Domains of CEACAM 1 Isoforms to Actin—To determine whether F-actin directly associates with the cytoplasmic domain of CEACAM1-L, we generated a GST fusion protein (GST-Cyto-L) containing the L-isoform cytoplasmic domain (74 amino acids) fused to the carboxyl terminus of GST and performed an F-actin co-sedimentation (spin down) assay. In the standard actin co-sedimentation assay, the protein is mixed with F-actin, the sample centrifuged, and the F-actin pellet analyzed by SDS-gel electrophoresis for any associated proteins. When we found that GST-Cyto-L did not co-sediment with pre-polymerized F-actin (Fig. 4A), we investigated the possibility that it could bind to G-actin in a competitive manner, reducing the amount of F-actin formed during the polymerization process. In this pre-polymerization assay, we found that GST-Cyto-L fusion protein did not bind to F-actin and did not prevent actin polymerization (Fig. 4A). Thus, these data agree with our immunoprecipitation results that demonstrated low binding of CEACAM1-L to the cytoskeleton in the absence of tyrosine phosphorylation. These results further confirm the study of Sadekova et al. (30) who showed that murine CEACAM-L did not bind to F-actin in a spin-down assay.

As a further control, we tested the binding of GST-Cyto-S to F-actin. In the alternatively spliced variant, the cytoplasmic S-isoform is only 14 amino acids long and has no tyrosines. Surprisingly, GST-Cyto-S bound F-actin strongly, especially when preincubated with G-actin in the pre-polymerization assay (Fig. 4A). Negative controls showed no binding of GST only or BSA to F-actin, and a positive control showed binding of α-actinin to F-actin (Fig. 4B). Because GST-Cyto-S bound to F-actin in the co-polymerization assay, we performed further studies on this fusion protein. When increasing amounts of the GST-Cyto-S fusion protein were used in the co-polymerization sedimentation assay, a KD of 7.0 μM was calculated (Fig. 4C). This analysis (Hill plot) shows the usual saturation curve over the expected concentration range for a membrane-expressed protein.

Because these studies suggested that the minimum sequence required for actin binding was present in the cytoplasmic S-isoform which partially overlaps the L-isoform, we synthesized peptides corresponding to this sequence and compared their binding by SPR to the analogous sequence in the L-form and to the binding of the GST-Cyto fusion proteins. The above studies also suggested the possibility that one or both of the isoforms bind G-actin but not in a competitive way (i.e., not blocking polymerization to F-actin). Therefore, both fusion proteins were tested for their ability to bind G-actin using SPR. SPR allows a direct measurement of binding and dissociation kinetics and calculation of KD from the ratio of the kinetic constants.

**SPR Studies on the L-isoform—G-actin was immobilized at a binding level of 3 ng/mm² on a Biacore CM5-rg chip, based on the assumption that an SPR response of 1000 relative units (RU) translates to 1 ng/mm² immobilized protein (44). Gel filtration analysis of GST-Cyto-L revealed a single species of 143 kDa, corresponding to tetratrams (GST alone shows a mixture of dimers, tetramers, and multimers). Binding of the GST-Cyto-L fusion protein to G-actin was measured at concentrations ranging from 0.25 to 2.5 μM (Fig. 5A). No binding of the GST control was observed at the highest concentrations tested (10 μM was assumed for monomeric GST, although it is a mixture of oligomers). Because GST constitutes the bulk of the fusion protein, this indicates that the 74-amino acid fusion domain is responsible for the binding of GST-Cyto-L to G-actin. The regular kinetics of association and dissociation indicate that the binding is specific. In addition, we showed that immobilized and regenerated G-actin binds DNase I (data not shown), a well established binding partner for native G-actin (45), and GST-Cyto-L bound equally well to immobilized G-actin after the DNase I binding experiments. The KD for the GST-Cyto-L fusion protein binding to G-actin was determined as 0.74 × 10⁻⁸ M (Table III).

Based on the work of Obrink and co-workers (28, 46) who showed binding of calmodulin to synthetic peptides derived from the extreme juxtamembrane cytoplasmic portion of rat CEACAM1-L and the entire cytoplasmic domain of rat CEACAM1-S, we decided to synthesize and further investigate the human equivalents of these peptides regarding a possible interaction with immobilized G-actin. Peptides corresponding to the cytoplasmic juxtamembrane sequence of the CEACAM1-L (Cyto-L-(419–432)) or the complete sequence of the CEACAM1-S cytoplasmic domain (Cyto-S-(419–432), including an amino-terminal biotin-Gly-Gly-sequence (Table I), were synthesized and tested for binding by SPR to immobilized G-actin.

High binding was observed for the wild type long peptide...
| Spot | Protein identified | (M + H)$^+$ | Residues | Sequence from data base$^c$ |
|------|-------------------|-------------|----------|---------------------------|
| 1    | Myosin            | 986.6       | 1718–1726| GALALEEKR                 |
|      |                   | 1220.7      | 1446–1455| KFDQLLAEER                |
|      |                   | 1226.6      | 1622–1632| DLEAHIDTANK               |
|      |                   | 1229.7      | 1165–1174| REQEVSILKK                |
|      |                   | 1286.7      | 1391–1401| LQKDRLEGSLQR              |
|      |                   | 1377.8      | 645–656  | TVGQQLYKEQLAK             |
|      |                   | 1378.6      | 1694–1694| EMEALEELDR                |
|      |                   | 1530.8      | 1817–1829| IALKQLQDNETK              |
|      |                   | 1540.9      | 1248–1260| KKVKEAIQELQVK             |
|      |                   | 1565.7      | 1900–1913| ELEDATETADAMNR            |
|      |                   | 1585.8      | 1391–1401| LQKDLEGLSQR               |
|      |                   | 1646.8      | 1485–1498| AQLEAMEQKAELER            |
|      |                   | 1844.0      | 1690–1697| ASREILAQAKENK             |
|      |                   | 1873.9      | 1343–1357| EQLEEEAAKRNLEK            |
|      |                   | 1929.0      | 1218–1234| AKQTLENERGELANEVK         |
|      |                   | 2245.1      | 1699–1717| RQAQQEQERDELADIANSSGK     |
| 2    | Vimentin          | 1088.5      | 207–216  | QVDVNASLAR                |
|      |                   | 1169.7      | 129–138  | ILAALEQQLK                |
|      |                   | 1533.8      | 222–234  | KVESLQEEIAFLK             |
|      |                   | 1557.9      | 410–423  | ISLPLFTPSLNLRR            |
|      |                   | 1587.8      | 100–112  | TNEKYEILQELQNLDR          |
|      |                   | 1682.8      | 424–438  | ETNLESLLPVYDTHSK          |
|      |                   | 1688.8      | 170–183  | VEVERDLAEDIRM            |
|      |                   | 1734.8      | 364–377  | LQDEIQMNKKEEMAR           |
| 3    | Cytokeratin 1     | 874.5       | 66–74    | SLVNGLGSK                 |
|      |                   | 1092.5      | 603–616  | SGSSGSSSGSGISGR           |
|      |                   | 1393.7      | 278–289  | TNAENEFTVIKK              |
|      |                   | 1475.7      | 212–223  | WELIQVQDITSTR             |
|      |                   | 1638.8      | 185–199  | SLNQFASFDIKVR             |
|      |                   | 1657.8      | 13–29    | SGGGFSSGAGIQNYQR          |
|      |                   | 2501.2      | 365–386  | SKAESAQLQSKYEELQITAGR     |
| 4    | β-Actin$^b$       | 644.4       | 63–68    | GILTLK                    |
|      |                   | 644.4       | 178–183  | LDLAGR                    |
|      |                   | 976.4       | 329–336  | IAAPPERK                 |
|      |                   | 945.5       | 29–37    | AVFPSIVGR                 |
|      |                   | 976.4       | 19–28    | AGFAGDDAPR                |
|      |                   | 1133.6      | 197–206  | GYSFTTAER                 |
|      |                   | 1181.6      | 316–327  | EITALAPSTMK               |
|      |                   | 1198.5      | 51–61    | DSYYGDEAQSK               |
|      |                   | 1515.7      | 85–95    | IWHHTFYNELR               |
|      |                   | 1790.9      | 239–254  | SYELPDGQVITIGNER          |
|      |                   | 1954.1      | 90–113   | VAPEEHVPLLLEAPLNPK        |
| 5    | γ-Actin$^b$       | 644.4       | 63–68    | GILTLK                    |
|      |                   | 644.4       | 178–183  | LDLAGR                    |
|      |                   | 923.6       | 329–336  | IAAPPERK                 |
|      |                   | 976.4       | 19–28    | AGFAGDDAPR                |
|      |                   | 998.5       | 184–191  | DLTDLMLK                  |
|      |                   | 1133.6      | 197–206  | GYSFTTAER                 |
|      |                   | 1161.6      | 316–327  | EITALAPSTMK               |
|      |                   | 1171.6      | 40–50    | HGGVYMGQM                |
|      |                   | 1198.5      | 51–61    | DSYYGDEAQSK               |
|      |                   | 1515.7      | 85–95    | IWHHTFYNELR               |
|      |                   | 1516.7      | 360–372  | QYEDESGPIVHR              |
|      |                   | 1790.9      | 239–254  | SYELPDGQVITIGNER          |
|      |                   | 1960.9      | 69–84    | YPIEHEGVTNWDDMEK          |
|      |                   | 2233.3      | 90–116   | VAPEEHVPLLLEAPLNPKANR     |
| 6    | Tropomyosin       | 744.5       | 106–112  | LATALQK                   |
|      |                   | 875.4       | 206–213  | SLEAQAEK                  |
|      |                   | 1314.8      | 168–178  | KLVIESDLER                |
|      |                   | 1460.7      | 77–90    | KATDAEADVASLNR            |
|      |                   | 1476.7      | 113–125  | LEEAEKADESER              |
|      |                   | 1644.9      | 36–49    | SKQLEDEVLSLQKK            |
|      |                   | 1688.8      | 214–226  | YSQKEDRYEEEIK             |
|      |                   | 1727.9      | 92–105   | IQLVEEELDRAQER            |
|      |                   | 1801.9      | 153–167  | HIAEADRKYEEVAR            |
|      |                   | 1960.0      | 134–149  | AQKDEERMRMLQELK           |

$^a$ aa, amino acids.
$^b$ Determined by position on two-dimensional gel.
$^c$ OWL nonredundant data base.
Positions of H9251 that only a small percentage of the peptide was binding (data not achieved even at this concentration, suggesting 1–3 CEACAM1. A, proteins with cytoplasmic domains from the L- and S-isoforms of H9262 880.5 to immobilized actin after 140 s injection time at a flow – Cyto-L-(419–432), demonstrating that the Cyto-L-scrambled had the same amino acid composition of the interaction. Under identical conditions, the control peptide was synthesized and tested to establish the specificity protein (1242-fold less), the off-rates are comparable. A control ably slower than that obtained for the GST-Cyto-L fusion protein. The pellets were run on SDS – actinin (419–432)) to G-actin at 0.5 (Fig. 6A), lower relative binding measured in 14-amino acid sequence adjacent to the cell membrane. Because Obrink and co-workers (47) have shown that pseudophosphorylated versions of the rat cytoplasmic domain of CEACAM1-S have lower binding to calmodulin than the wild type sequences and are potential sites for PKC phosphorylation, we also synthesized pseudophosphorylated and phosphorylated versions of the Cyto-L-(419–432) peptide (Table I). The peptides with amino acid substitutions at T425E and S429E (Cyto-L-T425E,S429E), as well as the three phosphorylated peptides (Cyto-L-ThrP-425, Cyto-L-SerP-429, Cyto-L-ThrP-425, and SerP-429) showed more than 80% decreased binding to G-actin compared with wild type Cyto-L-(419–432) (Table IV). Similar results were obtained for the pseudophosphorylated (Glu substituted or Thr or Ser) and the dual-phosphorylated peptides (Table IV). Mutation of the Ser and Thr residues to Ala (Cyto-L-T425A,S429A) also resulted in greater than 80% decreased binding (Table IV). The low binding of these peptides even at 1 mM prevented measurement of their KD values. We interpret these results as follows: modification of these key residues reduces the magnitude of binding to the G-actin-binding site (relative ΔRU) by lowering the amount of peptide in the correct conformation for binding.

Because tropomyosin was also immunoprecipitated with CEACAM1-L and is, in fact, found in the immunoprecipitates before and after pervanadate treatment, we performed analogous SPR binding studies with the GST-Cyto-L fusion protein to immobilized tropomyosin. 2500 RU of tropomyosin were immobilized, corresponding to 2.5 ng/mm² of tropomyosin on the chip surface. GST-Cyto-L bound to tropomyosin but with kinetic and KD constants lower than to G-actin (Fig. 5B and Table III). However, the relative binding measured in ΔRUs was similar for both tropomyosin and G-actin. Once again, the control experiments showed that GST itself had no binding to immobilized tropomyosin. Thus, the binding is specific and direct.

When the synthetic peptides from the CEACAM1-L cytoplasmic domain were tested (e.g. Fig. 6B), lower relative binding to tropomyosin was observed (Table IV) in every case for the mutated peptides versus the wild type peptide (Cyto-L-(419–432)). These results are similar to those obtained for the actin binding study (Table IV). Thus, it is likely that amino acid substitutions in the critical Ser and Thr residues lower the percent peptide in the correct conformation for binding. In addition, these data suggest that this peptide sequence shares a binding site for G-actin and tropomyosin. Indeed, the binding of Cyto-L-(419–432) to G-actin is inhibited by tropomyosin, and its binding to tropomyosin is inhibited by G-actin (Fig. 7). However, when tropomyosin is tested for its ability to inhibit the binding of the GST-Cyto-L fusion protein to actin, no decrease in binding was observed. This result suggests that there is a second G-actin-binding site in the CEACAM1-L cytoplasmic domain (i.e. both are allowed to bind simulta-
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Fig. 5. Binding of the GST-Cyto-L fusion protein to immobilized actin or tropomyosin. A, the GST-Cyto-L fusion protein (50 μl of 0.25 and 2.5 μM diluted in HBS buffer) was passed at 20 μl/min in the presence of 2 mM CaCl₂ over actin immobilized on a CM5 chip. GST (10 μM) was injected as a control. Each injection is followed by HBS (dissociation phase). B, the GST-Cyto-L fusion protein was passed over immobilized tropomyosin under the same conditions.

TABLE III

| GST-cyto fusion proteins and peptide binding to immobilized actin or tropomyosin | Actin | kₐ (1/ms) | kₐ (1/s) | Kₐ |
|---|---|---|---|---|
| GST-Cyto-L | 7280 | 5.4 × 10⁻⁵ | 0.74 × 10⁻⁴ |
| Cyto-S (419–432) | 5420 | 1.67 × 10⁻⁴ | 3.07 × 10⁻⁵ |
| Tropomyosin | 5.86 | 7.31 × 10⁻⁵ | 1.25 × 10⁻⁴ |
| GST-Cyto-L | 2108 | 2.18 × 10⁻⁴ | 1.03 × 10⁻⁵ |
| GST-Cyto-S | 2140 | 2.84 × 10⁻⁴ | 1.32 × 10⁻⁵ |
| Cyto-L (419–432) | 2.79 | 5.12 × 10⁻⁵ | 1.83 × 10⁻⁵ |

GST-Cyto fusion proteins (GST-Cyto-L, 2.5 μM; GST-Cyto-S, 5 μM; or Cyto-L (419–432) peptide, 1 mM) were injected onto either actin or tropomyosin immobilized onto a biosensor chip for 150 s at a flow rate of 20 μl/min. For examples of the sensorgrams see Figs. 5 and 6.

Because calmodulin is known to bind to the cytoplasmic domain of rat CEACAM1 peptides (46, 47), binding studies were also performed in the presence of 10 μM calmodulin (Fig. 7). Calmodulin showed 50–60% inhibition for the synthetic peptides versus 0–10% inhibition for the GST-Cyto fusion proteins binding to G-actin. For example, although 10 μM calmodulin reduced binding of the Cyto-L (419–432) peptide to actin by >50%, binding of the GST-Cyto-L fusion protein was <10% inhibited by 10 μM calmodulin (Fig. 7A). On the other hand, although 10 μM calmodulin inhibited binding of the Cyto-L (419–432) peptide to tropomyosin as much as to G-actin, binding of GST-Cyto-L fusion protein was increased more than 2-fold. This result is consistent with two binding sites for calmodulin, the second of which is positively correlated with the binding of tropomyosin. It should be noted that Obrink and co-workers (28) also found a second binding site for calmodulin in the cytoplasmic domain of rat CEACAM1-L.

**SPR Studies on the S-isoform—** We also synthesized a GST-Cyto-S fusion protein containing the 14-amino acid cytoplasmic peptide from CEACAM1-S. When analyzed by gel filtration, GST-Cyto-S was a single species with a mass of 59 kDa, corresponding to a dimer. GST-Cyto-S showed a 4-fold lower Kₐ for binding to G-actin compared with GST-Cyto-L (Table III). The binding to G-actin was <15% inhibited by 10 μM calmodulin (Fig. 7A), in agreement with the previous prediction that this domain interacts with calmodulin (47). Similar to our strategy for the CEACAM1-L cytoplasmic domain, peptides with a variety of substitutions were synthesized corresponding to the CEACAM1-S cytoplasmic domain (Table I). All of these peptides, including the wild type, showed negligible binding to G-actin with unmeasurable Kₐ values (Fig. 6A and Table IV).

Based on the observed decreased binding in going from the GST-Cyto-L fusion protein to the synthetic peptides in the CEACAM1-L case, it is likely that the binding constants for the short cytoplasmic domain peptides are below the threshold of sensitivity for this instrument. We conclude that their Kₐ values are <10⁻⁴ M.

The GST-Cyto-S fusion protein derived from the CEACAM1-S cytoplasmic domain showed a similar Kₐ for tropomyosin binding compared with GST-Cyto-L binding to tropomyosin (Table III), which is, in turn, 10-fold lower than for the binding of either fusion protein domain to G-actin (Table II).

The difference in size between Cyto-L and Cyto-S was predicted by Obrink, Weng, Sigmundsson, and Iyengar at the Eleventh International CEA 2000 workshop held in Bristol, UK, August 10–13, 2000.
As with the GST-Cyto-L fusion protein, binding of GST-Cyto-S to G-actin was increased in the presence of tropomyosin (Fig. 7A), and binding of GST-Cyto-S to tropomyosin was increased by G-actin (Fig. 7B). Calmodulin inhibited binding of the GST-Cyto-L fusion protein to tropomyosin similar to the synthetic peptide (Fig. 7B). The synthetic peptides derived from the CEACAM1-S cytoplasmic domain showed negligible binding to tropomyosin (Table IV), similar to the result obtained for these peptides binding to G-actin (Table IV).

G-actin is normally associated with both Ca\(^{2+}\) and ATP (43). To regenerate the BIAcore chip between binding studies, we used 6 M guanidinium HCl, a treatment that is likely to remove both Ca\(^{2+}\) and ATP from the immobilized G-actin. To ensure renaturation of the chip surface, we routinely included 2 mM CaCl\(_2\), but not ATP, in the post-regeneration buffer. When lower concentrations of Ca\(^{2+}\) were used (1, 10, and 100 \(\mu\)M), the same rank order of peptide binding was observed (data not shown). However, when EDTA (10 mM) was added to the injected protein, binding of the GST-Cyto fusion proteins and the synthetic peptide Cyto-L-(419-432) was inhibited by >50% for the fusion proteins and >40% for the peptide Cyto-L-(419-432) (Fig. 7), suggesting an important role for Ca\(^{2+}\) in the association of CEACAM1 cytoplasmic domains to actin, e.g. through stabilization of the native structure of actin on the BIAcore chip, similar to its function in CEACAM1 cytoplasmic domains binding to calmodulin (28). When the GST-Cyto fusion proteins or synthetic peptides were tested in the same way with tropomyosin, a similar result was obtained (Fig. 7B), although inhibi-
tropomyosin in the presence of inhibitors. The presence of 10 mM ATP with little or no change in the observed absolute amount of binding to G-actin increased in the absence of ATP, and the binding of GST-Cyto-L, GST-Cyto-S fusion proteins (50 μl of 2.5 μM, diluted in HBS + 10 μM CaCl₂ buffer), or Cyto-L-(419–432) peptide (50 μl of 0.5 μM, diluted in HBS + 10 μM CaCl₂ buffer) were passed at 20 μl/min over actin or tropomyosin immobilized on a CM5 biosensor chip. EDTA (10 mM), tropomyosin (TM, 10 μM), actin (ACT, 1 μM), or calmodulin (CaM, 10 μM), respectively, were added to the fusion proteins or the synthetic peptide prior to injection to inhibit binding to the immobilized ligand. Association of the fusion proteins or the peptide in the absence of inhibitors in each experiment served as a reference. A, GST-Cyto fusion proteins or peptides binding to immobilized actin in the presence of inhibitors. B, GST-Cyto fusion proteins or peptides binding to immobilized tropomyosin in the presence of inhibitors.

Fig. 7. Modulation of binding of CEACAM1 fusion proteins and peptides to immobilized G-actin or tropomyosin. GST-Cyto-L, GST-Cyto-S fusion proteins (50 μl of 2.5 μM, diluted in HBS + 10 μM CaCl₂ buffer), or Cyto-L-(419–432) peptide (50 μl of 0.5 μM, diluted in HBS + 10 μM CaCl₂ buffer) were passed at 20 μl/min over actin or tropomyosin immobilized on a CM5 biosensor chip. EDTA (10 mM), tropomyosin (TM, 10 μM), actin (ACT, 1 μM), or calmodulin (CaM, 10 μM), respectively, were added to the fusion proteins or the synthetic peptide prior to injection to inhibit binding to the immobilized ligand. Association of the fusion proteins or the peptide in the absence of inhibitors in each experiment served as a reference. A, GST-Cyto fusion proteins or peptides binding to immobilized actin in the presence of inhibitors. B, GST-Cyto fusion proteins or peptides binding to immobilized tropomyosin in the presence of inhibitors.

The dependence of the CEACAM1 GST-Cyto fusion proteins on the presence of both Ca²⁺ and ATP for binding to G-actin was also tested. The results show that for both fusion proteins the absolute amount of binding to G-actin increased in the presence of 10 mM ATP with little or no change in the observed Kᵩ values (data not shown). For example, GST-Cyto-S showed >3-fold higher binding to G-actin in the presence of 10 mM ATP versus the absence of ATP, and the binding of GST-Cyto-L increased >7-fold. These results suggest that both Ca²⁺ and ATP play important roles in the association of CEACAM1 cytoplasmic domains to actin, most likely through stabilization of actin in its native structure.

DISCUSSION

Actin Binding Studies—CEACAM1 is a cell-cell adhesion molecule that has distinct short and long cytoplasmic domains (48). When we transfected murine MC38 cells with human CEACAM1-L, we observed localization of CEACAM1-L to cell-cell interfaces with little or no co-localization with actin (Fig. 1C). Upon treatment with pervanadate virtually all of the CEACAM1-L was co-localized with actin (Fig. 1D). This result is similar to the findings of Sadekova et al. (30) who showed that microinjected murine CEACAM1-L cDNA in Swiss 3T3 cells caused accumulation of CEACAM1-L at cell-cell boundaries and co-localized with actin. A key difference in our study was that actin co-localization required pervanadate treatment, suggesting a requirement for a tyrosine phosphorylation event, perhaps on the CEACAM1 itself. As will be seen later, this information may explain why these researchers did not detect a direct interaction between murine CEACAM1-L and F-actin in vitro.

Because most, if not all, cell surface proteins are capable of transmitting outside-in signals upon activation, it is reasonable to ask which type of signal transduction systems are associated with CEACAM1-L. In the case of CEACAM1-L, its cytoplasmic domain has been shown to be phosphorylated on Ser/Thr residues by PKC (49) and on Tyr residues in its ITIM motif by Src kinases (19, 23). Attempts to disrupt cell-cell association by mutation of the Tyr residues to Phe in the ITIM motif failed to change the cell-cell adhesion phenotype (50), and the large number of Ser/Thr residues in the CEACAM1-L domain have discouraged investigators from performing further mutational analysis. Although some light was thrown on the problem when Obrink and co-workers (27, 28, 46) showed that the cytoplasmic domain of rat CEACAM1-S and the juxtamembrane residues of the CEACAM1-L cytoplasmic domain bound calmodulin, little was known about further downstream targets or the consequences of the PKC phosphorylation events. To investigate this matter further, we used a direct approach to isolate CEACAM1-L-associated proteins by immunoprecipitation of CEACAM1-L followed by two-dimensional gel separations and LC/MS/MS analysis of the separated proteins. This approach revealed a number of major and minor proteins associated with CEACAM1-L, especially after activation of the cells with pervanadate. Whereas the activation conditions cannot be construed as physiological, they did cause CEACAM1-L to co-localize with actin (Fig. 1D) and caused extensive phosphorylation of CEACAM-1 on its Tyr residues (Fig. 2B). Concomitantly with these changes, increased amounts of two major proteins at 200 and 45 kDa were observed in the CEACAM1-L immunoprecipitates (Fig. 2C and Fig. 3). Further analysis revealed the major proteins as actin and myosin, suggesting that CEACAM1-L associates with actomyosin filaments, especially at cell-cell boundaries.

Immunoprecipitation studies by Da Silva-Azevedo and Rutter (29) on rat small intestinal cells have shown an association of rat CEACAM1-L with actin. Our work further extends their studies by identifying actin, myosin, and tropomyosin association with human CEACAM1-L. In addition, Beauchemin and co-workers (30) microinjected murine CEACAM1-L cDNA into Swiss 3T3 cells and showed that the association between CEACAM1-L and F-actin depends on activation of Rho GTPases. However, this group concluded that the interaction between CEACAM1-L and actin was indirect based on negative results of an F-actin co-sedimentation assay with a GST fusion protein containing the murine L-cytoplasmic domain. Indeed, we found that a GST fusion protein containing the human L-cytoplasmic domain (GST-Cyto-L) also failed to associate with CEACAM1-L. In the case of CEACAM1-L, its cytoplasmic domain has been shown to be phosphorylated on Ser/Thr residues by PKC (49) and on Tyr residues in its ITIM motif by Src kinases (19, 23). Attempts to disrupt cell-cell association by mutation of the Tyr residues to Phe in the ITIM motif failed to change the cell-cell adhesion phenotype (50), and the large number of Ser/Thr residues in the CEACAM1-L domain have discouraged investigators from performing further mutational analysis. Although some light was thrown on the problem when Obrink and co-workers (27, 28, 46) showed that the cytoplasmic domain of rat CEACAM1-S and the juxtamembrane residues of the CEACAM1-L cytoplasmic domain bound calmodulin, little was known about further downstream targets or the consequences of the PKC phosphorylation events. To investigate this matter further, we used a direct approach to isolate CEACAM1-L-associated proteins by immunoprecipitation of CEACAM1-L followed by two-dimensional gel separations and LC/MS/MS analysis of the separated proteins. This approach revealed a number of major and minor proteins associated with CEACAM1-L, especially after activation of the cells with pervanadate. Whereas the activation conditions cannot be construed as physiological, they did cause CEACAM1-L to co-localize with actin (Fig. 1D) and caused extensive phosphorylation of CEACAM-1 on its Tyr residues (Fig. 2B). Concomitantly with these changes, increased amounts of two major proteins at 200 and 45 kDa were observed in the CEACAM1-L immunoprecipitates (Fig. 2C and Fig. 3). Further analysis revealed the major proteins as actin and myosin, suggesting that CEACAM1-L associates with actomyosin filaments, especially at cell-cell boundaries.

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tion. The reason for the lack of binding for the L-isofrom to F-actin in the co-sedimentation assay may be due to its dependence on tyrosine phosphorylation (see Fig. 1). However, it should be noted that both isoforms show amino acid sequence homology in the first 4–8 amino acids of their juxtamembrane domains. The similar sequences between the two isoforms in this region may explain their similar binding to G-actin, but their differential binding to F-actin must be due to the amino acid differences in the same region, the effects of the extended sequence of the long isoform (compared with the short form), or both.

The juxtamembrane region of the cytoplasmic domain of CEACAM1-L has an identical sequence over the first 4–8 amino acids of the CEACAM1-S cytoplasmic domain, depending on the actual start of the cytoplasmic domain and end of the transmembrane domain. A comparison of these regions is shown: CEACAM1-S-Cyto domain, FLHFGKTQGSGPLQ, and CEACAM1-L-Cyto domain, FLHFGKTQGRSDQ.

As shown above, we have indicated the last four amino acids of the predicted transmembrane domain in bold, and the amino acids shared between the two domains are underlined. In the gene for CEACAM1, the codon for the Ser/Arg (the first amino acid difference between the two isoforms) is completed by alternative splicing leading to the subsequent amino acid changes between the two domains, which in the case of CEACAM1-S leads to a stop codon after the Gln residue. With this information in mind, we synthesized peptides over these regions to explore their potential difference in binding G-actin. Our strategy for peptide synthesis was based on retaining a portion of the putative transmembrane domain (bold, as shown above) and including an amino-terminal biotin residue with a Gly-Gly linker. When tested in an SPR binding assay, the synthetic peptide derived from CEACAM1-L (Cyto-L-(419–432)) bound immobilized G-actin, but the peptide derived from CEACAM1-S (Cyto-S-(419–432)) bound G-actin poorly (Fig. 6). A likely explanation of these results is that the Cyto-S-(419–432) peptide has too low an affinity for detectable binding of G-actin in the BIAcore flow system. Nonetheless, the Cyto-L-(419–432) peptide has a $K_D$ for immobilized actin that is almost 1000-fold less than the GST-Cyto-L fusion protein (Table III). We can interpret this result in at least two ways. First, the shorter synthetic peptide has less conformers in the correct orientation compared with the longer GST-Cyto-L fusion protein. Second, the GST-Cyto-L fusion protein may possess a second actin-binding site. We believe that both possibilities contribute to the observed difference.

When we mutated residues within the Cyto-L-(419–432) sequence postulated to be PKC phosphorylation sites (47), we found that the mutated peptides had lower relative binding to immobilized actin compared with the wild type peptide, and $K_D$ values of $<10^{-4}$ m. These results suggest that amino acid changes within the peptide sequence lower the percent conformers capable of binding in a flow system. Because these amino acid changes were found to decrease calmodulin binding in the studies performed by Obrink and co-workers (47), it is noteworthy that the presumed phosphorylation of these key residues by PKC also leads to decreased G-actin binding, at least at this particular G-actin-binding site. Unfortunately, we were unable to demonstrate a direct binding of these peptides to calmodulin immobilized on a BIAcore chip, but neither were Obrink and co-workers able to do so. To demonstrate binding in their system, it was necessary to synthesize the peptides on a cellulose filter, a system that leads to an extremely high density of the bound ligand in a preferred orientation (which may more closely mimic the situation on the inside surface of the cell membrane).

**Tropomyosin Binding Studies**—The finding of tropomyosin in the CEACAM1-L immunoprecipitates was intriguing (Fig. 3), because the role of tropomyosin in non-muscle cells is unknown and the change in isoform expression from non-muscle to muscle type correlates with malignant transformation. In this case, the immunoprecipitates showed only TM2, a low molecular weight, muscle-type isoform (Table II). Direct binding of the cytoplasmic domains of CEACAM1 was explored by an SPR binding assay in which tropomyosin was immobilized to the hydrogel. Indeed, we obtained similar results for binding of the synthetic peptides and GST-Cyto-L fusion protein from the CEACAM1-L cytoplasmic domain to tropomyosin as we did for G-actin. The most striking differences were the 10-fold lower $K_D$ of the GST-Cyto-L fusion protein for tropomyosin versus G-actin and the negative inhibitory effect of G-actin on its binding to tropomyosin. Whereas tropomyosin is able to inhibit the binding of the GST-Cyto-L fusion protein to G-actin, the reverse is not true, and in fact, increased binding is observed. This suggests that there is a second actin-binding site on the long cytoplasmic domain. The emerging interaction model for the long cytoplasmic domain is shown in Fig. 8. In this model, the juxtamembrane region is able to bind actin or tropomyosin, and their binding is inhibited by calmodulin. In addition, the amino acid sequence differences between the two isoforms in this region leads to different relative binding to G-actin, at least as measured by synthetic peptide binding. Because this difference is not observed for the GST-Cyto fusion proteins, we believe that the presentation of the peptides in a preferred orientation by either the membrane or the GST protein plays a critical role. Potential phosphorylation by PKC on key Ser/Thr residues in this site decreases both calmodulin binding (47) and actin binding (this report). In addition, as predicted by Sadekova et al. (30), G-actin can bind at a more distal region of the CEACAM1-L cytoplasmic domain. The second G-actin-binding site, which has not yet been identified, may be influenced by other modifications, including phosphorylation of Tyr and Ser/Thr residues and the binding of other modulating proteins. Interestingly, the GST-Cyto-S fusion protein but not GST-Cyto-L co-sedimented with actin only if it was...
include during the polymerization step (Fig. 4A). These results suggest that neither isoform prevents the polymerization of G- to F-actin and that the S-isoform may be directly incorporated into newly forming actin filaments. In the case of the L-isoform, available evidence suggests that its association with F-actin is indirect (30). Based on our data, it is likely that the L-isoform must undergo additional modifications such as phosphorylation (Figs. 1–3) prior to binding F-actin. In the absence of phosphorylation, the L-isoform could serve as a G-actin-binding site but would dissociate during the polymerization step. This property may have distinct advantages for the differential coupling of cell adhesion events to the cytoskeleton.

A summary of the interactions of the short and long cytoplasmic isoforms of CEACAM1 with actin and tropomyosin is shown in Fig. 8. Both isoforms are capable of binding G-actin or tropomyosin. The stronger binding of actin may predominate, especially given that tropomyosin enhances G-actin binding. Calmodulin is able to inhibit binding except in the case of the long isoform binding to tropomyosin, where it increases binding.

The implications of the dual interactions of CEACAM1 cytoplasmic domain with G-actin and calmodulin are of potential importance. Tropomyosin and actin are usually found in a cooperative complex that is further modulated by caldesmon in nonmuscle cells (51) or troponins in muscle cells (52). Tropomyosins have a variety of isoforms that are strictly regulated in cells (53, 54) and are often dysregulated during tumorigenesis (55–57). The latter dysregulation may, in turn, affect other important interactions, perhaps explaining why the down-regulation of CEACAM1 in colon cancer (13) and other cancers (14) corresponds with a change in tropomyosin isoforms. In the murine colon carcinoma cell line used in this study, we found that only a short isoform of tropomyosin (TM2) co-precipitated with CEACAM1-L, a result consistent with the cancer phenotype (58, 59). Thus, it is possible that the cytoplasmic targets of the long or short isoforms of tropomyosin influence cell morphology changes consistent with the cancer phenotype. In our GST-Cyo-L cytoplasmic domain binding studies, a mixture of muscle tropomyosin isoforms was used. Further studies are required to determine whether both the short and long isoforms of tropomyosin bind equally well to the cytoplasmic domain of CEACAM1, and if these binding patterns, in turn, regulate actin binding to CEACAM1 isoforms. It will also be of some interest to see if the reintroduction of CEACAM1 into cancer cells re-establishes a more normal tropomyosin pattern, because transfections of CEACAM1 into tumor cells can restore a more normal phenotype in prostate (14), bladder (60), and breast (61) cancer cells.
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