

# Episialin (MUC1) Overexpression Inhibits Integrin-mediated Cell Adhesion to Extracellular Matrix Components

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**Abstract.** Episialin (MUC1) is a transmembrane molecule with a large mucin-like extracellular domain protruding high above the cell surface. The molecule is located at the apical side of most glandular epithelial cells, whereas in carcinoma cells it is often present at the entire surface and it is frequently expressed in abnormally large quantities. We have previously shown that overexpression of episialin reduces cell-cell interactions. Here we show that the integrin-mediated adhesion to extracellular matrix of transfectants of a melanoma cell line (A375), a transformed epithelial cell line (MDCK-*ras-e*) and a human breast epithelial cell line (HBL-100) is reduced by high levels of episialin. This reduction can be reversed by induc-

ing high avidity of the  $\beta 1$  integrins by mAb TS2/16 (at least for  $\beta 1$ -mediated adhesion). The adhesion can also be restored by redistribution of episialin on the cell surface by monoclonal antibodies into patches or caps. Similarly, capping of episialin on ZR-75-1 breast carcinoma cells, growing in suspension, caused adherence and spreading of these cells.

We propose that there is a delicate balance between adhesion and anti-adhesion forces in episialin expressing cells, which can be shifted towards adhesion by strengthening the integrin-mediated adhesion, or towards anti-adhesion by increasing the level of expression of episialin.

**E**PIESIALIN (also known as PEM, EMA, CA 15-3 antigen, etc.), encoded by the MUC1 gene, is a sialylated transmembrane glycoprotein with a large mucin-like domain consisting of 20-amino acid repeats which are rich in serines, threonines, and prolines (Ligtenberg et al., 1990; Wreschner et al., 1990; Gendler et al., 1990; Lan et al., 1990). The number of repeats varies from about 30 to 90 as a result of genetic polymorphism, causing a variation in the length of the extracellular domain of 1,000 to 2,200 amino acids. Because of the large number of proline residues, it has been suggested that the extracellular domain has an extended structure, which becomes rigid as a result of the addition of O-linked carbohydrate side chains (Hilkens et al., 1992). In a mucin-like domain the average length of a sequence of 20-amino acid residues is approximately 5-nm long (Jentoft, 1990). We and others have determined by electron microscopy that episialin protrudes 200–500 nm above the plasma membrane (Bramwell et al., 1986; Calafat, J., C. Patriarca, H. Janssen, H. Peterse, and J. Hilkens, manuscript submitted for publication). In contrast, the extracellular domain of most other membrane molecules, e.g., cell adhesion molecules, does not extend more than 30 nm above the plasma membrane (Hynes, 1992; Becker et al., 1989).

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Episialin is present at the apical side of most glandular epithelial cells. In carcinoma cells, in which polarized expression of the molecule is often lost, the level of expression can be increased more than 10-fold as compared to their normal counterparts (Hilkens et al., 1984; Zaretsky et al., 1990). We have previously established that overexpression of episialin in human melanoma cells (A375) and normal mammary epithelial cells (HBL-100) results in a decreased ability of the cells to aggregate (Ligtenberg et al., 1992a). We have proposed that a high level of expression of this extended molecule could hamper interactions with macromolecular structures on other cells (Hilkens et al., 1992). In the present report we show that high levels of episialin are also able to reduce integrin-mediated extracellular matrix (ECM)<sup>1</sup> interactions, and that the adhesion can be restored by activation of the appropriate integrins or by capping of episialin.

## Materials and Methods

### Cells

A375 human melanoma cells, *ras*-transformed Madin-Darby canine kidney

1. **Abbreviations used in this paper:** cDNA, complementary DNA; Col I, collagen I; Col IV, collagen IV; ECM, extracellular matrix; FN, fibronectin; GAM-FITC, goat anti-mouse fluorescein isothiocyanate; GARa-FITC, goat anti-rat fluorescein isothiocyanate; LM, laminin; mAb, monoclonal antibody; MG, Matrigel.

cells showing epithelial morphology (MDCK-*ras-e*; a kind gift from M. Mareel, Ghent, Belgium) (Behrens et al., 1989; Vlemminckx et al., 1991), HBL-100 human breast epithelial cells and ZR-75-1 breast carcinoma cells were grown in DME supplemented with 10% FCS. The transfectants of the A375 cell line and the HBL-100 cell line have been described previously (Ligtenberg et al., 1992a). The MDCK-*ras-e* cell line was transfected with the full length cDNA encoding episialin as has been described before (Ligtenberg et al., 1992a). From each cell line two independent clones with a high level of episialin expression were selected. As negative controls, parental cells, revertant cells, cells only transfected with the neomycin resistance gene or cells transfected with the vector containing the episialin cDNA in reverse orientation were used. Revertant cells lacking episialin were bulk selected from the positive transfectants by two to five cycles of cell sorting using the FACStar (Becton Dickinson & Co., Mountain View, CA). Cells were stained with mAb 139H2 or mAb 214D4 in an indirect immunofluorescence assay. Bulk selection was performed to avoid clonal selection. The transfectants are indicated by a "+" and the revertants by a "-".

The ZR-75-1 cells were cultured in two ways: passaging only the adherent cells and passaging both cells in suspension and adherent cells resulting in the ZR-75-1S cell line.

### Immunofluorescence

All antigens were detected by indirect immunofluorescence using FITC-labeled secondary antibodies unless otherwise stated. The fluorescence intensity was determined by FACScan analysis.

### Antibodies and Extracellular Matrix Components

mAb TS2/16 (which activates  $\beta$ 1-integrins) (Arroyo et al., 1992) was generously provided by Dr. Sánchez-Madrid (Universidad Autonomas de Madrid, Madrid, Spain). mAb AIB2 (anti- $\beta$ 1) (Werb et al., 1989) was a kind gift from Dr. C. H. Damsky (University of California, San Francisco, CA). mAb C17 (anti- $\beta$ 3) (Tetteroo et al., 1983) was kindly provided by L. Admiraal (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). mAbs J8H and GoH3 are directed against the integrin  $\alpha$ 6 subunit (Hogervorst et al., 1993; Sonnenberg et al., 1990). mAbs 139H2 (Hilkens and Buijs, 1988) and 214D4, which are both IgG1, are directed against the mucin-like domain of episialin. The mAb 232A1 (IgG1) is directed against the non-repeat part of episialin just upstream of the transmembrane region. Either mAb 123C3 (directed against the neural cell adhesion molecule; N-CAM) (Moolenaar et al., 1990) or normal mouse serum were used as a negative control. The optimal mAb dilutions were determined by FACScan analysis. F(ab)<sub>2</sub> and Fab fragments of mAb 214D4 were prepared by pepsin and papain digestion, respectively (Harlow and Lane, 1988). Pepsin and papain were purchased from Pierce Chemical Co. (Rockford, IL). mAbs 214D4 and GoH3 were labeled with FITC or Texas red according to the instructions of the supplier (Molecular Probes, Eugene, OR).

Fibronectin (FN) and collagen I (Col I) were purchased from Boehringer Mannheim (Mannheim, Germany), collagen IV (Col IV), laminin (LM), and matrigel (MG), which contains all major basement membrane components, were purchased from Collaborative Biomedical Products (Becton Dickinson Labware, Bedford, MA). Poly-L-Lysine hydrobromide (average mol wt  $\pm$  400,000) was purchased from Sigma Chem. Co. (St. Louis, MO).

### Cell Adhesion Assays

FN and LM were diluted in PBS, Col I and Col IV in 0.1 M acetic acid and MG in Hepes-buffered DME. Individual wells of microtiter plates (96 wells; Greiner GmbH, Trickenhausen, Germany) were coated with 100  $\mu$ l buffer containing the ECM components or mAbs at the following concentrations (unless otherwise stated): FN, 20  $\mu$ g/ml; LM, 20  $\mu$ g/ml; Col I, 20  $\mu$ g/ml; Col IV, 40  $\mu$ g/ml; MG, 50  $\mu$ g/ml; mAbs GoH3, TS2/16; and J8H, 10  $\mu$ g/ml for 12–16 h at 4°C. These components in these concentrations produced maximal binding of the cell lines tested. Subsequently, the wells were washed once with PBS and incubated with 1% BSA at room temperature for 1 h to block remaining protein binding sites.

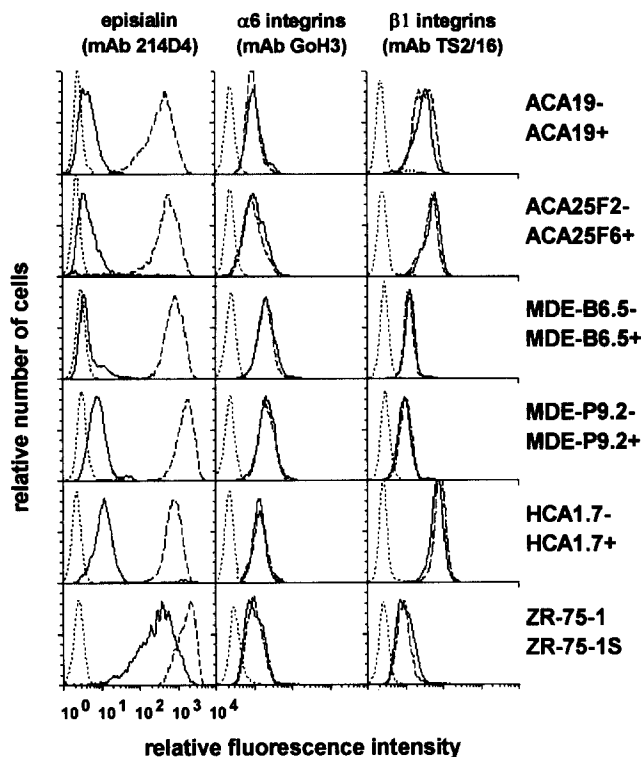
Cells from subconfluent, exponentially growing cultures were detached from the flask by a short treatment (<5 min) with 0.25% trypsin and 0.02% EDTA in PBS. This treatment did not affect episialin and integrin expression by any significant degree as tested by FACScan analysis. The cells were washed in DME containing 25 mM Hepes and 0.25% BSA (DHB) and finally resuspended in DHB at a concentration of  $1 \times 10^7$  cells/ml. The cells were radiolabeled with <sup>51</sup>Cr using 100  $\mu$ Ci/0.1 ml cell suspension, incubated at 37°C for 1 h, washed twice with DHB, and finally resuspended

at a concentration of  $0.5-1 \times 10^5$  cells/ml. Viability was checked by trypan blue staining. No experiments were performed when the viability of the cells had dropped below 95%. 0.1-ml aliquots of this suspension were added to individual substrate-coated wells and incubated at 37°C for 30 min. Nonadherent cells were removed by washing with PBS containing 1 mM Ca<sup>2+</sup> and 1 mM Mg<sup>2+</sup>. Bound cells were lysed with 2% SDS in 0.01 M NaOH and the amount of radioactivity in the lysate was determined in a gamma counter. After correction for spontaneous <sup>51</sup>Cr release, the number of adherent cells was calculated as the percentage of the total number of cells added per each well. Each assay was performed in triplicate and repeated at least three times. One representative experiment is shown.

To determine the effects of various mAbs on binding of the cells to the ECM components, labeled cells were preincubated with appropriate dilutions of the mAbs at 37°C for 15 min before addition to the wells.

### Generation of Mutant cDNA Lacking the Endodomain of Episialin

For deletion of the cytoplasmic tail of episialin, stopcodons were introduced just downstream of the region encoding the transmembrane domain using the PCR. The oligonucleotide 5'-AGTCTGGGGCTGGGGTG-3', which corresponds to a sequence located about 60-nucleotides upstream of the region encoding the transmembrane domain, was used as the upstream primer. The downstream oligonucleotide 5'-(GCCGTGG)CCAGCTACCC-TTAGTTGTTTCGG-3' was used to introduce two stopcodons (double underlined) by mutating two residues (bold typeface). The sequence between parentheses was added to the oligonucleotide to obtain a MscI restriction site (underlined) that facilitated cloning. The mutations and the generated



**Figure 1.** Expression level of episialin and integrin subtypes on various cell lines as determined by FACScan analysis. The solid lines represent the episialin negative revertant cell lines (for ZR-75-1 cells: the adherent variant) and the dashed lines the episialin positive transfectant cell lines (for ZR-75-1 cells: the suspension variant ZR-75-1S). The negative control is represented by the dotted line. mAb 214D4 was used to detect episialin. mAbs GoH3 and TS2/16 were used to detect  $\alpha$ 6 and  $\beta$ 1 integrins, respectively. Note that mAb TS2/16 also detects the  $\beta$ 1 integrins on the canine MDE cells. No significant difference in the expression level of integrins was detected. One out of three experiments is shown.

MscI site in the PCR product were checked by sequencing. The resulting MscI fragment was used to replace the 3' MscI fragment of the full-length cDNA encoding episialin present in the pGEM3Z vector (Ligtenberg et al., 1992b). In this way, the 195 nucleotides encoding the COOH-terminal 65 amino acids, were deleted without affecting the stop-transfer sequence (Arg-Arg-Lys) and the first amino acid of the cytoplasmic tail (Asn).

### Immunoprecipitation

Cells were lysed and episialin was immunoprecipitated with mAb 232A1 which is directed against a non-repeat domain just upstream of the transmembrane domain. Following standard SDS-PAGE (Laemmli, 1970), the antigens were blotted onto nitrocellulose. The mucin domain was detected by using mAb 139H2 and the cytoplasmic domain with polyclonal antiserum directed against the cytoplasmic domain of episialin (Ligtenberg et al., 1992b).

### Confocal Laser Scanning Microscopy

Detached cells were resuspended in Hepes-buffered DME at a concentration of  $4 \cdot 10^6$  cells/ml. MAb, either conjugated with FITC or Texas red, was added and the suspension was shaken at 37°C in a waterbath for 15 min. Control samples were incubated at 0°C. Subsequently, the cells were washed and resuspended in PBS at a concentration of  $10^5$  cells/ml. The cells were added to poly-L-lysine-coated slides and allowed to attach for 10 min. Non-attached cells were removed by rinsing with PBS and the attached cells were fixed using 2% paraformaldehyde in PBS for 10 min. The slides were gently rinsed in PBS with three changes over 5 min. A droplet of Vectashield (Vectashield Laboratories, Inc., Burlingame, CA) was added to each specimen to prevent rapid quenching of the fluorescence. Adherent growing cells were fixed with 2% paraformaldehyde in PBS for 10 min and permeabilized with 1% Triton X-100 and further treated as described above.

All incubations with antibodies were performed at room temperature for 60 min. Antibodies were diluted in PBS containing 1% BSA. Immunofluorescence was visualized by Confocal Laser Scanning Microscopy (Bio-Rad Laboratories, Herfordshire, UK).

## Results

### Expression Level and Growth Pattern of Episialin-transfected Cells

To examine whether episialin interferes with the adhesive properties of cells, we transfected full-length cDNA for episialin into the human A375 melanoma, human normal mammary gland HBL100 and the canine MDCK-*ras-e* cells. These cell lines were chosen for transfection, because they differ in adhesiveness (see below) while they express no episialin or at very low levels.

FACScan analysis of two representative transfected cell clones of each cell line showed that episialin is strongly expressed on the surface of these cell clones and that the levels of episialin are comparable to those found on the breast carcinoma cell line ZR-75-1 (Fig. 1). The effects of high levels of episialin on attachment became immediately clear for the A375 cells, as a large proportion of the transfected cells no longer attached to the culture dish and grew in suspension (Fig. 2). Neither the parental cells nor the cells transfected with only the neomycin resistance gene or the episialin

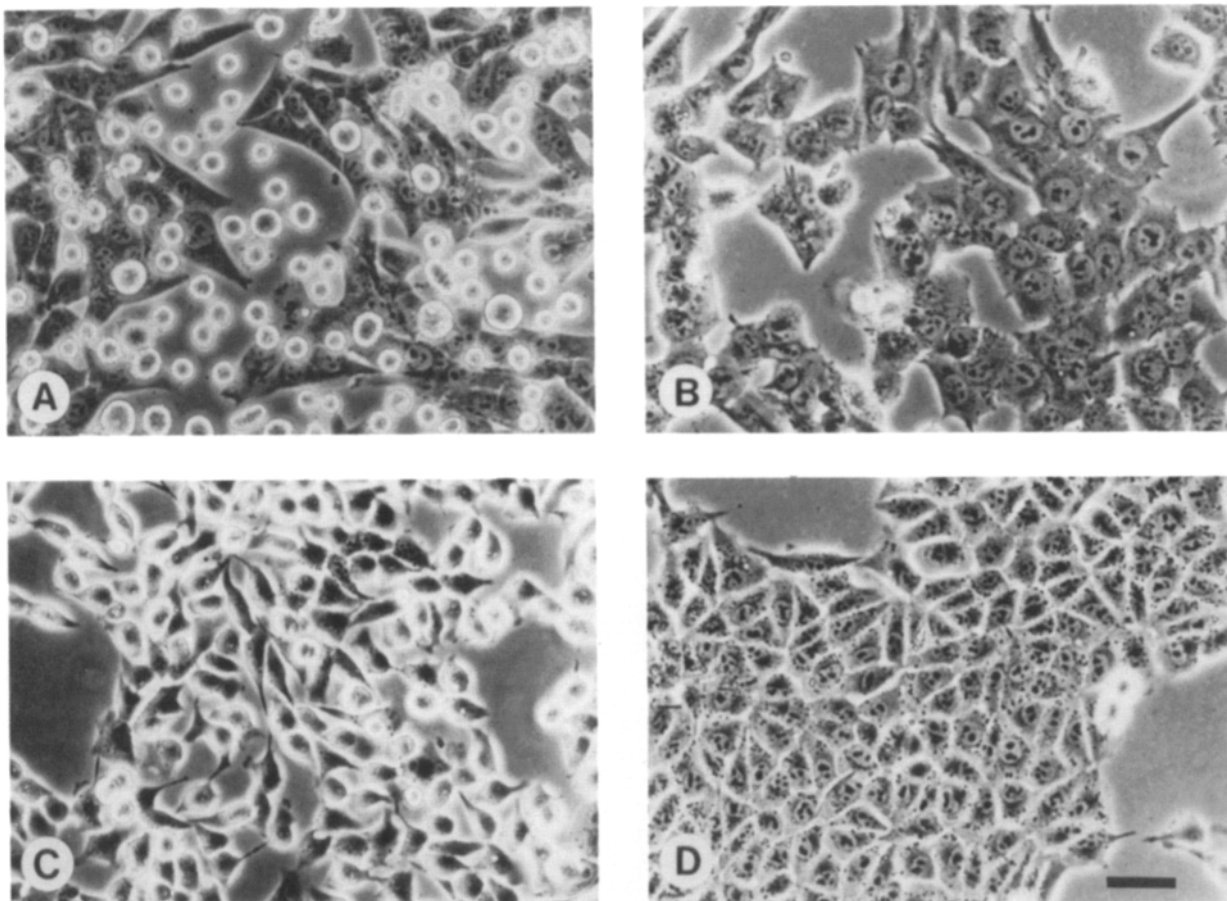


Figure 2. Growth pattern of episialin transfectant and revertant cell lines of A375 and MDCK-*ras-e*. Transfectants and revertants were cultured under standard conditions and are shown in the exponential growth phase. Expression of episialin affects the morphology of the cells. (A) ACA19<sup>+</sup>, (B) ACA19<sup>-</sup>, (C) MDE-P9.2<sup>+</sup>, and (D) MDE-P9.2<sup>-</sup>. Bar, 20  $\mu$ m.

cDNA in the reverse orientation showed this kind of growth behavior. In the MDCK-*ras-e* cells, episialin did not as clearly induce the growth in suspension, but rather affected the morphology of the cells. Specifically, the transfected MDCK-*ras-e* cells acquired a more fibroblastic morphology and failed to form epithelial sheets (Fig. 2). There was little or no detectable effect of episialin on the morphology or growth characteristics of HBL-100 cells (not shown). To exclude the possibility that we had selected cell clones of A375 and MDCK-*ras-e* cells with aberrant adhesive properties, revertant cells lacking episialin (indicated by a “-”) were isolated from the transfectants (indicated by a “+”) by cell sorting (with the exception of ACA25F2<sup>-</sup> which was obtained by cloning). Neither revertants nor parental cells expressed episialin at a significant level (Fig. 1), and indeed none of the revertants showed an aberrant growth pattern. Consistent with this, we found that the level of expression of integrins (only  $\alpha 6$  and  $\beta 1$  are shown) on the transfectants and revertants was not altered. Thus, it seems unlikely that accidental selection of aberrantly growing cell clones or the transfection process itself are responsible for the reduced adherence of the transfected A375 and MDCK-*ras-e* cells, and it can be concluded that the expression of episialin was responsible for this effect.

### Episialin Overexpression Affects Cell-ECM Interactions

To investigate the effect of episialin overexpression on the adhesiveness of these cells in more detail, the transfectants and revertants were compared for their ability to adhere to purified matrix components (laminin, fibronectin, collagen type I and IV, and Matrigel) using a <sup>51</sup>Cr-monitored cell adhesion assay. As shown in Fig. 3, adhesion to all different matrix proteins of both the A375 (ACA19<sup>+</sup> and ACA25F6<sup>+</sup>) and the MDCK-*ras-e* (MDE-B6.5<sup>+</sup> and MDE-P9.2<sup>+</sup>) transfectants was substantially reduced as compared with their revertants. It is noteworthy that the effect of episialin expression on cell adhesion of A375 cells is greater than that of MDCK-*ras-e* cells. This is most likely due to the fact that

non-transfected A375 cells adhere more weakly to matrix proteins than non-transfected MDCK-*ras-e* cells. Similarly, in the case of HBL100 cells which adhere the most strongly to matrix proteins, there was only a slight difference in adhesion between transfected and non-transfected cells. However, that adhesion was indeed weakened by episialin is shown by a greater requirement for matrix proteins of the episialin-positive transfectants than of the episialin-negative revertants to produce the same level of adhesion. For example, the coating concentration of laminin to yield 50% maximal adhesion was twofold higher for the transfected cells than for the revertants (Fig. 4). There was no difference in the expression of  $\alpha 6$  integrins (Fig. 1), which mediate the adhesion of these cells to laminin (Sonnenberg et al., 1990), between the transfectants and revertants. We conclude from these results that the ability of the three cell lines to interact with matrix proteins is reduced by the expression of episialin.

### Episialin Affects Integrin-mediated Adhesion

To verify the involvement of integrins in the adhesion of the episialin-negative revertants of A375 cells (ACA19<sup>-</sup>) to each ligand, antibody inhibition experiments were performed. As shown in Fig. 5 A the anti- $\beta 1$  mAb AIIB2 completely inhibited the attachment of the ACA19<sup>-</sup> cells to laminin and collagen types I and IV, while inhibition of adhesion to fibronectin was only partial. Combining the anti- $\beta 1$  with an anti- $\beta 3$  mAb (C17), however, resulted in an almost complete inhibition of attachment to fibronectin. Although adhesion to Matrigel was also partially inhibited by AIIB2, it was not further decreased by combining the AIIB2 mAb with C17. This indicates that in addition to  $\beta 1$  integrins, other, non- $\beta 3$  integrin receptors are involved in adhesion to Matrigel which, considering the complex mixture of adhesive glycoproteins present in Matrigel, is not unexpected. Furthermore, the results are consistent with  $\beta 1$ -mediated adhesion to laminin and collagen types I and IV, and both  $\beta 1$ - and  $\beta 3$ -mediated adhesion to fibronectin. As in the experiments shown in Fig. 3, also in these experiments, the episialin-positive A375 cells (ACA19<sup>+</sup>) did not bind to the various

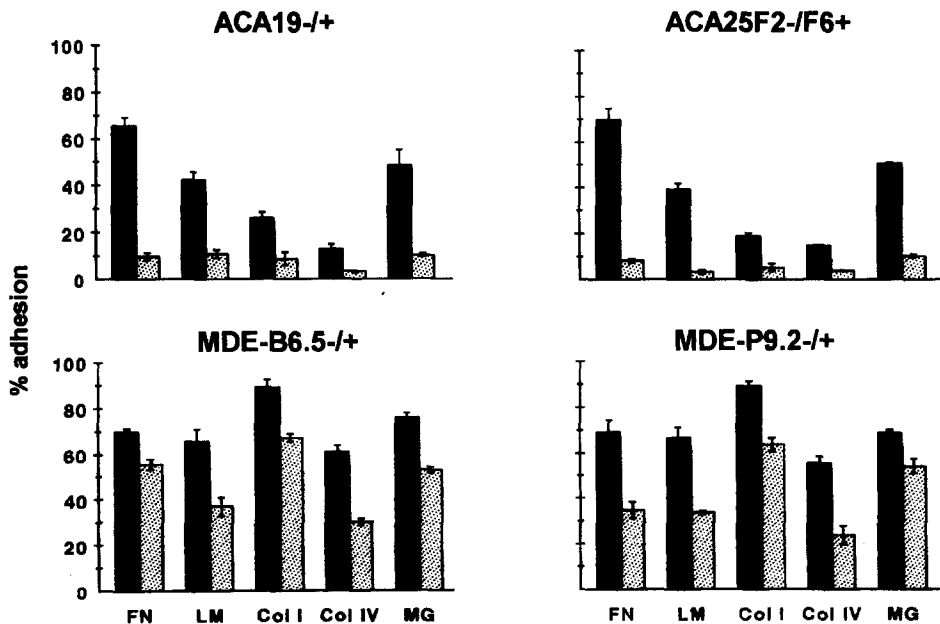
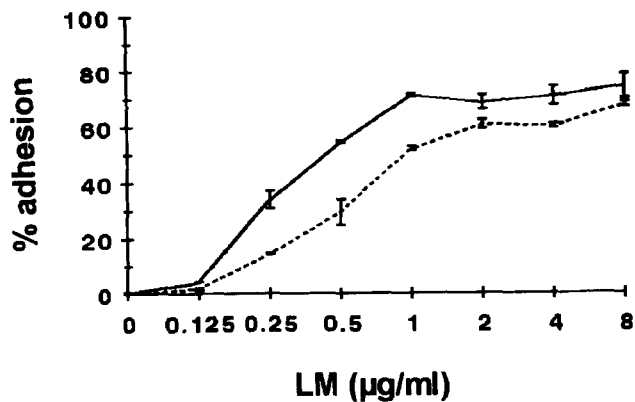


Figure 3. Adhesion of transfectant and revertant cells to various ECM components. The number of ACA19, ACA25F, MDE-B6.5, and MDE-E9.2 transfectant and revertant cells that bound to fibronectin, laminin, collagen I, collagen IV, and Matrigel is depicted as percentage of the total number of cells added per well. The cells were allowed to adhere for 30 min at 37°C. Solid bars represent the revertant cell lines, whereas the transfectant cell lines are represented by dotted bars. Percentage of adhesive cells were determined in triplicate. Error bars indicate the standard deviation. One out of four representative experiments is shown. Other sets of transfectants and revertants showed comparable results.



**Figure 4.** Adhesion of the HCA1.7 transfectant and revertant cell lines to laminin coated with different concentrations. The number of cells bound is depicted as percentage of the total number of cells added per well. The episialin-negative revertant is represented by the solid line, whereas the episialin-positive transfectant is represented by the dashed line. Percentages of adhesive cells were determined in triplicate. Error bars indicate the standard deviation.

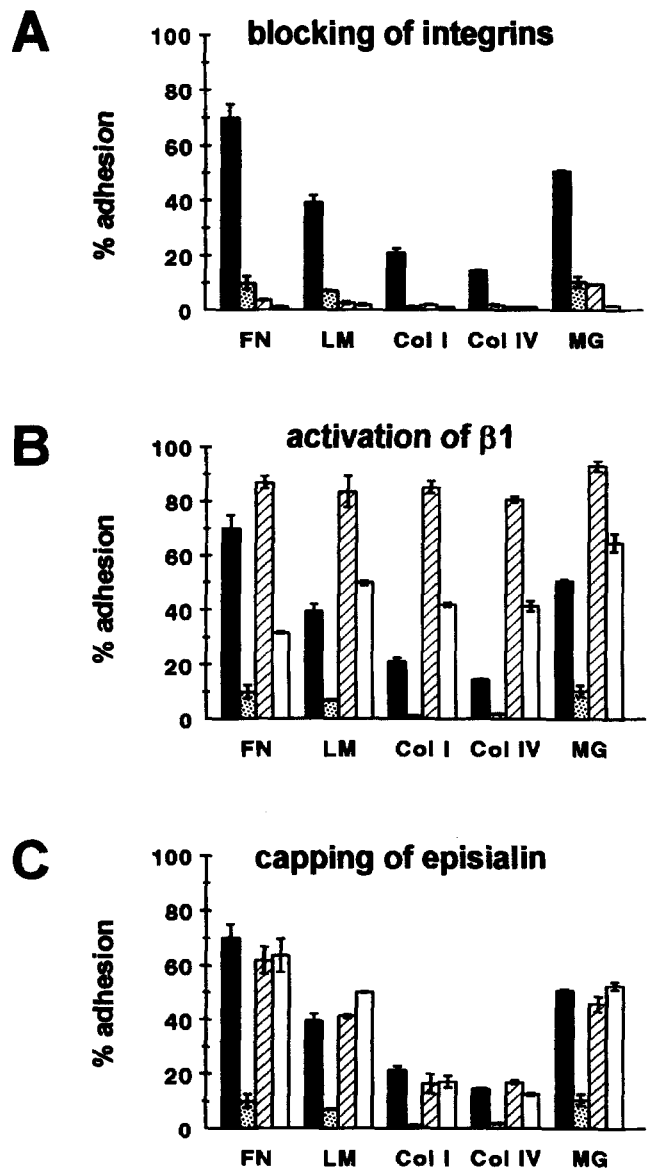
matrix proteins. Altogether, these data show that adhesion of episialin-negative revertants indeed is mediated by integrins and thus that overexpression of episialin affects integrin-mediated cell-matrix interactions.

#### Activation of $\beta 1$ Integrins Reverses the Anti-Adhesion Effect of Episialin

In a previous study, it has been shown that the adhesive activity of  $\beta 1$  integrins on A375 cells is low but can be readily increased by treatment of these cells with the anti- $\beta 1$  stimulatory antibody, TS2/16 (Arroyo et al., 1992). Since the episialin-positive A375 transfectants hardly adhered to the different matrix proteins, it was of interest to investigate whether they could be induced to adhere much better by adding mAb TS2/16. In Fig. 5 B it is shown that adhesion of the episialin-negative A375 revertants (ACA19<sup>-</sup>) to all substrates was enhanced to near maximal levels after stimulation with mAb TS2/16. These results are consistent with the notion that the  $\beta 1$  integrins on these cells are in a low state of activation. When TS2/16 was used to activate the episialin-positive A375 transfectants (ACA19<sup>+</sup>), adhesion was induced to comparable levels as for the untreated revertant cells, except for adhesion to fibronectin because this is mainly  $\beta 3$  mediated. That the TS2/16-stimulated transfectants did not adhere as strongly as the stimulated revertants is likely due to episialin. These results show that the negative effect of episialin on cell adhesion is not only dependent on its level of expression, but also on the activation state of the integrins.

#### The Anti-adhesion Effect of Episialin can be Reversed by Cross-linking of the Episialin Molecules

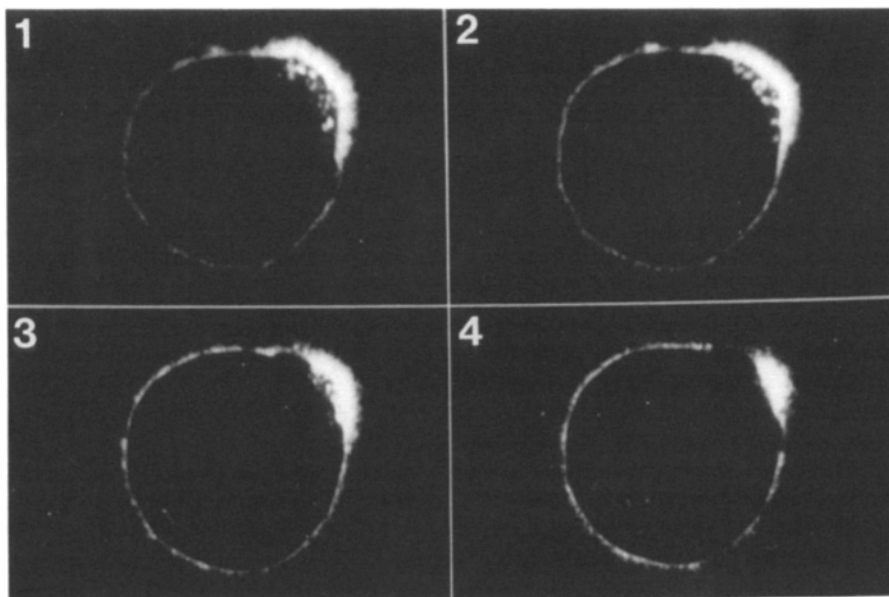
To obtain direct evidence that overexpression of episialin affects adhesion by preventing the interaction between integrins and the ECM components by steric hindrance, transfectants and revertants were preincubated with mAb 214D4 directed against the repeat domain of episialin. This mAb recognizes multiple epitopes on each episialin molecule as a result of the repetitive nature of the protein backbone, lead-



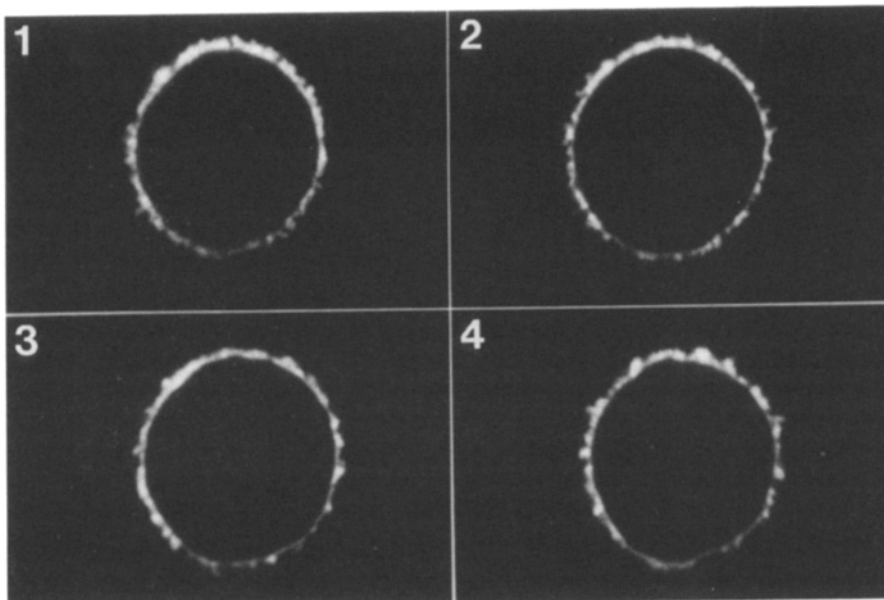
**Figure 5.** Inhibition or induction of integrin-mediated adhesion by antibodies against integrin subunits or against episialin. ACA19-transfectant and -revertant cells were preincubated with antibody for 15 min at 37°C. Subsequently, the cells were allowed to adhere for 30 min at 37°C. The number of cells bound is depicted as percentage of the total number of cells added per well. Percentages of adhesive cells were determined in triplicate. Error bars indicate the standard deviation. The bars represent the cell lines as follows: ■, untreated revertant cells; ▒, untreated transfectant cells; ▨, revertant cells treated with mAb; □, transfectant cells treated with mAb. Other sets of A375 transfectants and revertants showed comparable results. (A) mAb AIIB2 (blocking  $\beta 1$  integrin interactions) was used for blocking adhesion to LM, Col I, Col IV, and MG; mAbs C17 (blocking  $\beta 3$  integrin interactions) and AIIB2 were used for blocking the adhesion to FN. (B) mAb TS2/16 was used to activate  $\beta 1$  integrins. (C) mAb 214D4 was used to induce capping of episialin resulting in a higher level of adhesion.

ing to clustering of the episialin molecules at the cell surface in multiple patches or as one cap (Fig. 6 A). No cross-linking of caps between individual cells was observed.

When mAb 214D4 is added to the culture medium of



A

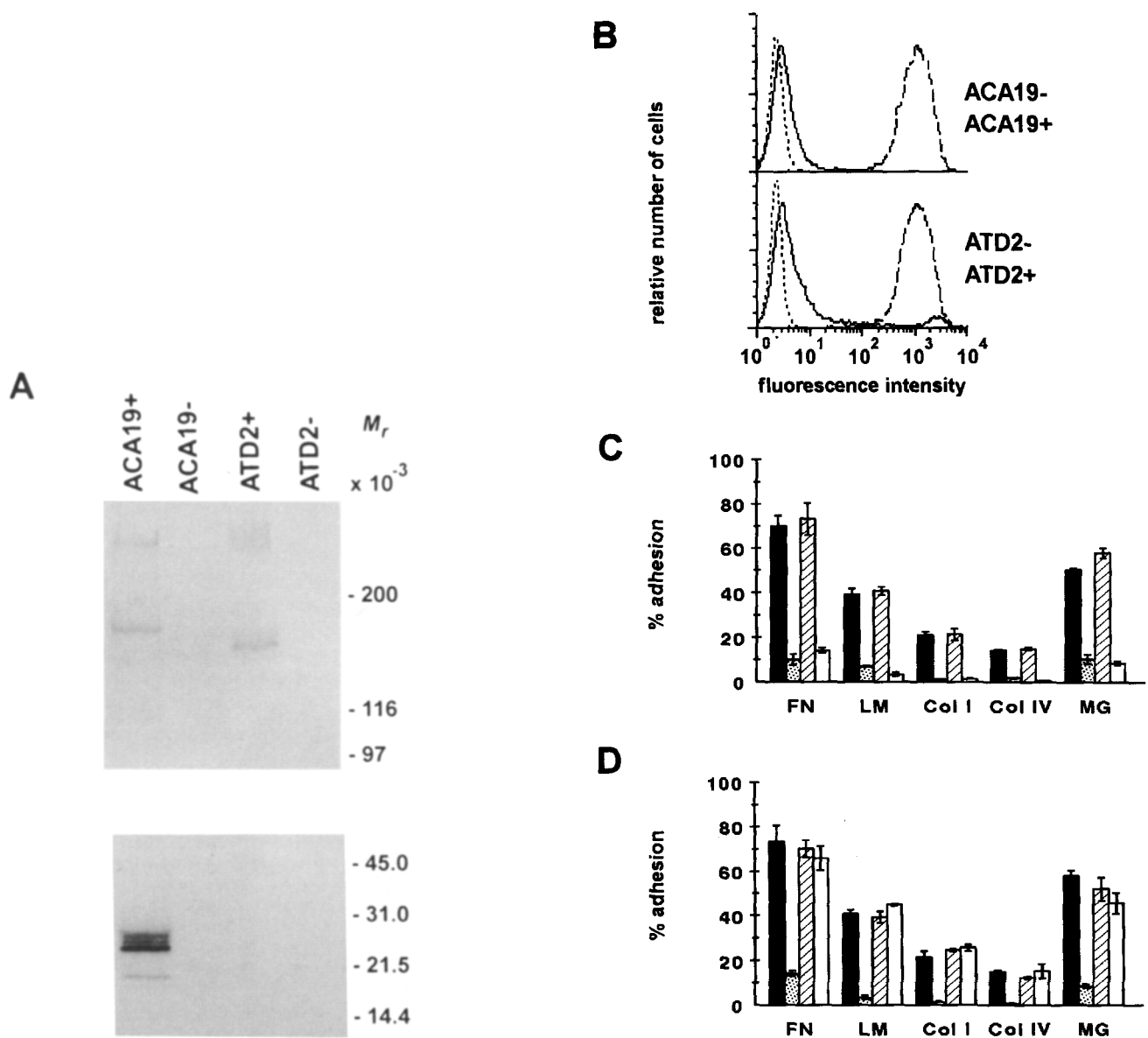


B

*Figure 6.* Capping of episialin on ACA19<sup>+</sup> cells after incubation with FITC-labeled mAb 214D4. Confocal laser scan photographs are shown of ACA19<sup>+</sup> cells incubated with FITC-labeled mAb 214D4 at 37°C (A), or at 0°C for 15 min (B). Each panel shows the same cell at four confocal planes 5- $\mu$ m apart.

ACA19<sup>+</sup> cells, of which a large proportion are growing in suspension, almost all of the free, floating cells attached to the plastic of the culture flask within 1 h, whereas the irrelevant mAb 123C3 had no effect. Adhesion to individual ECM components was also restored after addition of the mAb (Fig. 5 C). The same result was obtained after incubation of the cells with 214D4 (Fab')<sub>2</sub> fragments, but not by 214D4 Fab fragments (data not shown). The monovalent Fab fragments cannot cross-link the episialin molecules, which seems to be required for abolishing the anti-adhesion effect of episialin. Capping did not occur if ACA19<sup>+</sup> cells were incubated with mAb 214D4 at 0°C (Fig. 6 B). Treatment with

mAb 232A1, directed against a non-repeated extracellular epitope of episialin, did not induce capping or increased adhesion. Capping of episialin did not result in redistribution of integrins, a process known to affect adhesion (data not shown). Other mAbs directed against the repeat domain of episialin had a similar effect as mAb 214D4. These results demonstrate that a more or less uniform distribution of episialin over the entire cell surface is required for its effect on adhesion, probably because episialin can aspecifically mask the adhesion receptors, and that the binding potential of the integrins is not affected by the expression of episialin, per se.



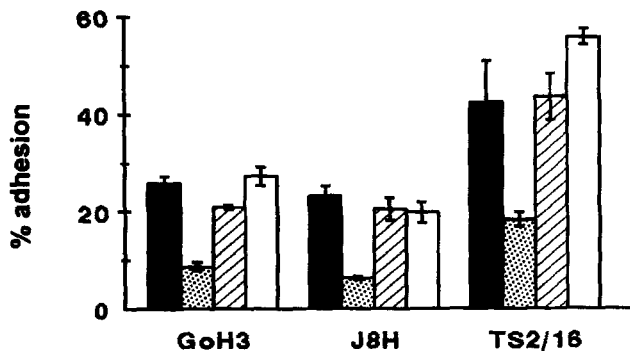
**Figure 7.** Analysis of the effect of the cytoplasmic tail of episialin on adhesion to ECM components. **(A)** Immunoprecipitation of the episialin deletion mutant which lacks its cytoplasmic domain as expressed by ATD2<sup>+</sup> cells and the full-length molecule as expressed by ACA19<sup>+</sup> cells. Cells were immunoprecipitated with mAb 232A1 recognizing an epitope just upstream of the transmembrane region. Upon routinely performed SDS-PAGE, episialin dissociates in the high molecular weight NH<sub>2</sub>-terminal part, containing the mucin-like domain, and a low molecular weight COOH-terminal part, containing the transmembrane and cytoplasmic domains. After SDS-PAGE, the cytoplasmic tail was detected using polyclonal antiserum against the cytoplasmic tail of episialin (12% gel, lower panel). The various bands are differently N-glycosylated (data not shown). The NH<sub>2</sub>-terminal part was detected by mAb 139H2 directed against the mucin-like domain (5% gel, upper panel): the upper band is the fully glycosylated form, whereas the lower band represents the episialin-precursor (Hilkens and Buijs, 1988). Episialin expression is absent in the revertant cell lines and no cytoplasmic tail can be detected in the ATD2 transfectant cell line. **(B)** Expression level of episialin on ATD2 and ACA19 transfectant and revertant cell lines as determined by FACS analysis. Both transfectant cell lines show comparable episialin expression. The solid lines represent the episialin-negative revertant cell lines and the dashed lines the episialin-positive transfectant cell lines. MAb 214D4 was used to detect episialin. The negative control is represented by the dotted line. **(C and D)** Adhesion of transfectant and revertant cells to various ECM components. The number of cells bound is depicted as percentage of the total number of cells added per well. Percentages of adhesive cells were determined in triplicate. Error bars indicate the standard deviation. **(C)** Comparison of the adhesion to ECM components of the ACA19 and ATD2 transfectant and revertant cell lines. The episialin-negative revertant cell lines are represented by the black bars (ACA19<sup>-</sup>) or hatched bars (ATD2<sup>-</sup>), whereas the episialin-positive transfectant cell lines are represented by the dotted bars (ACA19<sup>+</sup>) or open bars (ATD2<sup>+</sup>). **(D)** Effect of mAb 214D4 induced capping of episialin lacking its cytoplasmic tail. The episialin-negative revertant cell lines are represented by the black bars (ATD2<sup>-</sup> without mAb treatment) or hatched bars (ATD2<sup>-</sup> treated with mAb), whereas the episialin-positive transfectant cell lines are represented by the dotted bars (ATD2<sup>+</sup> without mAb treatment) or open bars (ATD2<sup>+</sup> treated with mAb).

### The Anti-adhesion Effect of Episialin Is Not Mediated by Its Cytoplasmic Tail

It is well established that association of integrins with the cytoskeleton is a prerequisite for adhesion (for a review see Sastry and Horwitz, 1993). Thus, competition for cytoskeletal components, resulting in less efficient integrin-mediated adhesion, could be an explanation for the episialin-mediated anti-adhesion effect, because episialin is also associated with the cytoskeletal network (Parry et al., 1990; Braga et al., 1992). Alternatively, the episialin cytoplasmic tail might interfere with signal transduction events that are involved in the regulation of the adhesive properties of cells. To exclude these possibilities, the cDNA part encoding the cytoplasmic tail of episialin was deleted and transfected into A375 cells resulting in ATD cell lines. Immunoprecipitation of episialin showed that the cytoplasmic tail was indeed deleted (Fig. 7 A). The expression level of the extracellular domain was tested by FACScan analysis (Fig. 7 B). The anti-adhesive effect of episialin was the same in both the ACA19<sup>+</sup> transfectants, expressing the full-length episialin, and the ATD2<sup>+</sup> transfectants, lacking the episialin cytoplasmic domain (Fig. 7 C), as was the effect of capping of episialin (Fig. 7 D). The effect of activation of  $\beta 1$  integrins and blocking integrin-mediated adhesion were also similar for both cell lines (data not shown). These results make it very unlikely that the cytoplasmic domain of episialin plays a significant role in the episialin-mediated anti-adhesion effect either by competition for cytoskeletal components or by interference with signal transduction events involved in adhesion processes.

### Episialin Inhibits Binding to Immobilized Antibodies

The results presented above suggest that the episialin molecules hinder the interaction of integrins with their ligands. Yet, the binding of the antibodies to integrins was not affected by episialin (Fig. 1). This apparent contradiction might be

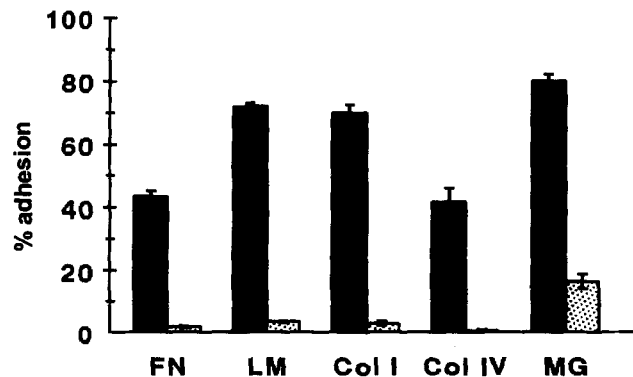


**Figure 8.** Binding of ACA19 transfectant and revertant cells to three different immobilized anti-integrin antibodies. mAbs GoH3 and J8H are directed against the  $\alpha 6$  chain, whereas TS2/16 is directed against the  $\beta 1$  chain. Soluble mAb 214D4 was used to induce capping of episialin. ACA19 transfectant and revertant cells were preincubated with mAb 214D4 for 15 min at 37°C. Subsequently, the cells were allowed to adhere for 30 min at 37°C. The number of cells bound is depicted as percentage of the total number of cells added per well. Percentages of adhesive cells were determined in triplicate. Error bars indicate the standard deviation. The bars represent the cell lines as follows: ■, untreated revertant cells; ▨, untreated transfectant cells; ▩, revertant cells treated with mAb; □, transfectant cells treated with mAb.

due to the additional physical immobilization of the integrin ligands on a solid substratum. To test whether the ability of the antibodies to diffuse affects the final outcome of the experiments, three different anti-integrin mAbs (GoH3, J8H, and TS2/16) were immobilized on plastic and the A375 transfectant and revertant cells were allowed to adhere before and after clustering of episialin. As shown in Fig. 8, the episialin-positive cells adhered less strongly to immobilized antibodies than their revertants. Capping of episialin to one pole of the cell restored the adhesion to the same level as the revertants (Fig. 8) as was shown for adhesion to the ECM components (Fig. 5 C). This supports our notion that overexpression of episialin hinders binding of integrins to immobilized ligands and that capping of episialin enhances this interaction by uncovering the integrins.

### Episialin Also Affects Adhesion of a Breast Carcinoma Cell Line

ZR-75-1 breast cancer cells express episialin at very high levels. A small percentage of these cells grow detached. Such cells are usually discarded during routine passaging. When the non-adherent cells were passaged together with the adherent cells, the number of cells growing in suspension increased during each passage until more than 80% of the cells were growing in suspension to become the ZR-75-IS cells. Episialin was significantly more strongly expressed on the latter cells whose adhesion to various ECM components was reduced as compared to the parental ZR-75-1 cells (Fig. 9). The expression level of e.g., the  $\alpha 6$  integrins, which are responsible for adhesion to laminin, was not affected (Fig. 1). If mAb 214D4 was added to floating ZR-75-IS cells, more than 90% of the cells adhered to the bottom of the culture flask within 2–4 h and cells spread within one day whereas other mAbs had no effect (Fig. 10). This indicates that the functional activity of the integrins is not affected by a high level of episialin. Moreover, episialin on adherent ZR-75-1 cells and ZR-75-IS cells (after capping) was mainly present at the apical site, whereas it was distributed over the entire surface of the suspension ZR-75-IS cells (Fig. 10). These results indicate that episialin cannot only reduce adhesion



**Figure 9.** Adhesion of adherent ZR-75-1 and suspension ZR-75-IS breast carcinoma cells. The number of cells bound is depicted as percentage of the total number of cells added per well. The ZR-75-1 cells are represented by the black bars, whereas the ZR-75-IS are represented by the dotted bars. Percentages of adhesive cells were determined in triplicate. Error bars indicate the standard deviation.



of the relatively artificial transfected cells, but also of a breast carcinoma cell line which naturally expresses it at high levels.

## Discussion

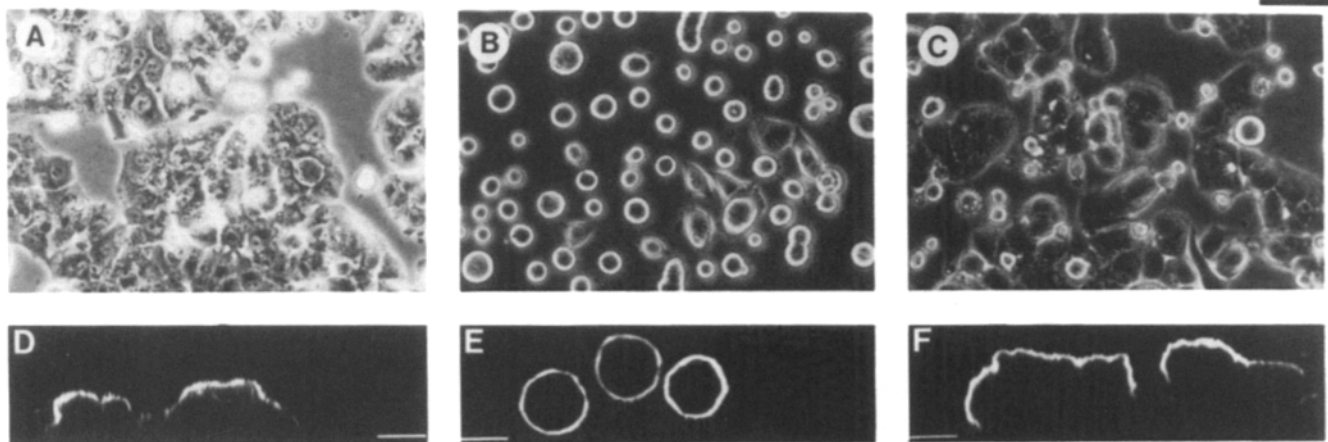
In this report, we show that a high level of expression of the mucin-like transmembrane molecule episialin (MUC1) can reduce integrin-mediated cell adhesion to various ECM components *in vitro*. The most simple explanation for this effect is that a high density of this extended and relatively rigid molecule hinders physically interactions of immobilized ligands with their receptors. For this phenomenon similar levels of episialin on the cells are needed as are present on carcinoma cell lines, suggesting that the reduction of adhesion is physiologically relevant.

The extent of this episialin-mediated reduction of cell-ECM adhesion is cell-type dependent. For example, the adhesion of A375 transfectants to various ECM components is reduced more strongly than that of the MDCK-*ras-e* transfectants and the HBL-100 transfectants, all expressing comparable levels of episialin. This can be explained by the differences in the levels of expression of the various integrins. For example, on HBL-100 cells the laminin receptors  $\alpha6\beta1$  and  $\alpha6\beta4$  are more strongly expressed than on the A375 cells and therefore their binding reaches maximal values at a lower concentration of laminin, as has been described before (Sonnenberg et al., 1990). In addition, the activation state of the integrins is likely to be important: adhesion of A375 transfectants could be restored by activation of the  $\beta1$  integrins with the stimulatory mAb TS2/16. This finding does not seem to be compatible with the notion that episialin physically hinders interactions of integrins with their ligands. We suggest that in unstimulated cells episialin reduces the number of possible integrin-ligand encounters, which renders adhesion more difficult, whereas the enhanced affinity of binding induced by mAb TS2/16 increases the number of productive integrin-ligand interactions resulting in adhesion.

Cells which do not adhere because of the presence of episialin can also regain their adhesive properties by clustering ("capping") of episialin to one pole of the cell. The most simple explanation is that uncovering the integrins on the cell surface allows them to interact with their ligands. This would imply that it is unlikely that the binding potential of the integrins is directly affected by episialin. Alternatively, the cytoplasmic domain of episialin might be involved in integrin inactivation, either by interfering with relevant signal transduction events or by competing for associated proteins that can bind to the actin filaments, as integrin molecules (for a review see Sastry and Horwitz, 1993) as well as episialin molecules (Parry et al., 1990; Braga et al., 1992) are linked to the cytoskeleton. However, involvement of the cytoplasmic domain is unlikely as A375 transfectants expressing episialin without a cytoplasmic tail showed comparable inhibition of adhesion.

Yet another possible explanation is that episialin interferes with adhesion by binding to transmembrane regulators of integrin-mediated cell adhesion via its transmembrane and/or ectodomain. However, this possibility seems less likely because adhesion of the episialin-positive A375 transfectants to immobilized antibodies was also reduced, which effect was abolished by clustering of the episialin molecules. Furthermore, not only integrin-mediated cell-ECM adhesion is strongly affected by episialin, but also E-cadherin-mediated cell-cell adhesion (Ligtenberg et al., 1992a). Taken together, these results indicate that episialin reduces adhesion in a non-specific manner by physically hampering binding of receptors to immobilized ligands.

Episialin also inhibits the adhesion of cells of a variant of the breast carcinoma cell line ZR-75-1 (ZR-75-1S), on which the density of episialin is higher than on the parental cells and which grow as single cells in suspension. mAb induced capping of episialin on these variant cells also resulted in their adherence. It might be argued that most breast carcinoma cell lines passaged *in vitro* show "normal" adhesion properties, despite the fact that these cell lines also express episialin at high levels. However, adherent cells are selected



**Figure 10.** Growth pattern and site of episialin expression of ZR-75-1 cells and ZR-75-1S cells. (A and D) ZR-75-1 cells; (B and E) untreated ZR-75-1S cells; and (C and F) ZR-75-1S cells upon incubation with an antibody, which can cap episialin, for 24 h. (A-C) Cells were photographed using phase contrast optics. (D-F) Immunofluorescence of cells after fixation and permeabilization using Texas red-conjugated mAb 214D4 directed against the episialin repeat domain. Shown are sections perpendicular to the substrate (except for the suspension cells). Episialin is mainly present at the apical site of the attached cells, whereas it is randomly distributed on the suspension cells. Bars: (A-C) 25  $\mu\text{m}$ ; (D-F) 10  $\mu\text{m}$ .

during culture. The percentage of ZR-75-1 cells growing in suspension can be simply increased by passaging both adherent and non-adherent cells together instead of discarding the floating cells with the spent medium.

Overexpression of episialin on cells and the resulting reduced adhesion of the cells may have secondary effects. We have shown that in vitro A375 transfectants with a high level of episialin are less susceptible to killing by cytotoxic lymphocytes due to reduced effector cell-target cell interactions (van de Wiel-van Kemenade et al., 1993). Disseminated cells in the circulation may thus escape immune surveillance more successfully.

Anti-adhesive effects have also been reported of other membrane-associated mucins. For example, leukosialin (CD43), which is mainly expressed on T lymphocytes and also has a rod-like extracellular domain, reduces cell-cell interactions (Jentoft, 1990; Ardman et al., 1992; Manjunath et al., 1993). Incubation of T lymphocytes with mAbs directed against the extracellular domain of leukosialin increases cell-cell aggregation, whereas Fab fragments do not (Cyster and Williams, 1992; De Smet et al., 1993). Cyster and Williams (1992) suggested that individual cells are agglutinated by these mAbs. Capping of leukosialin to one side of the cell and freeing of the integrins, as occurs with episialin, could be another explanation for the increased adhesion. An episialin-like mucin, epiglycanin, present on the murine mammary carcinoma cell line TA3/HA has similar effects on cellular interactions as episialin (for a review see Codington and Haavik, 1992). For instance, overexpression of epiglycanin on TA3/HA cells has been correlated with successful allotransplantation of these cells (Codington et al., 1978; Miller et al., 1982), probably because the MHC molecules are shielded by epiglycanin resulting in a reduced immune response against these cells. Capping epiglycanin also dramatically increases the adhesion of TA3/HA cells to ECM components (Kemperman et al., 1994). These observations suggest that mucin-like transmembrane molecules have an important common property: masking other cell surface molecules. These mucins may have a similar role in normal tissues. For example, the anti-adhesion effect of episialin, which is normally expressed at the apical side of glandular epithelial cells, may prevent inadvertent adhesion of the apical membranes facing each other in ductal and alveolar structures, as has been suggested before (Braga et al., 1992; Hilkens et al., 1992). In many infiltrating adenocarcinomas, episialin is distributed over the entire cell surface and its expression is often increased more than 10 times (Hilkens et al., 1984; Zaretsky et al., 1990). It remains to be established whether the episialin-mediated anti-adhesion effect, which we have determined in vitro for cell-cell interactions (Ligtenberg et al., 1992a) as well as for cell-ECM interactions contributes to invasion and metastasis in vivo.

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