The Tumor Growth-inhibiting Cell Adhesion Molecule CEACAM1 (C-CAM) Is Differently Expressed in Proliferating and Quiescent Epithelial Cells and Regulates Cell Proliferation

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ABSTRACT

The homophilic cell adhesion molecule CEACAM1 (C-CAM, BGP, CD66a) occurs as two coexpressed isoforms, CEACAM1-L and CEACAM1-S, in epithelia, endothelia, and leukocytes. CEACAM1-L can inhibit tumor growth; this effect is influenced by CEACAM1-S. To characterize the growth regulatory properties of CEACAM1, we analyzed the expression patterns of the isoforms, and here we demonstrate that both the expression levels and the S:L isoform ratios differ in proliferating and quiescent rat epithelial cells. Quiescent prostate NbE cells expressed more CEACAM1 than quiescent bladder NBT-II cells, a pattern that correlated with the expression levels in the parental tissues. In contrast, both the expression levels and the isoform ratios were strikingly similar in proliferating NbE and NBT-II cells, showing that a particular CEACAM1 expression pattern is compatible with cell proliferation. However, in confluent cells, CEACAM1 seemed to exert inhibitory effects on cell proliferation. Addition of anti-CEACAM1 antibodies to quiescent, confluent cells caused decreased expression of the cyclin-dependent kinase inhibitor, p27Kip1, stimulated growth factor-dependent DNA synthesis, and altered the S:L isoform ratio toward the ratio characteristic of proliferating cells. Taken together, our data suggest that CEACAM1 contributes to contact inhibition of cell proliferation in confluent cells but allows proliferation when expressed at different isoform ratios.

INTRODUCTION

Cell adhesion molecules mediate physical binding of cells in various adhesive assays. The majority of the molecules identified by this approach have been found to belong to three major protein superfamilies, the immunoglobulin superfamily, the cadherin superfamily, and the integrin superfamily (1). Many of these molecules have important roles in adhesive interactions in vivo, and in addition, a majority of the classical cell adhesion molecules functions as adhesion receptors because they are able to initiate or regulate cell signaling cascades (1, 2). Distinct cell adhesion receptors control both cellular proliferation and motility; in line with these functions, altered properties and expression patterns of several cell adhesion receptors have been observed in various types of carcinomas (3, 4). One cell adhesion receptor with such properties is CEACAM1 (formerly known as cell-CAM 105, C-CAM, BGP, and CD66a), which is a member of the CEA family, which makes up a well-defined subgroup within the immunoglobulin superfamily (3, 5). [The new nomenclature for genes and proteins belonging to the CEA family (5) is used in this report.] CEACAM1, which is abundantly expressed in epithelia, vessel endothelia, and leukocytes (6), can mediate adhesion by homophilic binding (7, 8) and influence tyrosine kinase-dependent signaling cascades (3, 9).

CEACAM1 occurs as several splicing isoforms (3), the two major of which, differing only in their cytoplasmic domains, are known as CEACAM1–4L [four extracellular immunoglobulin domains and a large cytoplasmic domain (L) of 71–73 amino acids] and CEACAM1–4S [four extracellular immunoglobulin domains and a small cytoplasmic domain (S) of 10–12 amino acids]. These two isoforms are coexpressed in most CEACAM1-expressing tissues, although at varying ratios (10). The L domain is highly conserved between species and contains two phosphorylatable tyrosine residues in immunoreceptor tyrosine-based inhibition motif sequences (3). The immunoreceptor tyrosine-based inhibition motif sequences are lacking in the S domain. After tyrosine phosphorylation, CEACAM1–4L can bind and activate src-family tyrosine kinases (11, 12) as well as the SH2-domain-containing protein tyrosine phosphatases SHP1 and SHP2 (13, 14). In addition, an increased intracellular calcium concentration leads to binding of calmodulin to the cytoplasmic domains of both the L and the S isoforms, which influences CEACAM1 dimerization (15, 16). Interactions of CEACAM1 with the actin filament system also have been observed (17, 18). Human CEACAM1 as well as CEACAM3 (formerly CGM1 or CD66d), CEACAM6 (formerly NCA or CD66c) and CEA are receptors for Opa-protein expressing bacterial strains of Neisseria gonorrhoeae and N. meningitides (9). Binding of such bacteria to CEACAM1–4L in human granulocytes activates the Jun-N-terminal kinase pathway and leads to intracellular bacterial uptake (9).

A number of reports have shown that CEACAM1 is down-regulated in various types of carcinomas (19–23), and it has recently been demonstrated that CEACAM1–4L can inhibit in vivo tumor growth of several different carcinomas (24–28). This important inhibitory signaling activity resides in the L domain and depends on the two tyrosine residues as well as on the two extracellular domains of the L domain (24, 28). CEACAM1–4S cannot inhibit tumor growth on its own, but it seems to influence the inhibitory activity of the L isoform in a significant way (29). Available data indicate that both the ratio of the two isoforms and the absolute expression levels are important for the tumor inhibition activity.

The tumor growth inhibitory effect of CEACAM1–4L was demonstrated by transfecting several tumor cells from different species with sense cDNA for CEACAM1 (24–28). Furthermore, it was found that transfection of a nontumorogenic, CEACAM1-expressing rat prostate epithelial cell line (NbE cells; Ref. 30) with antisense cDNA reduced CEACAM1 expression and induced tumor formation (25), demonstrating that endogenously expressed CEACAM1 also has tumor-suppressive activity. However, other cells, e.g., the bladder carcinoma-derived NBT-II cell line, give rise to tumors in syngeneic animals (31), despite that their expression of CEACAM1 (17). In addition, some human malignant tumors exhibit significant CEACAM1 expression (32–34). Thus, expression of CEACAM1 does not always suppress tumor growth. Ascertaining why CEACAM1 can inhibit tumor growth in some cells but not in others will give impor-
tant insights into the mechanisms of how CEACAM1 regulates cell signaling cascades. For this purpose, NbE and NBT-II cells make up an excellent experimental system.

In this report, we characterized the expression levels, the isoform ratios, and the surface distribution of CEACAM1 in NbE and NBT-II cells that were cultured under various conditions. We show for the first time that the expression levels and isoform ratios of CEACAM1 are different in proliferating and quiescent cells and that the two isoforms are regulated independently in both cell lines. Cell surface interactions participate in the control of CEACAM1 expression, and in both cell lines the cell surface organization of CEACAM1 itself seems to be an important regulating factor. We found that proliferating cells have a characteristic S:L isoform ratio that is much lower than that in quiescent cells, and we demonstrate that endogenous CEACAM1 can regulate p27Kip1 levels and DNA synthesis.

MATERIALS AND METHODS

Antibodies. Affinity-purified, rabbit polyclonal antibodies (oC16) against rat CEACAM1 were prepared and characterized as described by Odin et al. (6). The antipeptide rabbit polyclonal antibody, L2, which recognizes the cytoplasmic domain of the long isoform of CEACAM1, was described by Hunter et al. (16). Preimmune rabbit IgG was depleted of anti-CEACAM1 reactivity by absorption on a column of Sepharose-coupled purified rat CEACAM1 (6). The mouse Mab F7 has been described previously (17), and the mouse Mab 5.4 was generously provided by Dr. D. C. Hixson, Rhode Island Hospital, Brown University, Providence, RI. Both F7 and 5.4 are directed against the NH2-terminal immunoglobulin domain of rat CEACAM1. A mouse Mab, Sab-1, was produced by immunizing BALB/c mice with BALB/c 3T3 cells transfected with human CEACAM1 (BGP). Sab-1 recognizes human CEACAM1 (BGP, CD66a), CEACAM3 (CGM1, CD66d), CEACAM4 (CGM7), CEA (CD66e), CEACAM6 (NCA, CD66c), and CEACAM7 (CGM2), but it does not recognize rat CEACAM1 and does not bind to NbE or NBT-II cells. Subclass determination showed that both 5.4 and F7, as well as Sab-1, consist of IgG1-type immunoglobulins.

Cell Culture. Cells were grown in a 5% CO2 humidified atmosphere at 37°C. The NBT-II cell line was cultured in DMEM supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin (17). The NbE cell line (provided by Dr. S.-H. Lin, M. D. Anderson Cancer Center, Houston, TX, with permission from Dr. L. Chung, Department of Urology, Health Sciences Center, Charlottesville, VA) was cultured in 80% DMEM-20% Ham’s F12 medium supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin, 5 mg/ml insulin, 14 pg/ml triiodothyronine, 5 mg/ml transferrin, 0.24 mg/ml biotin, and 25 mg/ml adenine.

For some experiments, confluent cell layers were subjected to a limited trypsin treatment. The cells were treated with trypsin-EDTA (Life Technologies, Inc.) for 3 min, after which the trypsin was inactivated by addition of medium containing 10% FCS, the cell layers were washed gently, and fresh medium was added to the cultures. This caused the cells to round up slightly, but no cells were lost from the dishes. In another experimental series, confluent cell layers were cultured in the presence of antibodies (100 µg/ml) against CEACAM1 for 16 h; the cells were then harvested, and the CEACAM1 isoform ratios were determined.

Phase-Contrast and Fluorescence Microscopy. NbE and NBT-II cells were grown on glass coverslips and fixed at room temperature in buffer 3 (137 mM NaCl, 4.7 mM MgSO4, 1.2 mM CaCl2, 10 mM HEPES (pH 7.4)) containing 3% paraformaldehyde for 30 min, followed by incubation in buffer 3 containing 0.1% glycine for 30 min to quench reactive aldehyde groups. Permeabilization was performed with 0.1% Triton X-100 in buffer 3 for 20 min. Fixed cells were incubated overnight at 4°C with affinity-purified polyclonal anti-CEACAM1 antibodies (50 µg/ml), followed by incubation with FITC-conjugated swine antirabbit secondary antibodies (Dako) for 60 min at room temperature. The cells were then examined in a Nikon Labophot phase-epifluorescence microscope and photographed on Kodak Tri-X pan film at 400 ASA.

To study the effects of antibodies on the distribution of CEACAM1, cell cultures were incubated with polyclonal or monoclonal immunoglobulins (25 µg/ml in buffer 3) for 60 min at 37°C prior to fixation.

Flow Cytometry. Cells were removed from culture dishes by trypsinization. A fraction of 10^6 cells was resuspended in 25 µl of 3% FCS in PBS, and 50 µl of rat CEACAM1-specific antibodies (50 µg/ml) in 3% FCS in PBS were added. The cells were incubated for 60 min at 4°C, washed twice with 3% FCS in PBS, and finally incubated with FITC-conjugated secondary antibodies (Dako) for 60 min at 4°C. Background fluorescence was determined using nonimmune rabbit IgG or Mab Sab-1 instead of primary antibodies. For DNA quantification, the cells were fixed in 70% ethanol and then were incubated for 15 min at 37°C in PBS containing 0.1% Triton X-100, 100 µg/ml DNase-free RNase (Sigma), and 10 µg/ml propidium iodide. For simultaneous determination of CEACAM1 and DNA, the cells were stained with propidium iodide and an antibody labeling for CEACAM1. The stained cells were examined in a FACS Calibur flow cytometer (Becton Dickinson) and analyzed by CellQuest software (Becton Dickinson). In all calculations, we used the median values of the fluorescence intensities.

[^3H]Thymidine Incorporation. Thymidine incorporation was determined in cells growing in 96-well plates (5-mm diameter per well) or in 24-well plates (16-mm diameter per well). In both procedures, the cells were first cultured in standard medium containing 10% FCS for 48 h, and then in medium containing 0.5% FCS for another 24 h. The cells in 96-well plates were then cultured in standard medium containing either 0.5% FCS or 10% FCS for 12 h. [^3H]Thymidine (4 µCi/ml; Amersham) was added, and the cells were cultured for an additional 4 h. The wells were washed thoroughly and then transferred to glass fiber filters (Wallac), using a cell harvester (96 MACH III; Tomtec), and analyzed by a Micro Beta counter (Wallac). The cells in 24-well plates were used to examine the effects of different antibodies on DNA synthesis. In these experiments, the cells were first cultured for 10 h in standard medium containing 10% or 0.5% FCS. Monoclonal or polyclonal anti-CEACAM1 antibodies or control IgG (200 µg/ml), and [^3H]thymidine (1 µCi/ml; Amersham) were then added for an additional 6 h. The cells were washed thoroughly, treated with 10% trichloroacetic acid, washed, lysed with 0.1 M sodium hydroxide, transferred to liquid scintillation fluid, and counted in a liquid scintillation β-counter (Wallac).

Immunoblotting. Cells were harvested by either mechanical scraping or trypsinization. No differences in the amount of recovered CEACAM1 were seen between the cells that were trypsinized and those that were scraped, agreeing with previous observations that cell surface exposed CEACAM1 is insensitive to trypsin. The harvested cells were washed twice in ice-cold PBS and solubilized in lysis buffer [%NP-40, 0.1% SDS, 50 mM Tris-HCl (pH 7.5), 5 mM sodium P®, 1 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml chymostatin, 10 µg/ml pepstatin A, and 1000 KIU/ml Trasylol]. The samples were cleared by centrifugation for 30 min at 15,000 × g, and the protein concentrations of the supernatants were determined by a modified micro Lowry assay (17). The cell lysates were mixed with an equal volume of 2× SDS sample buffer, reduced with 50 mM DTT, and boiled for 5 min. Samples (75 µg protein) were electrophoresed on 10% polyacrylamide gels and transferred to PVDF membranes (Millipore). After blocking with 5% defatted milk powder in TBS for 60 min at room temperature, the membranes were incubated with L2 antiserum (1:500) for 60 min, washed twice in TBS containing 0.05% Tween 20, incubated with horseradish peroxidase-labeled swine antirabbit antibodies (Dako), and developed by ECL. The membranes were then stripped with 0.1 M glycine (pH 2.5) containing 0.05% Tween 20 at 56°C for 60 min, blocked with 5% defatted milk powder in TBS, incubated with oC16 antibodies, and developed by ECL as described above.

For analyses of the expression levels of p27Kip1 or cyclin E, NBT-II cell cultures were incubated in the presence of 10% FCS at 37°C for various times with or without 50 µg/ml of Mab 5.4 or Sab-1. The cells were washed twice with ice-cold PBS and solubilized in lysis buffer containing 0.1% β-mercaptoethanol but no sodium fluoride. The samples (40 µg of protein) were analyzed by SDS-PAGE (on 15% polyacrylamide gels), immunoblotting, and ECL as described above. The membranes were first developed with rabbit polyclonal antibodies against p27Kip1 (2 µg/ml; Santa Cruz Biotechnology) and then stripping with rabbit polyclonal antibodies against cyclin E (2 µg/ml; Santa Cruz Biotechnology).

RT-Triple-Primer PCR. Total RNA was isolated from NbE and NBT-II cells by guanidinium thiocyanate extraction, using the Qiagen RNAeasy mini-
kit (Qiagen). Samples containing 2 μg of RNA in a final volume of 20 μl were reverse transcribed by Moloney murine leukemia virus reverse transcriptase (MBI Fermentas), according to the recommendations of the manufacturer, using a 15-mer oligonucleotide primer (BP45, 5’-GGCATGGAAGTCAG-3’) that specifically hybridizes to the 3’-region of CEACAM1-L and CEACAM1-S. The second-strand cDNA synthesis and further amplification were performed in a final volume of 50 μl containing 2 μl of first-strand cDNA solution, 0.2 mM deoxynucleotide triphosphates, 1 μCi of [13P]dCTP (NEN), 5 units of Taq DNA polymerase (Pharmacia & UpJohn), 5 μl of 10× PCR buffer (supplied with the Taq polymerase), and 0.6 μM of each of the PCR primers. A common sense primer that recognized both CEACAM1 splice isoforms equally well (FP45, 5’-CTGGAGGTGTAGGGTTTGTGCTC-3’) recognized the alternatively spliced exon 7 present only in the L isoform, and the antisense primer for the S isoform (BP42, 5’-TCAGAGAGCCATCGTGCTC-3’) was constructed to anneal across the splice junction between exon 6 and exon 8. The PCR was initiated by heating the samples to 94°C for 60 s, followed by 28 cycles at 94°C for 45 s, 64°C for 45 s, 72°C for 60 s, and an extension at 72°C for 10 min. Equivalent aliquots from each PCR were separated electrophoretically on 2.7% agarose gels in Tris-borate EDTA buffer containing 2 μg/ml ethidium bromide; the gels were dried, and the radioactive PCR products were quantitated with a PhosphoImager using the ImageQuant analyses software (Molecular Dynamics).

RESULTS

Growth Pattern and CEACAM1 Expression in NbE and NBT-II Cells. Under standard culture conditions, both the NbE and the NBT-II cells grew as monolayers. Subconfluent cultures of both cell lines occurred as scattered colonies of associated cells. When the cells became confluent, they formed coherent layers of closely associated cells with a typical polygonal, epithelial-like morphology (hereafter referred to as 99% confluent cells). At this stage, mitotic cells were still observed. If the cells were maintained beyond that stage, they became more densely packed and tightly associated, but did not pile up, and no mitotic cells were seen (hereafter referred to as 100% confluent cells). The superconfluent (100%) monolayers NbE cells, in contrast to NBT-II cells, formed scattered dome-like structures in which the cells lost contact with the underlying support. The cells in these domes were initially part of the continuous confluent monolayer, but some dome-cells rapidly detached from the monolayers, resulting in micro-wounds in the remaining monolayer.

Analysis by indirect immunofluorescence demonstrated that both the NbE and the NBT-II cells expressed CEACAM1 on their surfaces (Fig. 1). Single cells in subconfluent cultures had a rather even distribution of CEACAM1 all over the cell surface. Most cells in the subconfluent cultures grew as colonies, and in such colonies CEACAM1 was highly concentrated in the lateral cell-cell contact areas (Fig. 1, a and f). A similar high concentration of CEACAM1 in the lateral cell-cell contact areas was seen in confluent (99%) cells of both types (Fig. 1, b and g). In superconfluent (100%) NBT-II cells, CEACAM1 remained localized to the lateral borders at apparent high concentration, and this staining was identical regardless of whether the fixed cells were permeabilized with (Fig. 1i) or without (Fig. 1h) nonionic detergent. Also in superconfluent (100%) NbE cells, significant CEACAM1 staining occurred at the lateral borders, but this was visible only when the cells were permeabilized (Fig. 1d). In nonpermeabilized, superconfluent NbE cells (Fig. 1c), no lateral staining was detected because at this stage the NbE cells became highly polarized and developed tight junctions that prevented access of the antibodies to the lateral borders. In addition to the lateral staining, just confluent and superconfluent cultures of both NbE and NBT-II cells exhibited significant CEACAM1 expression on their apical surfaces (i.e., the upper, free surfaces). This was particularly prominent in the confluent NbE cells (Fig. 1, b and c). However, the less polarized NBT-II cells also had a significant expression on their apical surfaces, which showed up as a diffuse staining of the whole cell bodies in Fig. 1, g and h. That this represented a true apical staining was demonstrated by focusing directly on the apical surfaces (not shown). The addition of anti-CEACAM1 antibodies caused rearrangement and clustering of CEACAM1 on the cell surfaces (Fig. 1, e and k). In confluent NBT-II cells, both lateral and apical CEACAM1 was rearranged (Fig. 1k), whereas in confluent
was obtained from the forward light scattering, which was recorded in the flow cytometer. The forward light scattering is proportional to the cellular volume. By this approach, we found that the NbE cells were larger than the NBT-II cells, the NbE:NBT-II forward light scattering ratio being 1.80, whereas the average size differences between subconfluent or confluent cells of either type were negligible. The surface concentration is, however, related to the surface area rather than the volume of the cells. Because the suspended cells were spherical, we could use the forward light scattering ratio of 1.80 to calculate that the ratio of the surface areas of the two cell types was 1.48. Accordingly, the fluorescence intensities of the NBT-II cells were multiplied by a factor of 1.48 to translate the fluorescence intensities of the two cell types to comparable surface concentrations of CEACAM1. This gave surface overall CEACAM1 concentrations (in arbitrary units) of 197, 159, and 1054 for 30, 99, and 100% confluent NbE cells, and 287, 297, and 120 for 30, 99, and 100% confluent NBT-II cells, respectively, when applied to the experimental data shown in Fig. 2. Thus, the surface concentrations of CEACAM1 were very similar in subconfluent NbE cells and NBT-II cells but differed almost 10-fold between the superconfluent cells.

The shift in CEACAM1 expression levels in superconfluent versus subconfluent NbE and NBT-II cells was confirmed by immunoblot analysis using an antibody that recognized both CEACAM1 isoforms (Fig. 3). However, when we compared these data with the immunoblot pattern obtained with an antibody specific for the L cytoplasmic sequence, it was obvious that the two CEACAM1 splice isoforms were regulated differently (Fig. 3). The results indicated that subconfluent and superconfluent cells had different isoform ratios. Because we did not have an antibody that was specific for the S isoform, we decided to analyze the expression patterns of the two isoforms in more detail at the mRNA level.

For this purpose we developed a competitive RT-triple-primer PCR assay that was able to distinguish between the two alternatively spliced messengers. Using this approach, we found that the L and S isoforms were coexpressed in both the NbE and the NBT-II cells (Fig. 4). Under all conditions, the expression of the S isoform dominated over that of the L isoform (Table 1). However, as was the case with the overall expression levels of CEACAM1, the ratios between the two isoforms differed grossly between subconfluent and superconfluent cells. Furthermore, in both cell types, the relative expression of the L isoform increased in subconfluent cells compared with superconfluent cells. This resulted in 30% confluent NbE cells and NBT-II cells having very similar isoform ratios.
Specific Cell Surface Interactions Are Involved in the Control of CEACAM1 Expression. The finding that the total expression levels and the isoform ratios of CEACAM1 depended on the confluence state in both cell types suggested that specific cell surface interactions participate in the regulation of CEACAM1 expression. Alternatively, local depletion of soluble factors might affect CEACAM1 expression levels because of cell crowding. To distinguish between these possibilities, we subjected confluent cell layers to short pulses of trypsin treatment and measured CEACAM1 expression at different times after the trypsin treatment. This treatment caused the cells to round up slightly, and cell-cell contacts were broken, but no cells were lost from the dishes; therefore, the cell layers remained at the same densities.

As mentioned above, trypsin treatment per se did not cause any detectable degradation or loss of surface-exposed CEACAM1. However, several hours after the trypsin pulse treatment, we observed transient changes in the surface expression of CEACAM1, such that the expression levels decreased in the NbE cells and increased in the NBT-II cells (Fig. 5). The effect was maximal between 3 and 24 h, and 48 h after the trypsin pulse the surface expression had returned to the same levels as seen before the trypsin treatment. The isoform ratios were measured 16 h after the trypsin pulse and were also dramatically changed. In both cell types, the S:L ratios decreased significantly and approached the values seen in subconfluent cells (Table 2). Thus, trypsin treatment of confluent NbE and NBT-II cells mimicked the effects seen in subconfluent cells with respect to both the total expression levels and the isoform ratios of CEACAM1. Because the trypsin treatment did not alter the cell density, these results suggested that specific molecular cell surface interactions participated in the regulation of CEACAM1 expression on the cell surface.

Because specific molecular cell surface interactions seemed to be involved in the regulation of the CEACAM1 expression levels, we asked whether CEACAM1-mediated cell-cell binding was included, i.e., was the surface expression levels of CEACAM1 regulated by CEACAM1-mediated homophilic adhesion and/or by the supramolecular organization of CEACAM1. To answer this question we treated the cells with antibodies against CEACAM1 to perturb the CEACAM1 surface organization, and measured the isoform ratios 16 h after addition of the antibodies. One polyclonal antibody and two different Mabs were used.

As demonstrated in Fig. 1, e and k, addition of anti-CEACAM1 antibodies caused rearrangement and clustering of CEACAM1 on the cell surfaces. In confluent NBT-II cells, both lateral and apical CEACAM1 was rearranged (Fig. 1k), whereas in confluent NbE cells, mainly the apically localized CEACAM1 was affected (Fig. 1e) because the antibodies did not gain access to the laterally localized CEACAM1. In both cell types, both antibodies changed the CEACAM1 isoform ratios in confluent cultures toward the ratios observed in subconfluent cultures, i.e., the S:L ratios decreased (Table 2). These results thus demonstrated that CEACAM1 on the cell surface participated in the regulation of its own expression levels.

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**Table 1** CEACAM1 isoform ratios in confluent and subconfluent NbE and NBT-II cells

<table>
<thead>
<tr>
<th>Confluence state</th>
<th>S:L ratio&lt;sup&gt;b&lt;/sup&gt;</th>
<th>NbE cells</th>
<th>NBT-II cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%</td>
<td>14.3 ± 2.9 (n = 13)</td>
<td>10.6 ± 1.0 (n = 14)</td>
<td></td>
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<tr>
<td>99%</td>
<td>4.7 ± 1.0 (n = 4)</td>
<td>3.7 ± 0.7 (n = 4)</td>
<td></td>
</tr>
<tr>
<td>30%</td>
<td>4.4 ± 1.3 (n = 12)</td>
<td>3.5 ± 0.6 (n = 11)</td>
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</table>

<sup>a</sup> The cells were grown to superconfluent (100%), just confluent (99%), or subconfluent (30%) states, as described in “Materials and Methods.”

<sup>b</sup> The ratios of the expression levels of the transcripts for the S and L CEACAM1 isoforms were determined by RT-triple-primer PCR as described in “Materials and Methods.” The data are given as mean ± SD for the indicated number of experiments.

<sup>c</sup> The significance levels were calculated relative to the 100% confluent cells of respective cell type, using Student’s t test.

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**Table 2** CEACAM1 isoform ratios in confluent NbE and NBT-II cells treated with trypsin or antibodies against CEACAM1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>S:L ratio&lt;sup&gt;b&lt;/sup&gt;</th>
<th>NbE cells</th>
<th>NBT-II cells</th>
</tr>
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<tbody>
<tr>
<td>Medium control</td>
<td>14.5 ± 2.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.3 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Trypsin pulse</td>
<td>8.2 ± 0.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.4 ± 1.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Preimmune IgG (control)</td>
<td>12.7 ± 1.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>11.0 ± 0.5&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Anti-CEACAM1 Pab&lt;sup&gt;d&lt;/sup&gt; Mab&lt;sup&gt;e&lt;/sup&gt; aCC16</td>
<td>8.0 ± 0.4&lt;sup&gt;f&lt;/sup&gt;</td>
<td>6.2 ± 0.6&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Mab Sab-1 (control)</td>
<td>12.8&lt;sup&gt;e&lt;/sup&gt;</td>
<td>11.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Anti-CEACAM1 Mab 5.4</td>
<td>8.5&lt;sup&gt;e&lt;/sup&gt;</td>
<td>7.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Anti-CEACAM1 Mab F7</td>
<td>6.5&lt;sup&gt;e&lt;/sup&gt;</td>
<td>6.8&lt;sup&gt;e&lt;/sup&gt;</td>
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<sup>a</sup> Superconfluent (100%) cell cultures were treated with a short trypsin pulse or with antibodies against CEACAM1 as described in “Materials and Methods.”

<sup>b</sup> The ratios of the expression levels of the transcripts for the S and L CEACAM1 isoforms were determined by RT-triple-primer PCR as described in “Materials and Methods.”

<sup>c</sup> The data are given as mean ± SD for the indicated number of experiments.

<sup>d</sup> Pab, polyclonal antibody.

<sup>e</sup> These data are from one single experiment utilizing two different monoclonal antibodies against CEACAM1.
Relation of CEACAM1 Expression to Cell Proliferation. The striking differences in the CEACAM1 expression levels and isoform ratios between subconfluent and superconfluent cells suggested a correlation between the CEACAM1 expression pattern and cellular proliferation. To investigate this idea further, we determined the proliferative status of the cells by measuring both DNA synthesis and DNA content. As shown in Fig. 6, serum-starved cells did not synthesize DNA if not stimulated with growth factors. Ten percent FCS resulted in a large increase in thymidine incorporation in subconfluent but not in superconfluent NbE and NBT-II cells provided that the monolayers were intact without cell-free areas. Analysis of the DNA content confirmed that at 100% confl uency essentially, only cells in the G1-G0 phase occurred, whereas both NbE and NBT-II cells in 30 and 99% confluent cultures were actively cycling and contained high proportions of cells in both the S-phase and the G2-M phase (Fig. 7). Flow cytometric determination showed that all cells in subconfluent cultures of their respective cell lines had the same CEACAM1 expression levels regardless of DNA content (Table 3). Thus, proliferating cells in all phases of the cell cycle expressed different amounts of CEACAM1 compared with nonproliferating cells, which means that the switch in CEACAM1 expression levels occurred when the cells entered/exited the cell cycle from/to the quiescent G0 phase. This conclusion was supported by the finding that trypsin treatment of confluent cell layers, which altered the CEACAM1 expression pattern (Fig. 5 and Table 2), was accompanied by a more than 2-fold stimulation of DNA synthesis (data not shown) and that proliferating NbE and NBT-II cells exhibited similar CEACAM1 expression levels and isoform ratios, regardless of whether the cells were subconfluent or confluent.

CEACAM1 Influences p27Kip1 Expression and DNA Synthesis. The possibility of a functional association between the CEACAM1 expression pattern and cell proliferation prompted us to analyze whether cell surface-associated CEACAM1 can influence proliferation. To that end, we treated the cells with antibodies that perturb the CEACAM1 surface organization and determined the effects on the DNA synthesis in confluent cell layers. Mabs 5.4 (data not shown) and F7 (17) and polyclonal antibodies (Fig. 1, e and k) caused similar rearrangements of CEACAM1. Because the dome formation in confluent NbE cell layers made it more difficult to obtain these cells in a quiescent state and because antibodies did not have access to the lateral borders of confluent NbE cells, we restricted this analysis to the NBT-II cells. The addition of both polyclonal anti-CEACAM1 antibodies and Mab 5.4, which recognize the extracellular domain of CEACAM1, caused a 2-fold increase of the DNA synthesis in confluent NBT-II cells in the presence of 10% FCS, whereas control IgG had no effect (Fig. 8a). This stimulation required the presence of growth factors in the FCS, because serum-starved cells that were continuously incubated in 0.5% FCS did not respond with DNA synthesis when the anti-CEACAM1 antibodies were added (Fig. 8a). Thus, it appears that CEACAM1 can indeed regulate mitogenic signaling pathways in NBT-II cells.

To investigate the mechanism of the CEACAM1-regulated DNA
CEACAM1 participates in cell cycle regulation.

**DISCUSSION**

It is well known that cell adhesion molecules are expressed dynamically during embryonic development and that the expression of distinct adhesion molecules varies significantly in different states of cellular differentiation. However, not much is known about the expression dynamics in differentiated cells. Here, we demonstrate for the first time that both the expression levels and the isoform ratios of CEACAM1 vary considerably in proliferating and quiescent, differentiated epithelial cells. We also found that proliferating cells have a characteristic CEACAM1 expression pattern and that CEACAM1 interactions can contribute to maintaining a quiescent state of confluent cells.

**The CEACAM1 Expression Pattern of Quiescent Cells Reflects That of the Parental Tissues.** The cell lines that were used originated from normal prostatic epithelium (NbE cells) and from a bladder carcinoma (NBT-II cells), respectively. One advantage with these cells is that they express endogenous CEACAM1, and thus they are not subject to aberrant signaling effects that can occur by overexpression in cells that lack endogenous CEACAM1 production. The morphology and the surface location of CEACAM1 of confluent NbE cells were the same as in the columnar epithelial cells of normal prostate glands, which have the highest concentrations of CEACAM1 at their apical surfaces (35). Subconfluent NbE cells grew as colonies of incompletely polarized cells, with CEACAM1 distributed all over the surface and the highest concentrations at lateral borders of contacting cells. This resembles the situation in castrated rats, in which the prostate degenerates and the glandular epithelium transforms into less polarized cells, with CEACAM1 distributed on the entire cell surface (35). The lower CEACAM1 expression in confluent NBT-II cells compared with confluent NbE cells correlated with the expression levels in normal bladder mucosa and normal prostate (36, 37). Confluent NBT-II cells were seemingly less polarized than confluent NbE cells and had the highest CEACAM1 expression in the lateral borders. The low degree of polarization is a characteristic pattern of the cuboidal, deeper cells of the transitional epithelium of the bladder mucosa (38, 39), which suggests that NBT-II cells originate from these cells. Hence, it seems likely that the different expression patterns of CEACAM1 in confluent, quiescent cultures of the prostate epithelial cell line and the bladder carcinoma cell line reflect mainly the origins of these cells, although it cannot be ruled out that the strikingly different expression dynamics of the NbE and the NBT-II cells to some extent depends on the malignant transformation of the latter cell type.

**Proliferating Epithelial Cells Have a Characteristic CEACAM1 Expression Pattern.** The two cell lines grew as monolayers that showed the characteristic properties of contact-inhibited growth. Because they exhibited vastly different patterns of CEACAM1 expression in the quiescent state, it was surprising to find that actively proliferating cells of both types acquired almost identical CEACAM1 expression patterns concerning surface concentration, isoform ratio, and surface location. Because all factors and conditions that promoted cell proliferation caused the same characteristic CEACAM1 expression pattern, it seems likely that this expression pattern is functionally associated with and is important for the proliferative state of rodent epithelial cells. Similar expression patterns have also been observed in proliferating, regenerating hepatocytes (37, 40) and in proliferating placental trophoblasts (41). Furthermore, it recently was reported that isoforms of CEACAM1 carrying the L cytoplasmic domain were strongly up-regulated in activated human T cells, and it was demonstrated that CEACAM1 under these conditions acted as a costimulatory receptor that enhanced T-cell receptor-mediated, CD3-triggered cell proliferation (42). Taken together, these data indicate that certain expression levels and ratios of the two CEACAM1 isoforms, characterized by a relatively high expression of the L isoform, are compatible with and may even promote controlled cell proliferation.

**CEACAM1 Regulates Cell Proliferation.** That CEACAM1 under certain conditions indeed can regulate cell proliferation was demonstrated by the stimulation of DNA synthesis in confluent, quiescent NBT-II cells by antibody-induced cross-linking of CEACAM1. This effect occurred only in the presence of 10% FCS, whereas cells in nearly serum-free medium remained quiescent. Proliferation of subconfluent cells, on the other hand, was effectively stimulated by FCS without surface perturbation of CEACAM1. On the basis of these

**Fig. 8.** Effects of anti-CEACAM1 antibodies on DNA synthesis (a) and expression levels of cyclin E and p27Kip1 (b) in NBT-II cells. a, confluent cultures of NBT-II cells in 24-well plates were incubated with various antibodies in the presence of 0.5% FCS (open columns) or 10% FCS (filled columns), and [3H]thymidine incorporation was determined as described in “Materials and Methods.” Means of duplicate incubations are given; bars, ranges. b, confluent NBT-II cells were incubated with Mabs for 3, 6, or 12 h in the presence of 10% FCS as described in “Materials and Methods.” Control cells were incubated for 12 h without antibodies. The expression of p27Kip1 (top) and cyclin E (bottom) was analyzed by immunoblotting as described in “Materials and Methods.” Control, medium without antibodies; Sub-1, mouse Mab that does not recognize rat CEACAM1; Mab 5.4, mouse Mab against rat CEACAM1; IgG, preimmune rabbit IgG; αCC16, polyclonal rabbit antibody against rat CEACAM1.
results, we suggest that cell surface-localized CEACAM1 in confluent cells inhibits growth factor-induced proliferation. Because the perturbation of CEACAM1 led to altered protein levels of the cyclin-dependent kinase inhibitor p27^Kip1, this effect presumably involves regulation of the activity of the cyclin E-dependent kinase cdk2.

The influence of CEACAM1 on proliferation of confluent cells suggests a role for CEACAM1 in contact inhibition. Contact inhibition of cell proliferation is a phenomenon that apparently can be mediated by several different cell-cell recognition mechanisms. It has, for example, been found that both N-CAM (43) and E-cadherin (44), as well as hyaluronan-mediated cell aggregation (44, 45), can contribute to contact inhibition of cell proliferation. N-CAM (46) and E-cadherin (44) have been demonstrated to interact with distinct growth factor receptors, and both E-cadherin- and hyaluronan-dependent growth arrest involves regulation of p27^Kip1. In the case of E-cadherin, it was found that this effect was due to an inhibition of the activity of the epithelial growth factor receptor, which when active cause down-regulation of p27^Kip1 expression. Thus, E-cadherin and CEACAM1 act in a similar manner in that they seem to regulate growth factor receptor-induced signaling pathways that influence p27^Kip1. Interestingly, many epithelial cells express both E-cadherin and CEACAM1. It will therefore be important to investigate in future analyses the mechanisms of how these two cell adhesion systems influence cellular proliferation and how they might interact.

Mechanisms for Signal Regulation by CEACAM1. There are at least two possibilities for how antibody perturbation could lead to altered cell proliferation. This could be a direct effect on growth factor receptor-induced signaling because of altered activity of the surface-located CEACAM1 molecules to which the antibodies bind. Such altered activity might release putative inhibitory effects on growth factor receptor-triggered signaling. Alternatively, it could be the result of a more indirect effect that has to do with the altered expression levels and isoform ratios of CEACAM1 that are caused by the antibodies. A change of CEACAM1 expression toward the pattern seen in proliferating cells might result in a release of the inhibitory effects, or even in a stimulatory effect, on growth factor receptor-induced signaling. It remains to be seen whether the regulatory effects of CEACAM1 on growth factor receptor activity and on CEACAM1 expression involve the same or distinct signaling pathways.

Clearly, interactions of the extracellular domain of CEACAM1 are important because antibodies added to the cells could release the growth inhibition. The extracellular domain interactions probably regulate the activities of the intracellular cytoplasmic L domain, which contains phosphorylatable tyrosine residues. It has been demonstrated that an intact L domain is necessary for the tumor-inhibitory properties of CEACAM1. In the context of contact inhibition, it is interesting to note that tyrosine-phosphorylated CEACAM1-L can recruit and activate the protein tyrosine phosphatases SHP1 and SHP2 (14) because increased plasma membrane-associated tyrosine phosphatase activity is a hallmark of contact-inhibited cells (47). Furthermore, it was shown that tyrosine phosphatases are involved in E-cadherin-mediated growth arrest (44), and it was suggested that this would cause dephosphorylation and deactivation of the epithelial growth factor receptor. Activation of SHP1/SHP2 by CEACAM1 might have similar consequences, or it might alternatively lead to altered CEACAM1 expression, which might interfere indirectly with cell cycle regulation, as discussed above.

However, tyrosine-phosphorylated CEACAM1-L can also bind and activate src-family tyrosine kinases (11, 12). A key question, therefore, is when and under what conditions tyrosine-phosphorylated CEACAM1 binds to and activates tyrosine kinases and when it binds to and activates tyrosine phosphatases. We have suggested that such a differential binding and activation of kinases or phosphatases could be influenced by the dimerization status of CEACAM1 (3, 48), such that monomeric and dimeric L domains preferentially bind and activate either phosphatases or kinases, respectively. We have found that the dimerization of CEACAM1 in NBT-II cells is regulated by the intracellular calcium concentration and by binding of calmodulin to the cytoplasmic domains of CEACAM1 (16). Furthermore, CEACAM1 monomers and dimers are in equilibrium with each other (16); therefore, dimerization is also regulated by both the CEACAM1 expression levels and the isoform ratios (3). We have also postulated that CEACAM1 dimerization might be regulated by CEACAM1-mediated cell contact formation as a result of homophilic binding between the extracellular domains of CEACAM1 (3). In such a scenario, CEACAM1 in subconfluent cells, which are characterized by a particular CEACAM1 expression pattern and low abundance of cell-cell contacts, might preferentially activate tyrosine kinases and allow cell proliferation, whereas CEACAM1 in superconfluent cells, with a different CEACAM1 expression pattern and high abundance of cell-cell contacts, might preferentially activate tyrosine phosphatases and inhibit cell proliferation.

CEACAM1 Expression in the Malignant State. The tumor growth inhibitory effect of CEACAM1 might be due to its contact-inhibiting properties. This is in agreement with the observed down-regulation of CEACAM1 expression in a variety of human, mouse, and rat carcinomas (19–23), but is in apparent conflict with reports that some human cancers have unaltered or even increased expression levels of CEACAM1 (32–34). However, in light of the present results, it is clear that certain expression levels and isoform ratios of CEACAM1 allow and might even stimulate cell proliferation. Therefore, it seems plausible that tumors that express CEACAM1 have different expression levels and isoform ratios than normal, nonproliferating tissues. Accordingly, it will be important to characterize factors that regulate CEACAM1 expression in malignant tumors. It has been found that IFN-γ induces CEACAM1 expression in human colon carcinoma cells (49) and that fibroblast growth factor-1 influences CEACAM1 expression in NBT-II cells (17). One component that affects tumor growth is the cells of the tumor stroma; therefore, the effects of tumor stromata on CEACAM1 expression should be investigated. In this context, it is interesting to note that NbE cells injected alone into nude mice do not form tumors, but when injected together with malignant, bladder-derived mesenchymal cells, they form typical carcinomas (30). This could be due to induction of altered CEACAM1 expression, which indeed has been demonstrated to result in formation of malignant tumors from NbE cells (25).

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The Tumor Growth-inhibiting Cell Adhesion Molecule CEACAM1 (C-CAM) Is Differently Expressed in Proliferating and Quiescent Epithelial Cells and Regulates Cell Proliferation

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