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CEACAM1, a Cell-Cell Adhesion Molecule, Directly Associates with Annexin II in a Three-dimensional Model of Mammary Morphogenesis*

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The epithelial cell adhesion molecule CEACAM1 (carcinoembryonic antigen cell adhesion molecule-1) is down-regulated in colon, prostate, breast, and liver cancer. Here we show that CEACAM1-4S, a splice form with four Ig-like ectodomains and a short cytoplasmic domain (14 amino acids), directly associates with annexin II, a lipid raft-associated molecule, which is also down-regulated in many cancers. Annexin II was identified using a glutathione *S*-transferase pull-down assay in which the cytoplasmic domain of CEACAM-4S was fused to glutathione *S*-transferase, the fusion protein was incubated with cell lysates, and isolated proteins were sequenced by mass spectrometry. The interaction was confirmed first by reciprocal immunoprecipitations using anti-CEACAM1 and anti-annexin II antibodies and second by confocal laser microscopy showing co-localization of CEACAM1 with annexin II in mammary epithelial cells grown in Matrigel. In addition, CEACAM1 co-localized with p11, a component of the tetrameric AIIt complex at the plasma membrane, and with annexin II in secretory vesicles. Immobilized, oriented peptides from the cytoplasmic domain of CEACAM1-4S were shown to directly associate with bovine AIIt, which is 98% homologous to human AIIt, with average K_D values of about 30 nM using surface plasmon resonance, demonstrating direct binding of functionally relevant AIIt to the cytoplasmic domain of CEACAM1-4S.

Carcinoembryonic antigen (CEA)[1] cell adhesion molecule-1 (CEACEAM1) is a type I transmembrane glycoprotein member of the CEA gene family (1), which, in turn, is a member of the Ig gene superfamily (2). CEACAM1 mRNA has four major slice forms that produce protein products with either three or four Ig-like ectodomains and either a long (72–74 amino acids) or short (12–14 amino acids) cytoplasmic domain. CEACAM1 was originally identified in liver and bile as a CEA cross-reactive antigen (3) and was later shown to be on the surface of epithelia of breast, gastrointestinal tract, kidney, and prostate (4) and in activated endothelial cells (5), T-cells (6–9), B-cells (10, 11), dendritic cells (12), and granulocytes (13, 14). Although the function of CEACAM1 is not completely understood, rat CEACAM1 was identified as a cell-cell adhesion molecule in hepatocytes (15), and murine CEACAM1 transfected into Chinese hamster ovary cells effected homotypic cell adhesion (16). Murine CEACAM1 also has long and short cytoplasmic domains of which the long form has been shown to be involved in the insulin clearance pathway (17). Human CEACAM1 plays a central role in mediating apoptosis during lumen formation in a three-dimensional model of mammary morphogenesis (18, 19) and participates in angiogenesis in endothelial cells (5). In cancer, CEACAM1 has been shown to be down-regulated in liver (20, 21), prostate (22–24), colon (25), lung (26), and breast carcinoma (27, 28). The down-regulation in colon cancer occurs at the early adenoma stage (29) and in >90% of colon cancers (25). Transfection of the CEACAM1 gene into prostate (30, 31), bladder (32), breast (33), or colon cancer (34) cell lines renders these cells less tumorigenic in nude mice. Thus, CEACAM1 has many of the properties of a tumor suppressor gene; however, no genetic abnormalities regarding this gene have been reported.

Studies have been performed on the cytoplasmic domains to determine their role in signal transduction. The long cytoplasmic domain of CEACAM1 has two tyrosines in an immunoreceptor tyrosine-based inhibitory motif that can be phosphorylated by Src kinases (14, 35) and once phosphorylated bind SHP-1 and SHP-2 phosphatases (36). This domain has been shown to bind actin (37, 38), tropomyosin (38), calmodulin (39, 40), and integrin β_3 (41). Its function appears to involve cytoskeletal reorganization and inhibition of mitogenesis associated with the differentiated phenotype. The short cytoplasmic domain also interacts with actin, tropomyosin, and calmodulin (38) and can be phosphorylated on serine and threonine residues by PKC isozymes (42). While its function is less well studied, it has been shown to play a role in lumen formation by an apoptotic mechanism in a three-dimensional model of mammary morphogenesis (18, 19).

Annexin II (calpactin I heavy chain, lipocortin II, p36) is a 36-kDa Ca^{2+}- and phospholipid-binding protein (43) with a wide tissue (44) and species distribution (45). All annexins have a conserved carboxyl-terminal core domain with Ca^{2+} and membrane binding regions and a unique amino-terminal tail (46, 47). Phosphorylation of Tyr^{23} by Src kinase (48, 49) has been shown both *in vivo* and *in vitro* and is increased upon stimulation with platelet-derived growth factor (50–52) and insulin (53) receptor activation. Conventional PKCs phosphorylate annexin II on Ser^{25} (54, 55). Annexin II and its associated light chain are down-regulated in a variety of cancers

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[1] The abbreviations used are: CEA, carcinoembryonic antigen; CEACAM1, CEA cell adhesion molecule-1; CEACAM1-4L, CEACAM1 with 4 Ig-like domains and a long cytoplasmic tail; CEACAM1-4S, CEACAM1 with 4 Ig-like domains and a short cytoplasmic tail; PKC, protein kinase C; GST, glutathione *S*-transferase; SPR, surface plasmon resonance; MS, mass spectrometry; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; LC, liquid chromatography; mAb, monoclonal antibody; GFP, green fluorescent protein.

TABLE I
Synthetic peptides from the cytoplasmic domain of CEACAM1-4S

All peptides start with the linker X, 11-mercaptoundecanoic acid. The amino acid sequences corresponding to the CEACAM1 short cytoplasmic domain are underlined. CEACAM1-4S wild type-reversed has the same sequence as CEACAM1-4S wild type for the first four amino acids (bold) followed by the remainder in reversed order. The complete sequence could not be reversed due to its low solubility in aqueous buffers. Wt, wild type.

	Name	Amino acid sequence
CEACAM1-4S wild type	Wt	X-FLHFGKTGSSGPLQ
Thr^{423} pseudophosphorylation	T423E	X-FLHFGKEGSSGPLQ
Ser^{425} pseudophosphorylation	S425E	X-FLHFGKTGESGPLQ
Thr^{423}, Ser^{425}seudophosphorylation	T423E,S425E	X-FLHFGKEGESGPLQ
CEACAM1-4S wild type reversed	Wt-reversed	X-**FLHF**QLPGSSGTKG

including prostate (56, 57), liver (58), and breast (59).

Annexin II light chain (p11, S100A10), a member of the S100 protein family, binds annexin II forming a heterotetramer (AIIt) comprising two annexin II and two p11 molecules (43). Annexin II is localized throughout the cytoplasm, whereas AIIt is found at the plasma membrane-actin cytoskeleton interface (60–63). Both annexin II and AIIt have been shown to bind actin (64–66), but only AIIt can bundle actin (65, 67). Annexin II promotes aggregation (68, 69) and fusion (70, 71) of phospholipid vesicles, may have a role in Ca^{2+}-dependent endo- and exocytosis (72, 73), and is found associated with detergent-resistant membrane microdomains (74–76). It has also been found to play a role in proliferation (77, 78) and cell surface fibrinolysis (79–83). Annexin II is thought to be involved in the secretory differentiation of cultured mammary epithelial cells (84). Annexin II is uniformly present throughout mammary gland during pregnancy, but in the lactating mammary gland, annexin II is concentrated in the epithelium of immature alveoli where it is localized to the apical membrane and microvillar extensions (85). Furthermore annexin II is associated with secretory vesicles of various sizes (85).

In this study we identified annexin II as yet another protein that interacts with the short cytoplasmic domain of CEACAM1-4S. This interaction was identified using a glutathione *S*-transferase (GST) pull-down assay and confirmed by cross-immunoprecipitations and confocal laser microscopy. Furthermore bovine AIIt was shown to directly bind immobilized, oriented peptides from the cytoplasmic domain of CEACAM1-4S by SPR. Analysis of the kinetics of CEACAM1 association with annexin II by SPR revealed k_{on} and k_{off} rates of 4.71×10^4 M^{-1} s^{-1} and 1.74×10^{-3} s^{-1}, respectively, and a K_D of 30 nM.

MATERIALS AND METHODS

Cell Culture—Normal mammary epithelial (MCF10F) and mammary carcinoma (MCF7) cells were cultured as described previously (18, 19), and cervical carcinoma (HeLa) cells were grown according to American Tissue Culture Collection (ATCC) instructions. Matrigel culture was performed as described previously (19).

GST Fusion Proteins and Synthetic Peptides—GST-CEACAM1-4S fusion proteins containing the 14 amino acids of the cytoplasmic domain of CEACAM1-4S were made as described previously (19). Mutant GST-CEACAM1-4S constructs were made according to the QuikChange site-directed mutagenesis kit (Stratagene) by introducing mutations into pGEX-CEACAM1-4S vector using CEACEAM1-4S(T423D) sense and antisense primers (5′-CATTTCGGGAAGGAC GGCAGCTCAGG and 5′-CCTGAGCTGCCGTCCTTCCCGAAATG), CEACAM1-4S(S425D) sense and antisense primers (5′-GGGAAGACCGGCGACTCAGGACCACTC and 5′-GAGTGGTCCTG AGTCGCCGGTCTTCC), and CEACAM1-4S(T423D,S425D) sense and antisense primers (5′-CATTTCGGGAAG-GACGGCGACTCAGGACCACTC and 5′-GAGTGGTCCTGAGTCGC-CGTCCTTCCCGAAATG). All GST fusion proteins were purified from BL21 cells using B-PER GST fusion protein purification kit (Pierce) according to the manufacturer's instructions and as described by Schumann *et al.* (38).

Synthetic peptides containing the complete CEACAM1-4S cytoplasmic domain were synthesized by Dr. Bruce Kaplan using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry (Table I). All peptides were synthesized with an amino-terminal 11-mercaptoundecanoic acid group for direct coupling to the gold surface of a J1 sensor chip (BIAcore) in SPR experiments. Chemically synthesized peptides were first analyzed by nanospray MS/MS on a Thermoquest LCQ IonTrap to verify the mass and amino acid sequence. The peptides were then purified on a Biocad Sprint system (Perseptive Biosystems) to >95% purity by reversed-phase HPLC on a self-packed Poros Oligo R3 column (Perseptive Biosystems).

GST Pull-down Assay—MCF7 (1×10^7 cells/sample) or HeLa (1×10^9 cells/sample) cells were lysed in 0.5% Nonidet P-40 lysis buffer with protease inhibitors (Roche Applied Science), 1 mM Na_2MoO_2, 1 mM Na_2VaO_4 at 2×10^6 cells/50 μl of the lysis buffer. GST-Sepharose 4B (Pierce) was washed with PBS to remove preservatives, and the cell lysates were precleared with 500 μl of 50% GST-Sepharose 4B at 4 °C for 1 h. GST fusion proteins were bound to 100 μl of 50% GST-Sepharose 4B. The amount of GST fusion proteins used was based on the binding capacity of the GST-Sepharose 4B as determined by the manufacturer. Precleared lysates were added to the GST fusion proteins-Sepharose 4B complex. The mixture was incubated at 4 °C on a rotator overnight. The samples were loaded onto Micro Bio-Spin columns (Bio-Rad) and washed with 5 column volumes of the Nonidet P-40 lysis buffer. The precipitated complex was eluted with SDS loading buffer and resolved on an SDS-polyacrylamide gel. The resulting gel was stained with colloidal Coomassie Blue (Pierce) or SYPRO Ruby (Molecular Probes) or was silver-stained (Bio-Rad) according to the manufacturers' instructions for mass spectrometry or transferred to PVDF membrane for Western blotting.

Mass Spectrometry—CEACAM1-4S co-immunoprecipitated proteins were excised from Coomassie Blue-stained SDS-polyacrylamide gels and in-gel digested with trypsin (Promega) as described previously (38). All solutions were freshly prepared prior to digestion using HPLC grade chemicals (Sigma) and 0.2 μm 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 NH_4HCO_3 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. Liquid chromatography mass spectrometry (LC/MS/MS) analyses were performed using a LabView-controlled microcapillary HPLC system (38). The standard gradient was from 2 to 92% MS buffer B (90% (v/v) acetonitrile, 0.014% (v/v) trifluoroacetic acid) over 60 min using low trifluoroacetic acid buffers 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 (0.02% (v/v) trifluoroacetic acid) for desalting and removal of contaminating components. The 150-μm-inner diameter × 350-μm-outer diameter on-line microspray needles (Polymicro Technologies) were pulled using a laser-based micropipette puller (Sutter) to a terminal inner diameter of ~5 μm. The tip was packed at 4000 p.s.i. using 5-μm C_{18} beads (Zorbax) as described previously (38). The packed tip was connected to a 75-μm-inner diameter × 350-μm-outer diameter transfer line using a polyether ether ketone 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 Thermoquest LCQ IonTrap mass spectrometer 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 were: full MS, 5×10^7; MS^n, 2×10^7; and zoom MS, 2.55×10^6. The default maximum injection time was 500 ms with a single microscan count.

Immunoprecipitation and Western Blotting—Immunoprecipitations were performed as described previously (38) with the following modifications. Cells were lysed in cell solubilization buffer at 2×10^6 cells/50 μl of buffer, and proteins were immunoprecipitated with mAb T84.1 for

CEACAM1 and anti-annexin heavy chain polyclonal antibody (BD Pharmingen) for annexin II. Following the immunoprecipitation, blots were incubated for 1 h with either mAb 4D1C2 (a kind gift from Dr. Wagener) (13) or anti-annexin II polyclonal antibody at a final concentration of 1 μg/ml. Following three Tris-buffered saline washes the blots were incubated with either goat anti-mouse or goat anti-rabbit IgG-horseradish peroxidase at a 1:3000 dilution (Bio-Rad) for 1 h and washed again. Detection was carried out using the SuperSignal chemiluminescent substrate and enhancer Kit (Pierce) according to the manufacturer's instructions.

In Vitro Binding Annexin II Assay—AIIt from bovine lung (Sigma) was resuspended in PBS with 0.5 mM dithiothreitol. For each pull-down assay 0.5 μg of AIIt was mixed with 5 μg of GST fusion proteins prebound to GST-Sepharose 4B for 1 h. The mixture was incubated for 1 h at 4 °C, loaded onto Micro Bio-Spin columns, and washed with 5-column volumes of PBS. The bound complex was eluted with SDS loading buffer, separated on an SDS-polyacrylamide gel, transferred to PVDF membrane, and immunoblotted with anti-annexin II mAb.

SPR—Biomolecular interaction analyses were carried out in HBS buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM $CaCl_2$, 0.005% (v/v) Surfactant P20) using the BIAcore® 2000 (BIAcore). Thiol-derivatized (amino-terminal 11-mercaptoundecanoic acid) CEACAM1-4S peptides (Table I) were immobilized directly on the gold surface of a J1 sensor chip (BIAcore). For immobilization of protein, 1–5 μg of protein in 100 μl of HBS buffer were injected at a flow rate of 5 μl/min. Subsequently the sensor chip was conditioned with two consecutive injections of 10 μl of 25 mM HCl. Binding studies and regeneration of the chip surface between injections were carried out at a flow rate of 20 μl/min. Samples were diluted in HBS buffer immediately prior to injection (AIIt, 191.5, 95.7, and 38.3 nM; or anti-CEACAM1-4S polyclonal antiserum and preimmune rabbit serum diluted 1:1000). Between sample injections the surface was regenerated with 10 μl of 25 mM HCl followed by two 10-μl injections of HBS buffer.

Immunofluorescence—Slides were prepared from paraffin-embedded sections as described previously (18) and incubated for 1 h with anti-annexin II or anti-p11 mAbs (BD Pharmingen) diluted according to the manufacturer's suggestions. Following two PBS washes, slides were incubated with Alexa-488-conjugated goat anti-mouse secondary antibody for 1 h. After two PBS washes Alexa-546 (Molecular Probes)-conjugated mAb T84.1 (1 μg/ml) was used to detect CEACAM1. Staining was visualized on a Zeiss Model 310 confocal microscope.

RESULTS

Identification of Annexin II Interaction with CEACAM1-4S—The cytoplasmic domain of CEACAM1-4S contains 12–14 amino acids (depending on the predicted end of the transmembrane and beginning of the cytoplasmic domains), including Thr^{423} and Ser^{425} that can be phosphorylated by PKC (42). Recently we have shown that transfection of the mammary cancer cell line MCF7 with the CEACAM1-4S isoform reverts the cells to a normal phenotype when grown in a three-dimensional culture (19). Furthermore we have shown that transfection of the cells with the pseudophosphorylation mutant (T423D) causes apoptosis when the cells are grown in Matrigel, and when transfected with the double mutant (T423D,S425D), the cells rapidly died even when grown on plastic.[2] To identify the proteins responsible for CEACAM1-4S-mediated apoptosis we performed GST pull-downs with a GST fusion protein composed of the 14 amino acids of the cytoplasmic domain of CEACAM1-4S or the double mutant (T423D,S425D) since it had the most profound effect on cells. MCF7 and HeLa cell lysates were incubated with GST alone, GST-wild type, or GST-double mutant fusion proteins, and the bound proteins were eluted with SDS and separated by SDS-PAGE, and the resulting bands were excised and subjected to in-gel digestion with trypsin. The band in Fig. 1*A* marked by an *arrow* was identified as annexin II by LC/MS/MS with high confidence (Xcorr value > 2.5, SEQUEST) and excellent sequence coverage of 43% (Table II). While other bands were also identified, the annexin II band was chosen for further analysis because it

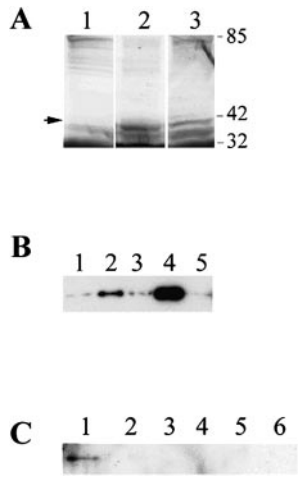

FIG. 1. **Identification of annexin II in CEACAM1-4S cytoplasmic domain GST-pull down assays.** MCF7 cell lysates were incubated with wild type or double mutant (T423D,T425D) GST fusion proteins, and bound proteins were eluted with SDS, separated by SDS-PAGE, and silver-stained. *A*, GST control (*lane 1*), GST-wild type (*lane 2*), and GST-double mutant (*lane 3*). The *arrow* indicates the band identified by LC/MS/MS as annexin II (Table III). *B*, the GST pull-down assay was repeated on HeLa cell lysates with additional mutants. Proteins from the SDS-polyacrylamide gel were transferred to a PVDF membrane and immunoblotted with anti-annexin II antibody. *Lane 1*, GST control; *lane 2*, GST-wild type; *lane 3*, GST-single mutant T423D; *lane 4*, GST-single mutant S425D; *lane 5*, GST-double mutant T423D,S425D. *C*, lysates from MCF7/CEACAM1-4S transfected (*lanes 1* and *2*), MCF7/vector control (*lanes 3* and *4*), and Jurkat (*lanes 5* and *6*) cells were immunoprecipitated with either mAb T84.1 (*lanes 1*, *3*, and *5*) or anti-CD4 mAb (*lanes 2*, *4*, and *6*), run on SDS-polyacrylamide gels, transferred to PVDF membranes, and immunoblotted with anti-annexin II antibody.

was absent in the GST only control and showed a higher intensity in the wild type *versus* the double mutant.

To confirm that annexin II was present in the GST pull-downs by a second method, the separated proteins were immunoblotted with anti-annexin II antibodies (Fig. 1*B*). As expected, a strong band for annexin II was observed in the GST pull-down for the wild type but not the double mutant GST fusion protein. To further define the binding specificity of annexin II to the CEACAM1-4S cytoplasmic domain, we also tested T423D and S425D single mutants. When GST pull-downs were performed with the single mutants, the S425D mutant bound the highest amount of annexin II with low binding for T423D (Fig. 1*B*). Thus, it appears that phosphorylation of Ser^{425} increases annexin II binding, while phosphorylation of Thr^{423} decreases it. Furthermore the T423D and double mutant both have lower binding compared with the wild type or S425D mutant, suggesting that differential phosphorylation of the CEACAM1-4S cytoplasmic domain plays a role in annexin II binding. This is intriguing because phosphorylation of the same residues affects the outcome of lumen formation in our cell-Matrigel studies.

The CEACAM1-4S interaction with annexin II was verified by immunoprecipitating cell lysates with anti-CEA mAb T84.1 from MCF7/CEACAM1-4S and MCF7/vector control cells that express CEA. Jurkat cell lysates, which do not express CEA or CEACAM1, were run as an additional control. Since mAb T84.1 reacts with both CEA and CEACAM1, the lysates were precleared with mAb T84.66, which only recognizes CEA. The resulting immunoprecipitates were immunoblotted with anti-annexin II antibody (Fig. 1*C*). The results demonstrate that annexin II is co-immunoprecipitated with CEACAM1. As a further control, when CD4 was immunoprecipitated from Jurkat cells followed by Western blotting with anti-annexin II, no annexin II was co-immunoprecipitated, demonstrating the

[2] J. Kirshner, D. Schumann, and J. E. Shively, unpublished data.

TABLE II
Peptides from annexin II identified by LC/MS/MS

An SDS gel band at 36–38 kDa was excised from an SDS gel (Fig. 1, *arrow*), reduced and *S*-alkylated, and digested with trypsin, and the resulting peptides were identified by LC/MS/MS. The sequence coverage was 43% (150/349 amino acids) with an Xcorr value (SEQUEST) >2.5.

Peptide sequence from data base[a]	$[M + H]^+$	Residues	No. of times detected
LSLEGDHSTPPSAYGSVK	1844.6	11–28	2
AYTNFDAERDALNIETAIK	2154.7	29–47	1
DALNIETAIK	1087.1	38–47	1
TKGVDEVTIVNILTNR	1772.7	48–63	1
GVDEVTIVNILTNR	1542.3	50–63	1
SALSGHLETVILGLLK	1650.9	89–104	2
ALLYLCGGDD	1039.5	139–145	1
TDLEKDIISDTSGDFR	1811.6	153–168	1
RAEDGSVIDYELIDQDAR	2063.7	179–196	1
AEDGSVIDYELIDQDAR	1908.1	180–196	1
WISIMTER	1035.1	213–220	1
SYSPYDMLESIR	1460.3	234–245	1
TNQELQEINR	1244.6	330–349	1

[a] OWL nonredundant data base.

specificity of the antibody used in the immunoprecipitation (Fig. 1*C*). Moreover, when annexin II was immunoprecipitated from MCF7/CEACAM1-4S cells followed by Western blotting with anti-CEACAM1-specific mAb 4D1C2 (this mAb reacts with CEACAM1 on Western blots but does not immunoprecipitate CEACAM1), CEACAM1 was identified in the immunoprecipitate (data not shown).

CEACAM1 Colocalizes with Annexin II and p11 in MCF10F Cells Grown in Matrigel—To test the interaction of CEACAM1 with annexin II in a biologically relevant system, we selected an *in vitro* model of mammary morphogenesis in which the "normal" mammary epithelial cell line MCF10F was grown in Matrigel. Under these conditions, the cells form acini with lumen in which CEACAM1 is localized between cells at 3–5 days in culture and to the apical portion of the acini at 12–14 days. When the resulting acini were stained for CEACAM1, annexin II, and p11 (the light chain of the AIIt complex), co-localization of all three antigens was observed at both the early (5 days) and late (14 days) stages of acinus development (Fig. 2). Strikingly CEACAM1 was also found to colocalize with annexin II in vesicles released from the acinus (Fig. 3) indicating a possible role of annexin II in CEACAM1 exocytosis. At this point, we became interested in AIIt, the biologically relevant form of annexin II, since it is known to be localized to the plasma membrane where CEACAM1 is found, while free annexin II is found in the cytoplasm.

AIIt Directly Binds to CEACAM1-4S—To determine whether AIIt directly associates with CEACAM1-4S, we performed an *in vitro* GST pull-down assay with commercially available bovine AIIt, which is 98.5% identical to human AIIt at the amino acid level. GST alone and GST fusion proteins (wild type and T423D, S425D, and T423D,S425D mutants) were mixed with bovine AIIt, and the complex was bound and eluted from GST-Sepharose. The results shown in Fig. 4 demonstrate that bovine AIIt binds the cytoplasmic domain of human CEACAM1-4S. Although both the wild type and mutant versions of CEACAM1-4S in the GST fusion proteins bound AIIt, there were variations in the extent of binding: the order of binding was T423D,S425D < wild type < T423D < S423D. The order of binding was somewhat different compared with that obtained using cell lysates where T423D,S425D < T423D < wild type < S425D (Fig. 1*B*). However, the results agree in that the S423D mutant binds the most, and the double mutant binds the least. The difference in results between the two assays is probably due to the fact that cell lysates contain other proteins (including monomeric annexin II) that modify or compete with the association or that AIIt itself may be modified by phosphorylation and/or Ca^{2+} binding. Reconstitution studies with purified proteins or depleted lysates are required to address this issue.

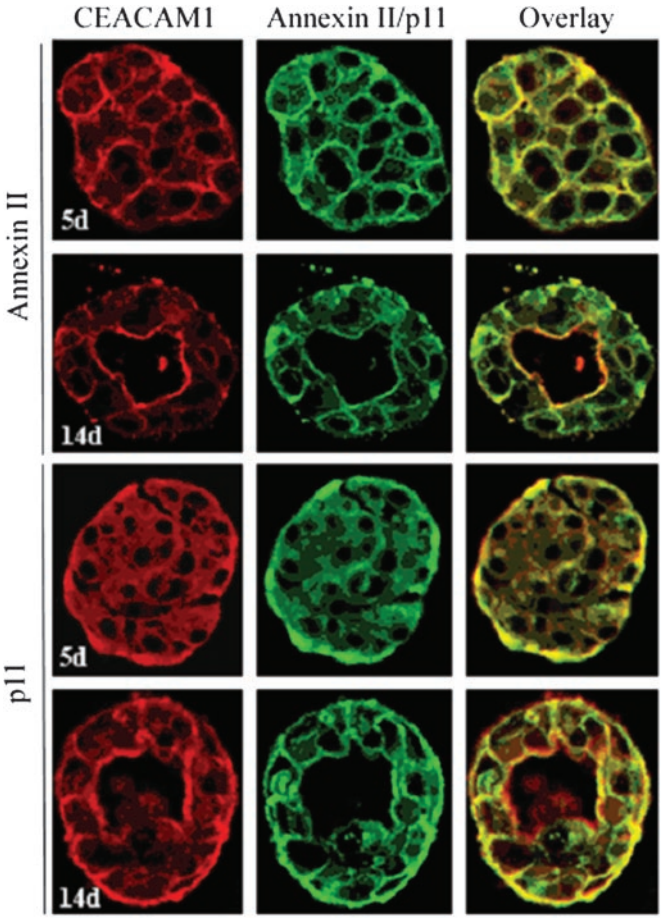

FIG. 2. **Annexin II and p11 co-localize with CEACAM1 in a normal mammary epithelial cell line grown in Matrigel.** The normal mammary epithelial cell line (MCF10F) was grown in Matrigel for 5 or 14 days (*d*), fixed, embedded in paraffin, and stained for annexin II, p11, and CEACAM1. CEACAM1 staining is shown in *red*, annexin II or p11 is shown in *green*, and co-localization of the two overlaid images is shown by *yellow* fluorescence.

To measure the kinetics of association of the cytoplasmic domain of CEACAM1-4S with AIIt, we used an SPR approach. When AIIt was immobilized on the biosensor chip, the GST fusion proteins failed to bind, perhaps due to denaturation or dissociation of AIIt during the regeneration phase on the BIAcore chip (a prerequisite of conditioning biosensor chips). The second approach was to immobilize each of the GST fusion proteins and use AIIt as a soluble ligand. Although AIIt showed good binding to immobilized GST fusion proteins, this approach

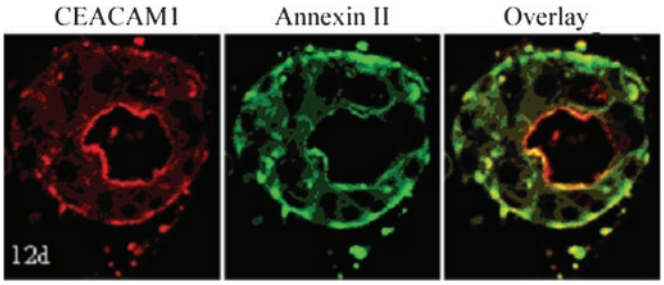

FIG. 3. **Co-localization of annexin II and CEACAM1 in exocytotic vesicles.** MCF10F cells were grown in Matrigel for 12 days (*d*), fixed, embedded in paraffin, and stained for annexin II and CEACAM1 expression. CEACAM1 is shown in *red*, annexin II is shown in *green*, and co-localization of the two is shown by *yellow* fluorescence. *Arrows* point to the co-localization of annexin II and CEACAM1 in exocytotic vesicles.

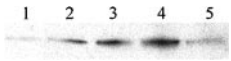

FIG. 4. **AIIt directly interacts with GST fusion proteins from the cytoplasmic domain of CEACAM1-4S.** Bovine AIIt was incubated with GST control and GST fusion proteins, and bound proteins were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with anti-annexin II antibody. *Lane 1*, GST control; *lane 2*, GST-wild type; *lane 3*, GST-single mutant T423D; *lane 4*, GST-single mutant S425D; *lane 5*, GST-double mutant T423D,S425D.

was abandoned due to a low but irreproducible background binding of AIIt to the immobilized GST control. Therefore, we explored a third approach in which oriented synthetic peptides corresponding to the CEACAM1-4S cytoplasmic domain were directly attached to the gold surface of the chip. Our synthesis strategy included a C_{11} alkyl chain at the amino terminus of the peptide to mimic the transmembrane region of CEACAM1 and a terminal thiol group for covalent attachment to the gold surface. The peptide sequences corresponded to the 14 amino acids of the cytoplasmic domain of CEACAM1-4S and the following mutants: T423E, S425E, and the double mutant T423E,S425E. In this study the pseudophosphorylation residue was glutamic acid (Glu) rather than aspartic acid (Asp) since the Asp-Gly sequence rearranges to a succinimide derivative during peptide synthesis. A control peptide including the reverse sequence of the wild type peptide (see Table I) was also synthesized. The peptides were immobilized on the gold surface of J1 BIAcore sensor chips at 1.5 ng/mm^2 based on the assumption that the SPR response of 1000 relative units translates to 1 ng/mm^2 of immobilized protein (38). Since the approach is novel, polyclonal antiserum raised to the cytoplasmic domain of CEACAM1-4S was used as a positive control and rabbit preimmune serum was used as a negative control for protein binding to peptide (Fig. 5*A*). A consistent binding of ~350 relative units was detected for a 1:1000 dilution of the polyclonal antiserum, while no binding was seen for the preimmune serum. Binding of bovine AIIt to the wild type peptide was measured at 38, 96, and 192 nM (Fig. 5*A*). The kinetics of association and dissociation of AIIt with the various CEACAM1 peptides *versus* the control peptide indicated that the binding was specific (Fig. 5*B*). While the kinetics of binding of the antibody to the immobilized peptides demonstrated a linear phase characteristic of mass limited binding, the kinetics of AIIt binding to the wild type peptide exhibited saturable binding. Analysis of the kinetics did not reveal major differences between the binding of AIIt to the wild type *versus* the mutant peptides (K_D values were in the range of 30–100 nM, Table III). We conclude that the intrinsic ability of AIIt to associate with CEACAM1-4S does not depend significantly on the phosphorylation status of its cytoplasmic domain. However, the results of the GST pull-down assays using cell lysates did reveal binding differences, suggesting that either additional components in the lysates affect the association or that modifications of AIIt directly affect the interaction.

DISCUSSION

Our previous studies indicated that CEACAM1-4S but not CEACAM1-4L was the biologically relevant isoform that mediated apoptosis during mammary morphogenesis (19). Since the cytoplasmic domain of CEACAM1-4S contains only 14 amino acids, it seemed likely that it would interact with a limited set of proteins during the apoptotic process. Since earlier studies (38, 42) had indicated that the phosphorylation status of Ser and Thr residues in CEACAM1-4S were important, we generated pseudophosphorylated mutants of CEACAM1-4S (T423D, S425D, and T423D,S425D) and demonstrated that the phosphorylation status of these residues indeed dictates the fate of the cells with phosphorylation of Thr^{423} leading to cell death for cells grown in Matrigel.[2] We also showed that phosphorylation of Ser^{425} was important since the double mutant T423D,S425D was lethal when transfected into either MCF7 or HeLa cells. In those studies we postulated that the cells in contact with the extracellular matrix survive (no Thr^{423} phosphorylation), while the cells in the center of the acinus die due to the differential phosphorylation of the cytoplasmic domain of CEACAM1-4S. To identify proteins interacting with the cytoplasmic domain, we compared GST fusions of the double mutant to the wild type sequence. Both MCF7 and HeLa cell lysates were used as sources of binding partners since both cell lines responded similarly to transfection with the double mutant. Using LC/MS/MS analysis of SDS gel bands from the GST pull-down assays, annexin II was identified as a major interacting protein for both the wild type and the double mutant. While other minor bands were also identified, we chose annexin II for further analysis because the band was absent in the GST control and was stronger for the wild type *versus* the double mutant, suggesting differential binding. The results were confirmed by Western blot analysis of the GST pull-downs and of immunoprecipitates using either anti-CEACAM1 or anti-annexin II antibodies.

Since neither GST pull-down assays nor immunoprecipitation studies on cell lysates prove that two proteins directly interact with each other, we performed the GST pull-down assay with commercially available bovine AIIt, which is 98% homologous to human AIIt. Similar to the results obtained with cell lysates, the S425D single mutant bound the most, and the double mutant (T423D,S425D) bound the least AIIt. However, the exact order of binding was different between the two assays. We interpret these results as follows. Since cell lysates contain other proteins that interact with and phosphorylate both CEACAM1 and either annexin II or AIIt, as well as free annexin II and p11, the binding of any two of the purified proteins could be different *in vivo*. To test this hypothesis, we must generate dose-response curves in which controlled amounts of actin, calmodulin, tropomyosin, annexin II, p11, phospholipids, and kinases are included or excluded from the assay. Most importantly, we must test the various phosphorylated forms of AIIt to determine their effect on the association. At this point, we cannot exclude the possibility that CEACAM1 interacts primarily with AIIt alone or in conjunction with one or more of their other interacting components at any given time during mammary morphogenesis. However, the confocal analyses shown in Fig. 2 suggest that AIIt may be the biologically relevant form of annexin II that interacts with CEACAM1.

The direct interaction of AIIt with the cytoplasmic domain of CEACAM1 was further analyzed by SPR. The fact that immobilized and acid-treated AIIt did not interact with the GST fusion proteins suggests that either the AIIt was denatured by

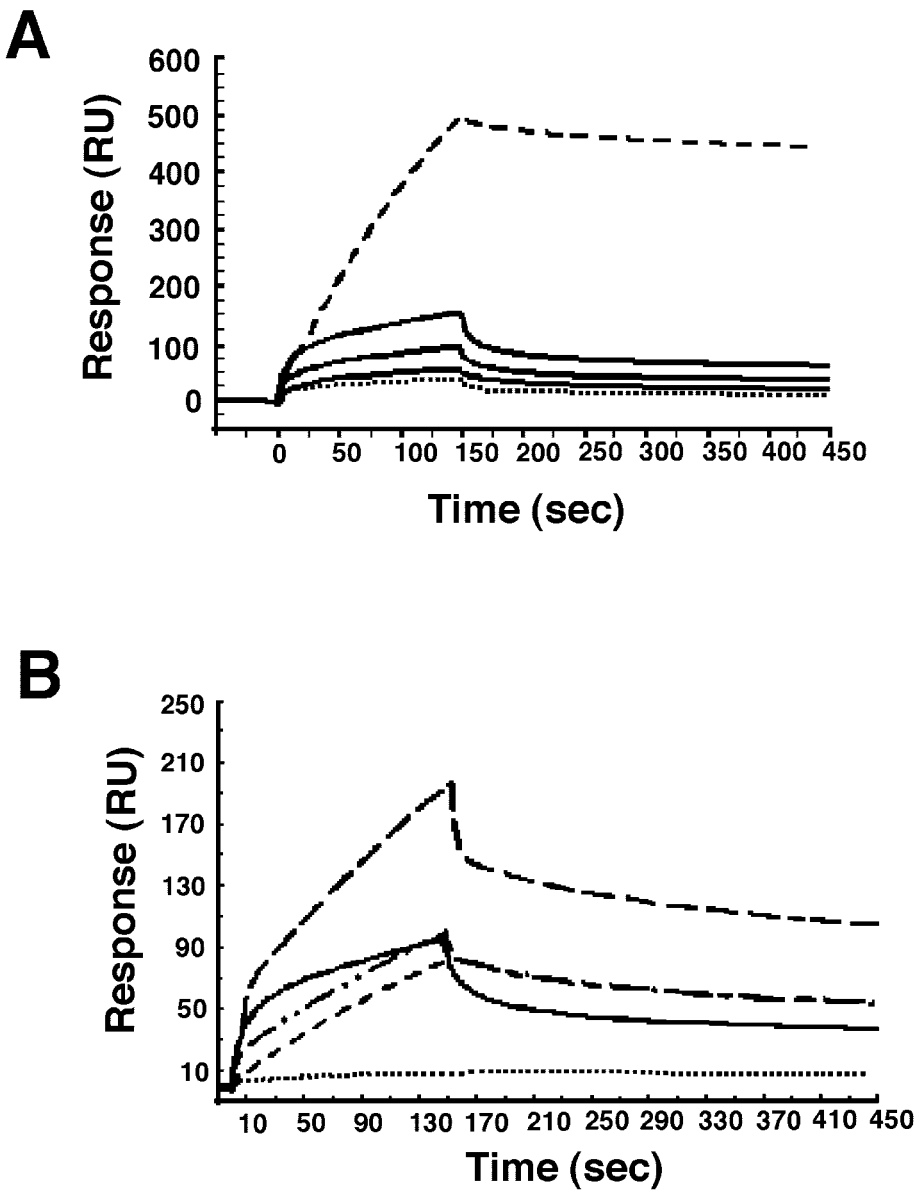

FIG. 5. **AIIt binding to immobilized, oriented peptides from the cytoplasmic domain of CEACAM1-4S.** Synthetic peptides corresponding to the cytoplasmic domain or the pseudophosphorylated derivatives of CEACAM1-4S were synthesized (Table I) and immobilized on the gold surface of a J1 BIAcore chip. Injections were performed in HBS buffer with 2 mM $CaCl_2$ at a flow rate of 20 μl/min followed by HBS buffer (dissociation phase) and 25 mM HCl (surface regeneration). *A*, AIIt at 38, 96, and 192 nM (*solid lines*) was passed over the wild type CEACAM1-4S peptide. Controls, polyclonal antiserum against the wild type peptide (diluted 1:1000, *dashed line*) and rabbit preimmune serum (diluted 1:1000, *dotted line*), were passed over immobilized wild type peptide. *B*, AIIt at 96 nM was passed over immobilized wild type-reversed peptide (*dotted line*), wild type peptide (*solid line*), single mutant T423E (*short-dashed line*), single mutant S425E (*dashed-dotted line*), and double mutant T423E,S425E (*long-dashed line*). *RU*, relative units.

TABLE III

Kinetic data for binding of AIIt to immobilized, oriented peptides from the cytoplasmic domain of CEACAM1-4S

AIIt (96 nM) was injected onto a surface of a biosensor chip with immobilized CEACAM1 peptides (Table I) for 150 s at a flow rate of 20 μl/min. For an example of the sensograms see Fig. 5, *A* and *B*.

Peptide	k_{on}	k_{off}	K_D
	$M^{-1}\ s^{-1}$	s^{-1}	M
Wild type	4.71×10^4	1.74×10^{-3}	3.69×10^{-8}
T423E	3.16×10^4	9.28×10^{-4}	2.94×10^{-8}
S425E	1.73×10^4	1.76×10^{-3}	1.02×10^{-7}
T423E,S425E	1.55×10^4	1.18×10^{-3}	7.60×10^{-8}

brief acid treatment or that the tetramer dissociated, disrupting the binding site. This may be further evidence that intact AIIt is required for the interaction. While immobilized GST fusion proteins interacted strongly with soluble AIIt as a ligand, we did not report these results due to a sporadic background binding of GST alone. Therefore, an alternative approach was devised. In this approach we immobilized oriented (with their carboxyl termini exposed) peptides that included a C_{11} alkyl chain at the amino terminus, mimicking the transmembrane milieu adjacent to the cytoplasmic domain of type I transmembrane proteins. For this study, the pseudophosphorylated peptides had a glutamic acid residue at Thr^{423} or Ser^{425} instead of Asp as was used for the GST fusion proteins. Glu had to be used, instead of Asp, due to the tendency of Asp-Gly sequences to cyclize during peptide synthesis. In this respect, we note that either Asp or Glu have been utilized interchangeably for pseudophosphorylation studies (86, 87). To validate our approach, we first demonstrated that antibodies raised to the cytoplasmic domain peptides of CEACAM1-4S bound with high affinity to the immobilized, oriented peptides, while control antisera did not. Based on this demonstration, we proceeded to AIIt binding studies. As expected, we observed concentration-dependent, saturable binding curves for AIIt with each of the immobilized peptides. Although minor changes in K_D were

observed for AIIt binding to the wild type *versus* the mutant peptides, the results were all in the mid nanomolar range (30–100 nM), suggesting that phosphorylation on the peptides leads to only minor changes in AIIt binding. This is in contrast to the extent of binding of AIIt to the mutant peptides observed in the *in vivo* GST pull-down assays. As discussed above, we believe that other components present in whole cell lysates affect the interaction and need to be identified in a systematic way. We are especially interested in testing phosphorylated forms of AIIt and the role of the lipid environment in the interaction. Since AIIt can be phosphorylated by both PKC (54, 55) and Src kinases (48, 49) it will be necessary to generate each of these species and repeat the binding assays. More importantly, we will need to determine the phosphorylation status of AIIt in the apical membranes of cells grown in Matrigel. Since AIIt is known to reside within lipid rafts (74–76) where both PKC and Src kinases have been shown to perform their signaling functions, it will be important to determine the effect of the lipid microenvironment on the AIIt-CEACAM1 interaction. It may be possible to duplicate the cholesterol- and sphingomyelin-rich environment in the SPR assays, rendering the results more relevant to *in vivo* conditions.

Other roles of annexin II include vesicle fusion and exocytosis (68–71), biological processes that also occur during mammary morphogenesis. Indeed our earlier studies with the CEACAM1-4S-ectoGFP fusion protein revealed that large amounts of CEACAM1-4S are secreted into the lumina of the acini formed by MCF7 cells transfected with CEACAM1-4S (19). Specifically we found vesicles positive for both CEACAM1-4S and AIIt secreted from mature acini (Fig. 3). Since AIIt has been implicated in exocytosis of secretory vesicles by cross-linking of the secretory granules and the plasma membrane (69, 88) it is possible that the CEACAM1-4S interaction with annexin II is required for the exocytosis of CEACAM1-containing vesicles. Further studies are required to explore this possibility.

Finally AIIt expression is associated with a differentiated phenotype, which, in the case of epithelial cells, involves cytoskeletal rearrangement to achieve a polarized phenotype and secretion of tissue-specific products. The interaction of AIIt with actin at the plasma membrane is similar to the interaction of CEACAM1 with both actin and tropomyosin (38). Since CEACAM1 binds polymerizing actin (38) and AIIt can bundle F-actin (65), the two may act in concert to rearrange the cytoskeleton at the apical membrane of epithelial cells. In fact, prior to this work, the only known binding molecules for AIIt at the plasma membrane were phospholipids. Since AIIt can dock to the cytoplasmic domain of CEACAM1 and both are localized to cholesterol/sphingomyelin-rich lipid microdomains, we propose that CEACAM1 is a major AIIt binding site in apical membranes. It will be of interest to determine whether other type I plasma membrane proteins serve a similar function. Since loss of differentiation is a hallmark of cancer, it is not surprising that both CEACAM1 and annexin II are down-regulated in many cancers. Thus, another prediction is that the complex of CEACAM1 and AIIt (perhaps with cytoskeletal associated proteins) generates cell cycle-inhibitory signals that maintain the differentiated state.

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