Ep-CAM: A Human Epithelial Antigen Is a Homophilic Cell–Cell Adhesion Molecule

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Abstract. The epithelial glycoprotein 40 (EGP40, also known as GA733-2, ESA, KSA, and the 17-1A antigen), encoded by the GA-733-2 gene, is expressed on the baso-lateral cell surface in most human simple epithelia. The protein is also expressed in the vast majority of carcinomas and has attracted attention as a tumor marker. The function of the protein is unknown. We demonstrate here that EGP40 is an epithelium-specific intercellular adhesion molecule. The molecule mediates, in a Ca2+-independent manner, a homophilic cell-cell adhesion of murine cells transfected with the complete EGP40 cDNA. Two murine cell lines were tested for the effects of EGP40 expression: fibroblastic L cells and dedifferentiated mammary carcinoma L153S cells. The expression of the EGP40 protein causes morphological changes in cultures of transfected cells—increasing intercellular adhesion of the transfectants—and has a clear effect on cell aggregating behavior in suspension aggregation assays. EGP40 directs sorting in mixed cell populations, in particular, causes segregation of the transfectants from the corresponding parental cells. EGP40 expression suppresses invasive colony growth of L cells in EHS–matrigel providing tight adhesions between cells in growing colonies. EGP40 can thus be considered a new member of the intercellular adhesion molecules. In its biological behavior EGP40 resembles to some extent the molecules of the immunoglobulin superfamily of cell adhesion molecules (CAMs), although no immunoglobulin-like repeats are present in the EGP40 molecule. Certain structural similarities in general organization of the molecule exist between EGP40 and the lin-12/Notch proteins. A possible role of this adhesion molecule in formation of architecture of epithelial tissues is discussed. To reflect the function of the molecule the name Ep-CAM for EGP40 seems appropriate.

The human pan-carcinoma antigen epithelial glycoprotein (EGP)1 40 (alias 17-1A antigen, KSA, ESA, etc.) is a 40-kD transmembrane glycoprotein that is expressed on the baso-lateral surface of the majority of simple cuboidal or columnar, pseudo stratified columnar and transitional epithelia (Spurr et al., 1986; Edwards et al., 1986; Momburg et al., 1987; Bumol et al., 1988). This epithelial molecule is encoded by the GA733-2 gene (Linnenbach et al., 1993), and the protein seems to be quite conserved in evolution, since a recently identified murine EGP40-related protein showed 86% homology with the amino acid sequence of the human molecule (Bergsagel et al., 1992). The presence of sequences related to the GA733-2 gene was reported for monkey, hamster, and chicken genomes (Linnenbach et al., 1993), and therefore it is likely that there exist

1. Abbreviations used in this paper: CAM, cell adhesion molecule; EGP, epithelial glycoprotein; TC, trypsin/Ca2+; TE, trypsin/EDTA.

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nal epithelia (Momburg et al., 1987; Bumol et al., 1988), EGP40 was suggested to be involved in either intercellular or cell-substrate adhesions of epithelial cells (Thampoe et al., 1988; Simon et al., 1990).

Here we report that EGP40 functions as a Ca$^{2+}$-independent cell adhesion molecule that mediates aggregation and "sorting out" of EGP40 cDNA-transfected cells, and induces morphological changes in multicellular structures (cell aggregates, colonies in matrigel) produced by the transfectants. Cell-cell connections mediated by EGP40 suppress the invasive growth of colonies of transfected cells embedded in matrigel. Our data indicate that EGP40 is an important epithelial adhesion molecule that may play a role in the organization of epithelial tissues, in cell segregation in multilayered epithelia and in supporting the architecture of tumor tissues.

**Materials and Methods**

**Cells and Tissue Culture**

The E-cadherin transfected LUN.6 cells, MC7-7 human mammary carcinoma cells and HBL-100 normal mammary epithelium immortalized by SV-40 transformation were kindly provided to us by Dr. J. Hilken's (The Netherlands Cancer Institute, Amsterdam). L cells (clone L929) were obtained from the American Type Culture Collection (Rockville, MD). Murine mammary carcinoma cells L153S were kindly provided by Dr. E. Schuring (University of Leiden) (Schuring et al., 1990). All cell lines were cultured in DME with 10% fetal calf serum.

**Expression Vectors**

For the expression of EGP40, three kinds of plasmids were constructed as follows. The Smal-BglII fragment from the G473-2 CDNA (kindly provided by Dr. A. Linnenbach (The Wistar Institute, Philadelphia, PA); Szala et al., 1990) was subcloned in vectors of the pUC series (Morgenstern and Land, 1990) under the control of either constitutive (SV-40 or rat $\beta$-actin) or inducible (MMTV) promoters.

**Transfection of Cells**

For transfection, 2-5 x 10$^6$ cells were grown in DME supplemented with 10% fetal calf serum in 35-mm dishes. Transfection was performed using DOTAP (Boehringer Mannheim, Mannheim, Germany). 5 $\mu$g of the plasmid mixed with plasmid PSV2neo (Spivak et al., 1984) in a 1:1 ratio were used for each transfection. After 18 h the DOTAP/DNA-containing medium was replaced by fresh medium. The cells were grown for an additional 24-48 h, and then transferred to 2 x 100-mm dishes and cultured in complete medium containing either 300 $\mu$g/ml (for L153S cells) or 800 $\mu$g/ml (for L cells) of G418 (GIBCO BRL, Gaithersburg, MD). After 14-18 d, G418 resistant clones were isolated, and their reactivity with the anti-EGP40 mAb 323A3 (Edwards et al., 1986) was analyzed by flow cytometry. Positive clones were recloned and used in subsequent experiments. Revertant clones were selected from the 7-10th passage of the transfectants. All the revertant clones selected had lost the EGP-cDNA containing vector, but still contained the vector with the selection marker (not shown).

**Cell Aggregation Assay**

Cells were detached by two different methods, either by TC (HANK's buffer with 0.01% trypsin and 1 mM CaCl$_2$) or by TE (0.01% trypsin, 0.1% EDTA) treatment according to Nose et al. (1988). Aggregation of cells was carried out 24-well tissue culture plates (Nunc, Roskilde, Denmark). To prevent cell adhesion to the bottom of the wells, 1 ml of 1% agarose (GIBCO BRL) was poured into each well before experiments. 5 x 10$^5$ cells suspended in 2 ml of HCMF (Hank's solution containing 100 mM Hepes, 1% BSA, and 100 $\mu$g/ml DNase I) buffer, either containing 1 mM CaCl$_2$ or Ca-free, were placed in each well, and the plate was incubated on a rotating platform (80-100 rpm) at 37°C and 5% CO$_2$. At distinct time points 200-$\mu$l samples were analyzed in a Coulter counter to determine the number of particles. The extent of aggregation was represented by the index N$_t$/N$_0$, where N$_t$ is the number of remaining particles at the incubation time point t, and N$_0$ is the initial number of particles corresponding to the total number of cells.

Otherwise the degree of cell aggregation was calculated after Shimoyama et al. (1992) as $D = (N_0 - N_t)/N_0$.

**Labeling of Cells**

For cell sorting experiments cells were labeled using fluorescent dyes that incorporate into membrane lipid bilayers, either PKH-2 (green fluorochrome) or PKH-26 (red fluorochrome) from Zynaxis Cell Science Inc. (Malvern, PA). These fluorochromes provide a stable labeling of living cells and do not interfere with either cell surface proteins or with cell behavior and interactions (Horan et al., 1990). Cells were labeled using the Cell labeling kit according to the manufacturer's protocol.

**Cell Sorting**

The analysis of cell sorting in mixed populations was performed as described elsewhere (Nose et al., 1988, Benchimol et al., 1989; Friedlander et al., 1989). Cells dissociated with TC were washed three times in Dulbecco's PBS and labeled as described above. The labeled cells were washed three times in 50% FCS in DME, resuspended in DME containing 0.8% FCS/1 mg/ml DNase (Boehringer Mannheim), mixed at various ratios depending on the experiment, and allowed to aggregate as described for the aggregation assay. After 1-2 h of aggregation, the cell-cell aggregates suspension was analyzed under a BRC-600 confocal microscope (Bio Rad Laboratories, Richmond, CA). Images from different areas of the preparation were taken, and the number of cells of each color in the aggregates was determined.

**Immunofluorescence Microscopy**

Cells grown on either glass slides or in multi-well chamber slides (Nunc) were fixed for 10 min in -20°C MeOH, quickly rinsed in -20°C acetone and allowed to dry. The preparations were blocked in 5% skim milk solution in PBS for 30 min at 37°C and indirect immunofluorescent staining was performed using a specific mAb and an anti-mouse FITC conjugate to detect the bound mAb. The preparations were analyzed with the BRC-600 confocal microscope.

**Colony Growth in EHS-Matrigel**

Cells were detached with Trypsin-EDTA to obtain a single-cell suspension. 200-300 cells (in 20 $\mu$l of medium) were mixed on ice with 200 $\mu$l of cold EHS-matrigel solution (Sera Biochemicals, Paramus, NJ) and plated into wells of a multichamber slide (Nunc). Matrigel was allowed to polymerize for 30 min at 37°C and complete culture medium was layered on top of the gel formed. After the embedding, the cells were controlled by microscopy. The majority was represented by single cells, no more than 1% of doublets were observed. After the cells in matrigel had produced colonies (3-12 d, depending on the experiment), all colonies were counted and scored, according to structural organization (loose or tight, four different grades, see Fig. 8), and according to type of growth as invasive or non-invasive, respectively.

**Results**

**Establishment of Cell Lines Expressing EGP40**

To study the function of EGP40, we have transfected two cell lines with EGP40 cDNA: L cells, a fibroblast cell line widely used in cell adhesion studies (Nose et al., 1988), and L153S cells, a murine mammary carcinoma cell line that has lost its epithelial phenotype (Schuring et al., 1990). The cells were subcloned three times before transfection, to minimize irrelevant cell heterogeneity in future clonal selection. To obtain stable transfectants, the fragment of EGP40 cDNA containing the entire coding sequence was subcloned downstream of the constitutive SV-40 and $\beta$-actin promoters or the inducible MMTV promoter in p33G, p36G, and p35G vectors, respectively. The constructs were introduced into cells by co-transfection with a neomycin resistant vector. After selection with G418, several clones producing EGP40 were iso-
lated for each type of vector used. As was determined by im-
munoblotting (Fig. 1 a) the 40-kD EGP40 protein was
expressed in all the selected, G418-resistant clones. The pro-
itin detected corresponds to the major molecular form of
EGP40 described in most epithelial cell lines analyzed
(Thampoe et al., 1988). A lower molecular weight band, de-
tected in all transfectants, corresponds to a non-glycosylated
form of EGP40, that is synthesized by human epithelial/car-
cinoma cells and by the transfectants in the presence of
triacamycin (not shown). This suggests that the early, non-
glycosylated precursor of EGP40 is abundantly produced in
transfectants. Two other forms of EGP40 present in T47D
mammary carcinoma cells (Fig. 1 a) and also detected in
other cell lines at various ratios to the major form (Thampoe
et al., 1988; Gourevitch, M. M., S. V. Litvinov, D. Scholl,
A. Tsubura, A. J. Haisma, and J. Hilgers, manuscript sub-
mitted for publication), were not found in the transfected
cells. Indirect immunofluorescence on living cells using the
323A3 mAb showed that EGP40 is present at the surface of
the transfectants (Fig. 1, b and c).

Generally, cells transfected with the EGP40 cDNA under
the control of the β-actin promoter expressed higher amounts
of EGP40 than cells transfected with the constructs contain-
ing the SV-40 promoter. We selected clones with low and
high levels of EGP40 expression for each cell line trans-
fected. With respect to the level of EGP40 at the plasma
membrane, the highest expressing clones were comparable
with mammary epithelium cell lines (Fig. 1 c). EGP40 was
expressed consistently by all cells of every clone (Fig. 1 b),
similar to the homogeneous expression of EGP40 in most
human epithelial cell lines analyzed.

EGP40 expression in clones transfected with constructs
under control of the MMTV promoter could be induced by
relatively low concentrations of dexamethasone (10⁻⁶ M).
After induction the number of EGP40 molecules at the cell
membrane increased up to 40 times (Fig. 1, a and c), as was
determined by flow cytometry. Non-induced clones ex-
pressed very low levels of EGP40.

Morphological Changes in Cultures of
Transfected Cells

The morphology of both transfected cell lines was altered by
the expression of EGP40. Whereas the parent L cells did not
form tight intercellular connections, the EGP40 transfectants
were often connected to each other, forming cell clusters. In
such clusters large areas of cell membranes were involved
in the formation of cell–cell contacts. The difference in
cell–cell interactions, while not very explicit at low cell
density, was clearly seen when high density cultures of the
parental and transfected cells were compared (Fig. 2). The
transfected cells sometimes formed multicellular aggregates,
in which cells were tightly interconnected (marked by black

Figure 1. Expression of EGP40 in cells transfected with EGP40 cDNA. (a) Immunoblot analysis of cell lysates of human mammary carci-
noma cells T47D (lane 1), murine L153S cells (lane 2), and the transfectant clones: C1.2 (lanes 3 and 4), C2.4 (lane 5), C67.21 (lane 6),
and L67.10 (lane 7). The EGP40 cDNA was introduced into transfectants under the control of the MMTV (C1), SV-40 (C2), β-actin
(C67 and L67) promoters. All lanes are samples containing equal cell equivalents. Cells C1.2 were either non-treated (lane 3) or pretreated
(lane 4) with 10⁻⁸ M dexamethasone for 24 h. Cells were lysed as described in Material and Methods, samples were electrophoresed in
10% gel and the immunoblotting was performed with anti-EGP40 mAb 323A3. Blot probed with the mAbs was incubated with the
anti-mouse alkaline-phosphatase conjugate. The major 40-kD molecular form of EGP40 (black arrows), minor molecular forms (arrow-
heads) and a non-glycosylated precursor form of EGP40 (white arrow) are marked. (b) Representative profiles of EGP40 expressed in
transfected cells and MCF-7 mammary carcinoma cells as analyzed by flow cytometry. Living cells were incubated with 323A3 mAb and
goat anti-mouse IgG-FITC conjugate and analyzed by flow cytometry. (c) Indirect immunofluorescence (as above) on the same transfectant
cell lines treated (white bars) or non-treated with 10⁻⁸ M dexamethasone. Human MCF-7 mammary carcinoma cells and the parental
L153S cells served as positive and negative controls respectively.
Figure 2. Morphology of the parental (a) and EGP-transfected (b) L cells. Note tight multicellular aggregates formed by the transfected cells in a high cell density culture (black arrows) in contrast to the parental L cells, that at similar density grow as a culture of not interconnected cells. Bar, 50 μm.

Figure 3. Morphology of the parental (a) and EGP-transfected (b) L153S cells. Note that intercellular adhesions are increased in the culture of transfected cells resulting in appearance of multicellular aggregates. Bar, 150 μm.

arrows in Fig. 2 b). We had never observed these types of cell–cell interactions in the cultures of the parental L cells. Although the connections between cells in EGP40-transfected clones were increased, the morphology and growth characteristics of these cells were different from L cells transfected with E-cadherin cDNA (LUN6.1): no "epithelialization" of the cell monolayer due to polarization of cells, typical for E-cadherin transfectants, was observed in cultures of EGP40-transfected L cells. Additionally, in contrast to E-cadherin transfectants, the contact growth inhibition at high cell density was less explicit in cultures of EGP40-transfectants. None of the selected revertant clones did show the enhanced intercellular contacts observed in the original transfectant clones (not shown).

The morphological changes in cultures of L153-transfectants were even more distinct. Similar to cultures of the L cell transfectants, the connections between EGP40-transfected L153S cells were increased, and the cells very often produced multicellular aggregates in contrast to the parental cell line (Fig. 3). The same morphological changes were observed in all clones transfected with EGP40 cDNA under the control of constitutive promoters, but not in revertant clones or in control clones obtained by transfection with the pSV2neo only. Similar effects of EGP40 expression on cell morphology were also observed in clones of L153S cells transfected with EGP40 under the control of the MMTV promoter, but only when dexamethasone was present in the culture medium. The effect was reversible, and in the absence of the drug the cells became morphologically similar to the parental cells within 72 h. No changes in cell morphology were observed in cultures of the parental cells in the presence of dexamethasone.

We studied the subcellular localization of EGP40 in the transfected cells using anti-EGP40 mAb 323A3. In highly differentiated epithelial cells EGP40 mainly accumulates in areas of intercellular contacts, but not on free domains of the cell membrane, as shown in Fig. 4 a for human mammary carcinoma cells, MCF-7. The resembling subcellular localization of the molecules was found in the transfected cells. In L153S transfectants a substantial staining with anti-EGP40 mAb was found in cytoplasm, and presumably was associated with an overexposed precursor molecules of EGP40. When the two of transfected L153S cells had established an intercellular contact, the EGP40 was detected in areas of the contact, but not in a free domains of cell membrane, as shown for both substrate-attached (Fig. 4 b, see...
Figure 4. Immunolocalization of EGP40 in zones of intercellular contacts. Indirect immunofluorescent staining of human mammary carcinoma MCF-7 cells (a) and transfected L153S (b and c) and L (d) cells was performed using mAb 323A3. The EGP40 molecules were localized in transfectants at the areas of cell-cell adhesion (white arrowheads) similar to human epithelial cells. Bars, 10 μm.

Also Fig. 8) and suspension (Fig. 4 c) L153S transfectants. In multicellular colonies (aggregates) the protein was also present only at the zones of intercellular contacts, but not at the outer surface (not shown). In the transfected L cells a larger part of the EGP40 molecules was found at the free domains of cell membrane, but still, certain accumulation of the EGP40 at intercellular boundaries was observed (Fig. 4 d).

These findings suggest that EGP40 may mediate intercellular adhesions, including primary intercellular contacts.

Aggregating Behavior of EGP40 Transfectants

To test the involvement of EGP40 in intercellular adhesion, we examined the aggregating properties of the EGP40 transfectants as described for other adhesion molecules, i.e., cadherins (Nose et al., 1988) and carcinoembryonic antigen (Benchimol et al., 1989). As was confirmed with immunofluorescence using anti-EGP40 antibody 323A3, neither the trypsin/Ca2+(TC) nor trypsin/EDTA (TE) treatments removed EGP40 molecules expressed at the surface of the transfectants. Cultures of the transfected and parental cell lines were dissociated by TC treatment to monocolonial suspensions and allowed to aggregate in suspension culture.

L cells aggregate poorly in suspension culture. Several EGP40-transfected clones were tested, and the transfectants always aggregated more efficiently than cells of the parental clone or the revertants (Fig. 5 a). In these experiments the E-cadherin–transfected L cells (LUN6.1 clone) served as a reference standard for aggregation. The EGP40-transfected L cells tested always aggregated more slowly than the same L cells expressing E-cadherin, although differences in aggregation levels of EGP40 transfectants and parental cells were apparent after 60 min.

The aggregation of EGP40-transfected L cells in contrast to E-cadherin transfectants was not Ca2+ dependent. When the same cell lines were dissociated by gentle pipetting in the presence of EDTA and allowed to aggregate in the absence of Ca2+, no cell aggregation was observed during 90 min of observation for either the parental L cells or E-cadherin transfectants (Fig. 5 b). However, the aggregating behavior of EGP40 transfectants was not affected by either EDTA treatment or the absence of Ca2+ in the aggregating medium.

Figure 5. EGP40-mediated cell aggregation. Cells were detached and dissociated by TC treatment and were allowed to aggregate in suspension culture. The results presented are scored from six parallels in each experiment. (a) Kinetics of aggregation of parental L cells and L cells transfectants expressing EGP40 (L-EGP) and E-cadherin (L-E cadh) in presence of Ca2+. (b and c) Aggregation of L and L153 transfectants in the presence (black bars) or absence (hatched bars) of Ca2+ in aggregating medium. The following cell lines were compared in aggregation assay: (b) EGP40-transfected L cells (clone L67.10) and the parental L (L929) cells, and (c) L157S cells expressing EGP40 (clone C67.21) and C67.21REV, an EGP40-negative revertant clone. LUN6.1, a clonal cell line of L cells transfected with E-cadherin, served as a reference cell line in these experiments.
Differences in aggregating properties were also revealed by comparing the parental L153S cells or EGP40-negative revertant clones with several EGP40-transfected L153S clones (Fig. 5 c). For these aggregation assays the cells were detached by the TC treatment that removes the majority of molecules involved in aggregation of the parental L153S cells, since such a treatment largely reduced the capability of these cells to aggregate.

In the presence of Ca²⁺ in the medium, the aggregation of the transfectants was significantly faster than that of the parental cells. The differences in aggregation were significant after 60 min. However, in the absence of Ca²⁺, in contrast to L cell transfectants, both the parental and transfected L153S cells were not aggregating.

To investigate the basis for such differences between L and L153S transfectants, we analyzed by flow cytometry (using the 323A3 mAb) the presence of EGP40 at the cell membrane after incubating the cell suspension for 1 h at 37°C in the presence and absence of Ca²⁺. Indeed, the number of EGP40 molecules at the surface of L153S-transfectants decreased dramatically in Ca²⁺-free medium, in contrast to the L cell transfectants, where the amount of plasma membrane-associated EGP40 was constant irrespective of the presence or absence of Ca²⁺ in the medium (not shown). For some human mammary carcinoma cells we had previously observed internalization of EGP40 molecules in the absence of Ca²⁺ and it is very suggestive, that a similar internalization occurs in L153S cells that by their origin are mammary carcinoma cells. The reduced presence of EGP40 at the cell membrane in the absence of Ca²⁺ seems to be the reason for the Ca²⁺ dependency of EGP40-mediated adhesion in cultures of L153S transfectants.

**Cell Aggregating Behavior in Prolonged Suspension Culture**

Both the transfected and parental L153S cells were capable of aggregating when been detached using the trypsin–EDTA treatment. Such cells were allowed to aggregate in a prolonged suspension culture. After 24 h, multicellular aggregates were formed by L153S cells. These aggregates had a loose structure, and the cells were attached to each other only through small domains of membrane, whereas the major part of the cell membrane was not involved in intercellular contacts (Fig. 6). The EGP40 transfectants, although not aggregating much faster in the aggregation assays, after prolonged suspension culture formed more condensed multicellular structures (Fig. 6 b). The surface of these aggregates resembled aggregates formed by epithelial cells. The transfected cells in the aggregates were connected by large areas of cell membrane (Fig. 6 d).

EGP40-transfected L cells were also capable of forming aggregates in a 24-h suspension culture in contrast to the parental cell line (not shown).

**EGP40 Mediates Homophilic Cell Interactions and Cell Sorting**

Intercellular adhesion molecules are known to mediate cell sorting, that is, a selective aggregation of cells expressing a certain type of molecules from a mixed population of cells. A sorting function was reported for several cadherins (Nose et al., 1988; Friedlander et al., 1989) and for carcinoembryonic antigen, a member of the immunoglobulin superfamily of cell adhesion molecules (CAMs) (Benchimol et al., 1989). We investigated whether the binding mediated by EGP40 is homophilic and whether the molecule directs "sorting out" of EGP-positive cells in mixed populations.

We first examined whether transfectants adhere to the parental cells when mixed. To distinguish between the two cell lines different fluorochrome dyes were used. When monolayer suspensions of the two cell lines were mixed at equal density in suspension culture, the L cell transfectants that express EGP40 aggregated on their own and did not incorporate parental L cells into aggregates. In a 2-h assay no more than 10% of cells in aggregates were found to be parental cells (not shown). This result strongly suggests that EGP40 mediates homophilic interactions.

In similar experiments we tested the L153S cells, since both the parental and EGP-transfected cells are capable of aggregating in suspension cultures. After being dissociated with the TE treatment that preserves the intrinsic aggregating mechanisms of L153S cells, the cells were labeled and allowed to aggregate for 2 h in the presence of Ca²⁺. As shown in Fig. 7, the transfectants and the parental cells aggregate independently. In control experiments, where the aggregation of the same cells, labeled with the two different dyes was studied (both the parental and transfectant cells were tested), no cell segregation was observed. To exclude a possible influence of the fluorescent dyes on cell aggregation, different combinations of labels as well as aggregation of labeled and unlabeled cells were studied. No differences were observed that might be related to the effects of the label on the cell aggregation. EGP-transfected L153S cells have shown higher aggregating activity than the parental cells; the analysis of the aggregates formed by cells in mixed culture revealed that multicellular aggregates (15 or more cells) were formed mainly by the transfectants. Aggregates formed by the parental cells only rarely exceeded 15 cells (Fig. 7 a) and mainly did not include transfectant cells.

Since aggregates of EGP40-transfected L153S cells rarely...
Figure 7. Homophilic cell–cell binding mediated by EGP. (a) Quantitative analysis of segregation of EGP-transfectants from the parental L153S cells. C67.21 cells labeled with PKH2 were mixed with L153S cells labeled with PKH26 in a 1:1 ratio and were allowed to aggregate for 2 h. Aggregates that were composed from >5 cells were randomly selected and the percentage of each was determined. (b) Quantitative analysis of co-aggregation of EGP40-transfected L cells (clone L67.10) with EGP-transfected L153S cells C67.21 (black bars) and the revertant clone C67.21REV cells (hatched bars). The experiment was performed similar to that described above. Aggregates that were composed from >20 cells were randomly selected and the percentage of each cell type was determined.

if EGP40 molecules indeed mediate homophilic cell–cell interactions it would imply that two different cell lines, both aggregating through EGP40-mediated adhesions, will adhere to each other. We tested combinations of EGP40 transfected L153S and L cells, since intercellular adhesions in both cell lines are mediated by EGP40. A revertant clone from L153S transfectants and the parental L153S cells served as controls in these experiments. In a mixed culture, L cells expressing EGP40 were much stronger aggregating with EGP40-expressing transfected L153S cells than with the parental L153S cells or revertants that are both EGP-negative (Fig. 7 b).

Mixed with cells where intercellular adhesions are mediated by CAMs other than EGP40, neither L cell nor L153S

Figure 8. EGP40-transfectants and the parental L153S cells in mixed culture. L153S and C67.21 (EGP40-positive transfectant clone of L153S cells) single-cell suspensions were mixed in 1:1 ratio, and the cells were co-cultivated for 24 h. After fixation, the immunostaining using 323A3 mAb was performed. Note the presence of EGP40 at the boundaries of two EGP40-positive cells (white arrowheads), but in contact area of positive and negative cell (contour arrowheads). The transfectants and the parental cells can be easily be discriminated: p, an example of the parental cell, t, an example of the transfected cell. Bar, 10 μm.
cell EGP40-transfectants formed mixed aggregates. L cells transfected with E-cadherin (LUN-6) and HBL-100 epithelial cells (E-cadherin-positive, EGP40-negative) were tested in these experiments as co-aggregating cell lines (not shown).

In conclusion, we have established that EGP40 mediates homophilic binding and can direct cell sorting in mixed populations.

**The Effect of EGP40 on Cell Colony Growth in EHS-Matrigel**

To analyze the effects of EGP40 on cell adhesion we followed the growth of colonies produced by single cells in EHS-matrigel. L cells do not form multicellular colonies in matrigel. Normally the dividing cells migrate from each other, and the resulting colony looks like a group of single cells (Fig. 9).

Cells transfected with EGP40 showed clear changes in morphology of the colonies formed under these conditions. The effect of EGP40 on growth of L cells in matrigel was already quite distinct after the first 3 d of culture, and persisted during the next 9 d, until the experiment was discontinued on the 12th day. More than 50% of the colonies produced by the EGP-transfected L cells represented multicellular aggregates, and 30-60% of these were formed by tightly bound cells (Fig. 9). Tight colonies, designated as type III and IV, displayed a decrease in cell proliferation rate, since no expansion of the colony size was observed after the sixth day. At the 12th day the type III and IV colonies consisted of smaller number of cells than colonies of the types I or II. We tested several clones expressing different levels of EGP40 under these conditions, and the occurrence of the tight colonies formed by cells was proportional to the level of EGP40 expression by the cells of the corresponding clone (see data on the levels of EGP40 expression in clones, as analyzed by flow cytometry, shown in Fig. 10 b). The colonies of low-expressing, revertant or negative clones are formed mainly by disconnected cells similarly to the colonies of the parental L cells. The higher was the level of EGP40 present at the membrane of transfected cells, the higher the percentage of organized multicellular colonies among the colonies formed.

Between days 3 and 12 the morphology of the colonies generally remained the same, and it was possible to discriminate tightly bound colonies (defined as non-invasive) from what can be defined as "invasive" colonies, where cells were migrating into the matrigel away from the main cell mass. The majority (>90%) of colonies formed by wild-type L cells or by revertant clones were invasive, in contrast to only 50-60% of colonies formed by transfectants expressing high levels of EGP40 (Fig. 10 a). The invasiveness of colonies correlated negatively to the level of EGP40 expression (see Fig. 10 b).

L153S cells and EGP-transfected clones from these cells were both equally highly invasive in matrigel (not shown). No substantial differences were observed in growth of these cell lines in matrigel.

**Discussion**

In the present study we have analyzed the biological function of the human 40-kD glycoprotein EGP40 (after Simon et al., 1990). Most epithelial tissues, including simple cuboidal and columnar, pseudo-stratified columnar and transitional epithelia, express EGP40 quite homogeneously, and the molecule is located at the lateral and sometimes basal domains of cell membranes (Varki et al., 1984; Edwards et al., 1986; Moldenhauer et al., 1987; Momburg et al., 1987). Among adult epithelial tissues EGP40 is not expressed in epidermal keratinocytes, gastric parietal cells, thymic cortical epithelium, myoepithelial cells and hepatocytes (Edwards et al., 1986; Moldenhauer et al., 1987; Momburg et al., 1987). Among adult epithelial tissues EGP40 is not expressed in epidermal keratinocytes, gastric parietal cells, thymic cortical epithelium, myoepithelial cells and hepatocytes (Edwards et al., 1986; Moldenhauer et al., 1987; Momburg et al., 1987). However, the protein is expressed by carcinomas that originate from squamous epithelia (Moldenhauer et al., 1987), and a number of tissues negative in adult express EGP40 during embryonic development, among them the epithelium of
the skin (the basal layer), hepatocytes, and thymus epithelium. Therefore EGP40 can be considered a pan-epithelial molecule.

The results presented here demonstrate that EGP40 can function as a Ca\(^{2+}\)-independent homophilic intercellular adhesion molecule. When expressed in murine cells, EGP40 mediates primary intercellular contacts, cell aggregation in suspension cultures, homotypic cell sorting in mixed populations of aggregating cells and suppresses the invasive growth of L cell colonies in EHS-matrigel. EGP40-mediated cell-cell adhesions were shown to have a strong effect on growth and morphology of transfected cells.

The localization of the protein at adjacent cell borders in both normal and malignant epithelial tissues is consistent with an adhesion function in these in vivo situations as well. A murine protein, highly homologous to EGP40, is also expressed mainly in epithelial tissues (Bergsagel et al., 1992), as would be expected for a molecule that is supposedly essential for epithelial tissues and therefore conserved during evolution.

With respect to the role of EGP40 in epithelial tissues, the cell sorting mediated by this molecule is of particular interest. Actually, in the L153S transfected/parental cells model the EGP40-directed segregation of cells was more pronounced than the influence of the molecule on the aggregating behavior of the transfectants. The aggregation of L153S cells might be directed by other molecules, and it is feasible that EGP40-mediated adhesiveness reduces other adhesions between the transfectants as a result of large amounts of EGP40 appearing on the membrane. The suggested diminishing effect of EGP40 on other intercellular contacts could explain why transfected and non-transfected L153S cells aggregate separately in mixed cultures like cells expressing different types of adhesion molecules. Similar effects on cell segregation in mixed populations were demonstrated previously for carcinoembryonic antigen (Benchimol et al., 1989), another Ca\(^{2+}\)-independent adhesion molecule.

In normal epithelium EGP40-mediated cell adhesions are present in a context of other adhesion molecules, and an effect of EGP40 on cell sorting may be crucial for tissue morphogenesis. Thus, in glandular epithelia, where two layers of epithelia are present, EGP40 is not expressed in the basal layer (Varki et al., 1984; Edwards et al., 1986). EGP40 may be important for sorting of mature ductal and alveolar epithelia from stem cell/myoepithelium.

In some other organs EGP40-directed sorting might play a role as well. Thus, in the pancreas segregation of B cells from non-B Islet cells was reported to be directed by Ca\(^{2+}\)-independent cell adhesion molecules, whereas E-cadherin mediates connections between both types of cells (Rouiller et al., 1991). EGP40 is expressed in pancreas Islet cells (Edwards et al., 1986; Moldenhauer et al., 1987).

Some data suggest that EGP40 is functionally active in many tumors as well. First, the cDNA encoding for EGP40 that was used for transfections in this study was cloned from colorectal carcinoma cells (Szala et al., 1990). This cDNA apparently encodes a functionally active molecule and does not differ from EGP40 cDNAs cloned from other sources (Strnad et al., 1989; Perez and Walker, 1989; Simon et al., 1990). EGP40 is located at the intercellular boundaries in many carcinomas similar to the molecule's location in normal tissues (Edwards et al., 1986; Momburg et al., 1987;).

In most adenocarcinomas, EGP40 expression can be found at all stages of tumor development (Momburg et al., 1987), suggesting that the molecule continues to mediate intercellular adhesions during tumor progression. Indeed, we have found EGP40-mediated cell-cell adhesions in some poorly differentiated carcinoma cell lines. Thus, the aggregation of COV362 cells (ovarian carcinoma cell line) can be inhibited by the presence of the anti-EGP40 mAb 323A3 in the aggregation medium. The effect most likely is caused by the cross-linking of the EGP40 molecules by the mAb with their subsequent internalization, leading to a reduced presence of EGP40 at the surface of the cells. These findings illustrate that EGP40 adhesions are the key intercellular adhesions for some carcinoma cells.

Figure 10. The suppression of invasive cell growth by EGP40 expression. Cell lines: a negative revertant clone of L cells (L67.7R), and L cell transfectants expressing EGP40 (low-expressor clone L67.12 and three high EGP40-positive clones L67.3, L67.4, and L67.10) or E-cadherin (LUN.6). Colonies formed by L cell transfectants in EHS matrigel, as described for Fig. 9, were scored on the 12th day of experiment according to the type of growth (a). Colonies were considered as invasive when >5 cells from a colony were found to migrate into the matrigel from the node. (b) The relative level of EGP40 expression as determined for each cell line by flow cytometry using mAb 323A3. A high percentage of non-invasive colonies formed by transfected cells positively correlated with a high level of EGP40 expression.
The intercellular connections mediated by EGP40 can have a suppressive effect on invasion of cells, as was demonstrated in the present study by a reduced invasive growth of L cells in matrigel. It is conceivable that in the course of tumor progression the suppressive role of EGP40 as intercellular CAM is increasing, since the E-cadherin adhesions, the main type of intercellular adhesions of epithelia, have often been found inactivated in many late stage carcinomas (Shimoyama and Hirohashi, 1989; Shimoyama et al., 1989; Navarro et al., 1991).

In EGP40-negative epithelial tissues the de novo expression of the molecule in tumor cells might be critical at early stages of tumorigenesis (squamous carcinomas, hepatocellular carcinomas). If the introduction of EGP40 into cell interconnections indeed is capable to diminish the strength of tumor-normal cell adhesions, as we suggested, the reduced interconnections with the normal surrounding tissue could provide some growth advantages to the tumor cells.

The place of EGP40 among other families of adhesion molecules—cadherins, integrins, selectins, and Ig-like CAMs—is not clear. The comparison of the predicted amino acid sequence of EGP40 did not reveal significant similarities with other cell adhesion molecules. The Ca²⁺-independent, homophilic adhesions mediated by EGP40 make the molecule functionally similar to some Ig-like CAMs, the carcinoembryonic antigen (Benchimol et al., 1989), N-CAM (Rao et al., 1992) and LI (Miura et al., 1992) in particular, although an Ig-like repeat is not present in the EGP40 molecule. The overall composition of the EGP40 molecule shows certain similarities with the organization of proteins encoded by the lin21/Notch gene family. These proteins are involved in intercellular signaling and cell-cell interactions that are important for differentiation and segregation of cells (for review see Greenwald and Rubin, 1992). The EGF-like repeats in a cysteine-rich domain of EGP40 are followed by a cysteine-poor domain in the extracellular region of the molecule (Lin-1993) similar to the organization of the extracellular domains of the lin21/Notch family of molecules.

In conclusion, we have shown that EGP40 is a homophilic cell adhesion molecule that may be important for cell segregation. In a view of its adhesion function and the presence in epithelial tissues only, we suggest to introduce the name EpiCAM for this molecule.

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