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Metastasis-association of the rat ortholog of the human epithelial glycoprotein antigen EGP314

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Screening for surface molecules expressed by metastasizing rat tumors had revealed evidence for metastasis-association of a molecule also expressed on epithelial cells. The similarity to the expression profile of the panepithelial glycoprotein EGP314 prompted us to isolate and sequence the gene and to explore functional features of the molecule in transfected tumor lines. The molecule D5.7A, named according to the antibody, D5.7, used for selection, indeed, is the ortholog of EGP314 with 92% and 80% identity to the murine and the human molecules. Like EGP314, D5.7A has a particular cleavage site, a small cleavage product being resolved under reducing conditions from the membrane anchored part of the molecule. Transfection of a low metastasizing fibrosarcoma, pheochromoblastoma and adenocarcinoma revealed that expression of D5.7A facilitates tumor progression. Depending on the origin of the tumor, D5.7A transfectants either metastasized via the lymphatic system (pheochromoblastoma, adenocarcinoma) or hematogeneously (fibrosarcoma). Particularly after proteolytic cleavage, D5.7A facilitated cell – cell adhesion and provided a proliferative signal upon crosslinking. Thus, the rat ortholog of EGP314 is involved in metastasis formation. Importantly, its functional activities apparently rely on proteolytic cleavage. These findings provide a first evidence on how a panepithelial marker can be involved in tumor progression.

Keywords: rat; metastasis; epithelial glycoprotein; adhesion

Introduction

Tumor progression involves a series of events, frequently summarized as metastatic cascade, which includes detachment from the primary tumor, migration through the extracellular matrix, penetration through the basal membrane, adaptation to the circulation pressure, attachment to the endothelia of the vessel wall and settlement and growth in distant organs (Fidler and Radinsky, 1990; Mareel *et al*., 1991). To perform these distinct functions, metastatic cells frequently display an array of altered gene products (Bishop, 1991; Mareel *et al*., 1993), which include cell-cell and cell-matrix adhesion molecules (Evans, 1992; Glinsky, 1993; Zetter, 1993), matrix-degrading enzymes, their activators, inhibitors and receptors (Matrisian, 1992; Ossowski, 1992; Liotta *et al*., 1983; Magnatti *et al*., 1986). Furthermore, there is increasing evidence that metastasis formation is rather the consequence of altered regulation of gene expression than of the activation of 'metastasis-specific' genes, which have not been described so far (Mareel *et al*., 1993). In line with the hypothesis that tumor progression relies on dysregulated expression of physiological gene products is the finding that the implements for tumor progression will be provided not only by the tumor cell, but also by the neighboring tissues (Mareel *et al*., 1993).

We have described a set of monoclonal antibodies (Matzku *et al*., 1989), which were raised against the metastatic variant of a pancreatic adenocarcinoma (BSp73ASML) of the rat (Matzku *et al*., 1983). Five antibodies were found to selectively recognize BSp73ASML as well as other metastasizing tumor lines, but neither the non-metastasizing variant (BSp73AS) nor other locally growing rat tumors (Matzku *et al*., 1983; Claas *et al*., 1996). Despite the restriction to the metastatic phenotype, the corresponding molecules were detected on non-transformed tissues and cells, whereby the expression pattern varied between widespread distribution and restriction to few selected tissue layers (Claas *et al*., 1996). Interestingly, all of these molecules were expressed or upregulated during implantation and placentation and some were differentially expressed during organogenesis (Claas *et al*., 1996). Three of these molecules have already been identified and described. The first one has been shown to be a variant isoform of the adhesion molecule CD44, CD44v4-v7 (Günthert *et al*., 1991). D6.1A is a tetraspanin molecule and the ortholog of the human tumor antigen CO-029 (Szala *et al*., 1990a). Likely by association with integrins or additional membrane structures the molecule can initiate activation of the clotting cascade (Claas *et al*., 1998). C4.4A is a phophatidyl-inositol anchored molecule with partial sequence homology to uPAR (Rösel *et al*., 1998). Here we report on the cloning and characterization of a molecule, named D5.7A, the ortholog to the murine and human panepithelial antigen EGP314, also known as EGP40, GA733-2, ESA, KSA, 17-1A antigen and Ep-CAM (Bergsagel *et al*., 1992; Bjork *et al*., 1993; Bumol *et al*., 1988; Edwards *et al*., 1986; Fernsten *et al*., 1990; Göttlinger *et al*., 1986; Lando *et al*., 1993; Larson *et al*., 1988; de Leij *et al*., 1994; Momburg *et al*., 1987; Nelson *et al*., 1996; Perez and Walker, 1989; Ross *et al*., 1984; Simon *et al*., 1990; Spurr *et al*., 1986; Stein *et al*., 1994; Strnad *et al*., 1989; Szala *et al*., 1990b). As revealed by transfection of non-metastasizing tumor cells, D5.7A appears to be a cell-cell adhesion molecule

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which may facilitate tumor progression by its growth promoting capacity upon ligand interaction. Interestingly, functional activity of D5.7A largely depends on proteolytic cleavage of the molecule.

Results

The D5.7A molecule has originally been described to be expressed by three gastrointestinal tumors of the rat, BSp73ASML, RG and PROG, which are known to metastasize via the lymphatic system. The molecule has not been detected on non-metastasizing rat tumor lines. A search for physiological expression of the molecule revealed that it was strongly expressed on epithelial cells (Claas *et al*., 1996). The same pattern of expression has been described for the panepithelial glycoprotein EGP of the mouse and the human epithelial glycoprotein antigen/major gastrointestinal tumor-associated protein GA733-2/EGP314 (Bergsagel *et al*., 1992; Momburg *et al*., 1987; Zaloudik *et al*., 1997). Since expression of GA733-2/EGP314 is used as diagnostic and therapeutic tool (Fagerberg *et al*., 1995; Li *et al*., 1997), it was of interest to evaluate, whether D5.7A is, indeed, the rat ortholog, which would offer a possibility to elaborate functional activities of the molecule.

Identification of D5.7A as the rat homolog of the panepithelial antigen in human and mouse

COS-7 cells were repeatedly transfected with plasmid DNA of a cDNA library derived from the metastatic rat colon carcinoma line RG. D5.7A positive cells were first selected by panning, thereafter by fluorescence staining and FACS sorting, i.e. the Hirt extraction procedures were followed by three rounds of transformation of *E. coli* using pools of decreasing numbers of colonies for the transfection of COS-7 cells. Finally, COS-7 cells transfected with the cDNA from one bacterial colony were used for sequencing the molecule.

The analysis of the sequenced DNA suggested an open reading frame for 315 AA. The potential start codon is flanked by a consensus sequence for the initiation of translation. The 5′ untranslated region spans 122 nucleotides. Two poly(A)$^+$ addition sites are found within the 447 bases of the 3′ untranslated region. The full length nucleotide sequence of the D5.7A revealed a strong homology to the human and mouse panepithelial glycoprotein (Figure 1).

D5.7A is a type I transmembrane protein with a cytoplasmic tail of 26 AA and an extracellular domain of 268 AA (Figure 2). Within the extracellular part there is an EGF-like domain (AA 26–48) and a thyroglobin-like repeat (AA 60–122). Corresponding to the human and the mouse molecule (Bergsagel *et al*., 1992; Bjork *et al*., 1993; Szala *et al*., 1990b). D5.7A has 12 conserved cystein residues two consensus sequences for phosphorylation and two potential N-glycosylation sites.

As already described, D5.7A shows a molecular weight of 42 kD. After tunicamycin treatment the molecular weight is reduced to 32 kD (Claas *et al*., 1996). Interestingly, after surface biotinylation of BSp73ASML, trypsine treatment and precipitation with D5.7 a second 35 kD and a small 7 kD protein became visible when separated under reducing conditions (Figure 3). The same phenomenon has been described for EGP314 and is due to cleavage of a small part of the molecule in the N-terminal region, which is linked to the remaining part via disulphide bonds (Schön *et al*., 1993).

D5.7A and metastasis formation

EGP314, the human ortholog of D5.7A, is used as a marker for metastasizing tumor cells in the bone marrow and in pleural/peritoneal exudates. However, the observation does not qualify the molecule as being actively involved in the process of tumor progression. To see whether D5.7A facilitates metastasis formation, we transfected three weakly or non-metastasizing rat tumor lines, a pancreatic adenocarcinoma (BSp73AS), a fibrosarcoma (BSp6S) and a pheochromoblastoma

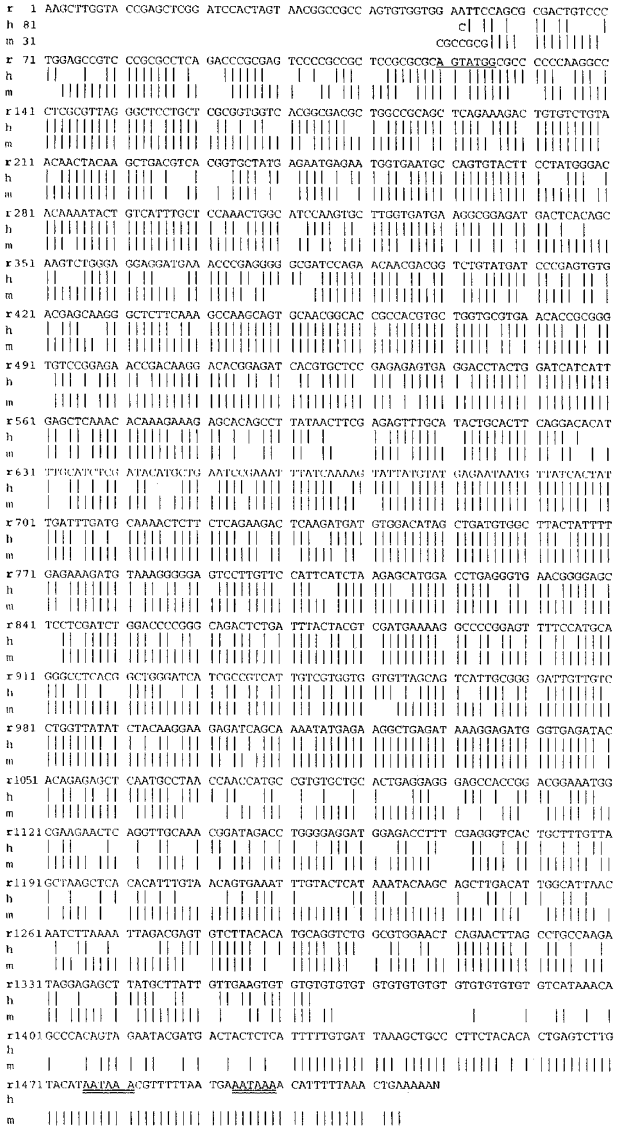

Figure 1 Nucleotide sequence of D5.7A. The nucleotide sequence of D5.7A is shown. Homologous nucleotides in the human and the mouse EGP314 cDNA are indicated by vertical lines. The consensus sequence for the initiation of translation (Kozak, 1989) is underlined and the two consensus sequences for addition of the poly(A)$^+$ tail are double underlined

A. Aminoacid sequence of D5.7A

```
                leader peptide          EGF-like repeat                 |
  1 MAPPKALALG  LLLAVVTATL  AAAQKDCVCN  NYKLTSRCYE  NENGECQCTS
                Thyreoglobin-like repeat
 51 YGTQNTVICS  KLASKCLVMK  AEMTHSKSGR  RMKPEGAIQN  NDGLYDPECD
                                                    |
101 EQGLFKAKQC  NGTATCWCVN  TAGVRRTDKD  TEITCSERVR  TYWIIIELKH

151 KERAQPYNFE  SLHTALQDTF  ASRYMLNPKF  IKSIMYENNV  ITIDLMQNSS

201 QKTQDDVDIA  DVAYYFEKDV  KGESLFHSSK  SMDLRVNGEL  LDLDPGQTLI
                          Transmembrane region      |
251 YYVDEKAPEF  SMQGLTAGII  AVIVVVVLAV  IAGAVVLVIS  TRKRSAKYEK

301 AEIKEMGEIH  RELNA
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B. Orthology of D5.7A to murine and human EGP314

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D5.7A   1  MAPPKALAFGLLLAVVTATLAAAQKDCVCNNYKLTSRCYENENGE  CQC   48
           ||||..|||||||..|||:||||.:|||:||||. .|: |:| :  |||
mEGP    1  MAPPQVLAFGLLLAAATATFAAAQEECVCENYKLAVNCFVNNNRQFTCQC   50
           ||.|.|||||||||||||||||:||||:||||...|  || ||  |||
hEGP    1  MAGPQALAFGLLLAVVTATLAAAQRDCVCDNYKLATSCSLNEYGEFTCQC   50

D5.7A  49  TSYGTQNTVICSKLASKCLVMKAEMTHSKSGRRMKPEGAIQNNDGLYDPE   98
           || |.|||||||||.|||||||||. || ||| |||||:|||||||||:
mEGP   51  TSVGAQNTVICSKLAAKCLVMKAEMNGSKLGRRAKPEGALQNNDGLYDPD   100
           ||||||||||||||||.||.|||||||||||:|||| ||||||||||:
hEGP   51  TSYGTQNTVICSKLASLCLAMKAEMTHSKSGRRIKPEG IQNNDGLYDPD   99

D5.7A  99  CDEQGLF  KAKQCNGTATCWCVNTAGVRRTDKDTEITCSERVRTYWIII   146
           ||| |||  :| ||||||..|||||||||||||||||||||||||||||
mEGP  101  CDESGLFTFLALQCNGTSMCWCVNTAGVRRTDKDTEITCSERVRTYWIII   150
           |||||||  ||||||||||||||||||||||||||||||||||||||||
hEGP  100  CDEQGLFFTKAKQCNGTATCWCVNTAGVRRTDKDTEITCSERVRTYWIII   149

D5.7A 147  ELKHKERAQPYNFESLH  TALQDTFASRYMLNPKFIKSIMYENNVITID  194
           |||||.|..||: .||:  ||||..:..|| |.|||..||:|||||||||
mEGP  151  ELKHKAREKPYDSKSLRFTTALQKEITTRYQLDPKFITSILYENNVITID  200
           |||||||. ||: :||:  ||||:.|.|||.||.||||.||||||||||
hEGP  150  ELKHKERESPYDHQSLQTFTALQEAFTSRYKLNQKFIKNIMYENNVITID  199

D5.7A 195  LMQNSSQKTKDDVKIADVAYYFEKDVKGE  SLFHSSKSMDLRVNGELLD  242
           |:|||||||:|||||||||||||||||||  |||| ||.||| |||| ||
mEGP  201  LVQNSSQKTQNDVDIADVAYYFEKDVKGFTESLFH SKKMDLTVNGEQLD  249
           ||||||||||||||||||||||||||||||  |||||||||||||||| ||
hEGP  200  LMQNSSQKTKDDVDIADVAYYFEKDVKGEFTSLFHSSKSMDLRVNGEPLD  249

D5.7A 243  LDPGQTLIYYVDEKAPEFSMQGLTAGIIAVIVVVVLAVIA  GIVVLVIS  290
           ||||||||||||||||||||||||.||:|||||||||:||:|  ||||||||
mEGP  250  LDPGQTLIYYVDEKAPEFSMQGLKAGVIAVIVVVVIAVVAFTGIVVLVIS  299
           ||||||||||||||||||||||||||||||||||||| |||||  ||||||||
hEGP  250  LDPGQTLIYYVDEKAPEFSMQGLTAGIIAVIVVVSLAVIAFTGIVVLVIS  299

D5.7A 291  TRKRLAKYEKAEIKEMGEIHRELNA  315
           : ||:|||||||||||||:||||||
mEGP  300  RKKRMAKYEKAEIKEMGEMHRELNA  324
           |||: ||||||||||||||||||||
hEGP  300  TRKKSAKYEKAEIKEMGEIHRELNA  324
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Figure 2 The D5.7A molecule. (**a**) Amino acid sequence of D5.7A: The amino acid sequence of D5.7A potentially spans 315 AA. The molecule contains an EGF like domain (underlined) and a thyroglobin like repeat (double underlined). The leader peptide is underlined by dots (von Heijne, 1986; Kozak, 1987); the transmembrane region is printed in bold. The two potential N-glycosylation sites are printed in italics and the two potential phosphorylation sites in bold italics. (**b**) Similarity of D5.7A to the human and murine panepithelial glycoprotein (HUSAR, Bestfit program): The AA sequences of D5.7A and the AA of the human and of the murine panepithelial glycoprotein are aligned for conformity. Vertical lines: identical amino acids, double point: structurally related AA. Conserved cystein residues are printed in bold. The Bestfit program revealed 80% identity and 87% similarity to the human and 92% identity and 95% similarity to the murine protein

(BSp3A) with D5.7A cDNA. Clones, which showed a high level of surface expression (Figure 4) were tested for *in vivo* growth characteristics after intrafootpad injection and excision of the primary tumor together with the draining lymph node (Table 1a). After excision of the mock-transfected lines animals were cured with the exception of two rats, which succumbed with a local recurrence and one rat, which developed metastases in distant lymph nodes. Instead, 4/5 BSp73AS-D5.7A-, 4/5 BSp6S-D5.7A- and 5/10

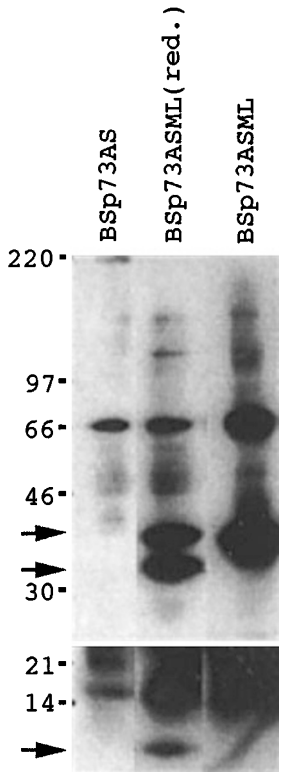


Figure 3 Immunoprecipitation of D5.7A. BSp73ASML and (as negative control) BSp73AS cells were biotinylated, trypsinized, lysed in RIPA buffer and precipitated with D5.7. The precipitates were separated on a linear gradient gel (4–20%) under reducing and non-reducing conditions. While under non-reducing conditions only one band of a 42 kD protein was visible, under reducing conditions three bands of a 42 kD, a 35 kD and, after longer exposure, of a 7 kD protein were seen. D5.7A proteins are indicated by arrows

BSp3A-D5.7-bearing rats developed metastasis. BSp73AS-D5.7A and BSp3A-D5.7A bearing rats developed lymph node and lung metastasis. BSp6S-D5.7A settled exclusively in the lung. Lung metastasis grew very rapidly with 3–10 nodules completely displacing the lung tissue. The nodules were barely vascularized and, likely as a consequence, displayed large necrotic areas (data not shown). The involvement of D5.7A was further supported by the observation that the growth of metastatic nodules was slowed down and/or started with delay in animals which received concomitantly with BSp73AS-D5.7A an intravenous injections of 200 μg D5.7, the application of antibody being repeated twice per week (Table 1b).

Thus, D5.7A facilitates tumor progression. Depending on the origin of the primary tumor, metastatic spread follows predominantly the lymphatic (adenocarcinoma and pheochromoblastoma) or the hematogeneous route (sarcoma).

Involvement of D5.7A in cell–cell adhesion and proliferation

Metastasis formation is known to be frequently accompanied by loss of cell–cell and alterations in cell-matrix adhesion molecules. Analyzing adhesion of D5.7A transfected tumor lines, irrespective of their origin, towards extracellular matrix proteins did not reveal evidence for D5.7A being involved in adhesion.

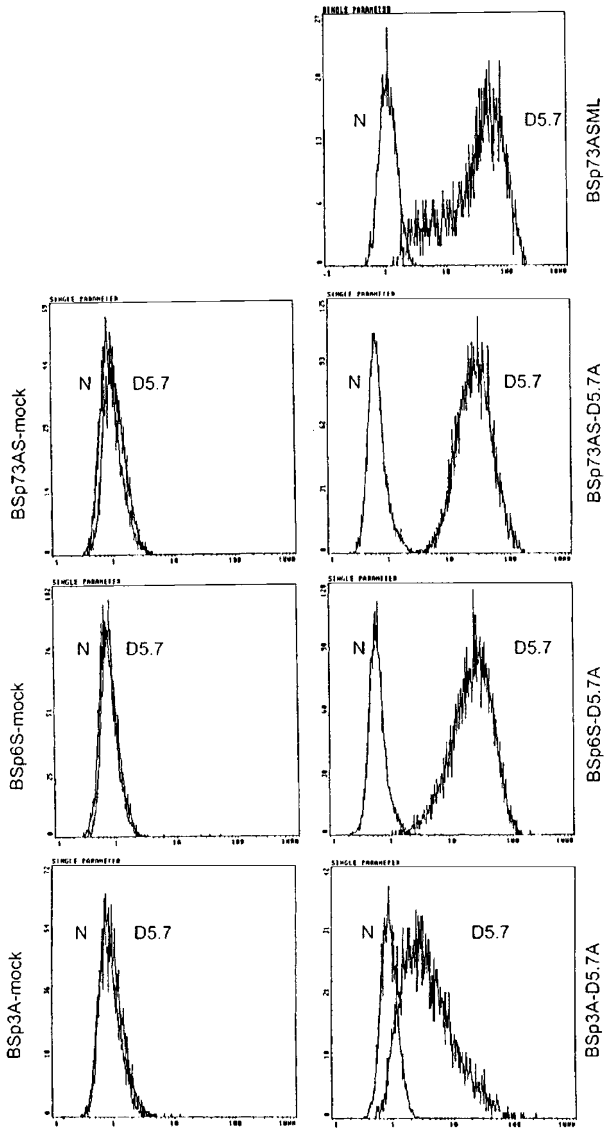


Figure 4 D5.7A surface expression in D5.7A cDNA transfected and constitutively D5.7A$^+$ cells. BSp73ASML cells, BSp73AS, BSp3A and BSp6S cells transfected with the empty vector or with the vector containing D5.7A cDNA were stained with D5.7. Overlays of the negative control (non-binding antibody plus second, dye-labeled antibody) (N) and D5.7 (D5.7) stained cells are shown

The same accounted for constitutively D5.7A-positive tumor lines, where we tried to interfere with adhesion by the D5.7 mAB. Furthermore, there was no evidence for D5.7A being involved in migration on or penetration through matrigel as determined in a transwell-migration assay. Both, adhesion to extracellular matrix proteins and incapability to penetrate through matrigel were not influenced by protease pretreatment of D5.7A cDNA transfected tumor cells (data not shown).

However, D5.7A *supported* cell–cell adhesion (Figure 5a and b). A higher percentage of cells adhered to a monolayer when either the monolayer or the added cells or both, the monolayer and the added cells expressed D5.7A. It should, however, be mentioned that adhesion particularly of BSp73ASML was most strongly increased when the monolayer also was D5.7A-positive. The involvement of D5.7A in cell–cell adhesion was sustained by the observation that binding was well inhibited by D5.7. Interestingly, cell–cell adhesion of D5.7A$^+$ cells was strengthened by protease pretreatment (0.5% trypsine for 30 min at 37°C). Again the effect was stronger when both partner cells expressed D5.7A. Protease treatment has been shown to cleave EGP at a special site without resulting in degradation of the whole molecule (Schön *et al*., 1993). Indeed, as revealed by flow cytometry binding of D5.7 was unaltered after trypsine treatment whereas, for example, the glycoprotein CD44 could no longer be detected (Figure 5c). Because adhesion between D5.7A$^+$ cells has been stronger than between D5.7A$^+$ and D5.7A$^-$ cells, it became tempting to speculate that D5.7A may also mediate homophilic binding. The hypothesis is supported by the fact that binding has been strengthened by trypsine treatment, which removes many cell surface proteins.

An additional hint for partial homophilic binding of D5.7A was provided by a cell aggregation assay using trypsinized cells. Litvinov *et al*. (1994) described that EGP mediates homophilic cell-cell adhesion, which has been demonstrated by cell sorting and aggregation. In a modified version of the aggregation assay (Table 2), we noted the BSp73ASML cells when incubated in Ca^{2+} free medium on a rotating platform had formed doublets or small aggregates of 2–5 cells after 30 min of incubation at 37°C. When BSp73ASML cells were trypsine treated, they formed larger aggregates. BSp73AS cells did not adhere one to each other at all and this was independent of trypsine treatment. BSp6S cells behaved differently to BSp73AS cells. They formed small aggregates. However, aggregation had been lost after trypsine treatment. When BSp6S cells were transfected with D5.7A cDNA a higher percentage of aggregates was seen. Distinct to BSp6S cells, protease pretreatment reduced, but did not abolish aggregation of BSp6S-D5.7A cells.

We interpret the results of the monolayer adhesion and the aggregation assays in the sense that D5.7A may take part in homophilic as well as heterophilic cell–cell adhesion and that both phenomena are strengthened by proteolytic cleavage.

Expression of EGP also has been associated with signal transduction (Fornaro *et al*., 1995) and cell proliferation (Tsubura *et al*., 1992). Thus, we finally explored whether D5.7A may facilitate tumor progression by providing a growth promoting stimulus. Indeed, crosslinking of D5.7A supported the proliferative activity of D5.7A$^+$ tumor cells (Figure 6a). Notably, the proliferative activity of D5.7A$^+$ tumor lines was increased after protease pretreatment (Figure 6b).

Taken together, D5.7A has been shown to facilitate the spread of tumor cells. The molecule apparently is involved in homophilic as well as heterophilic cell–cell interactions whereby ligand binding can stimulate tumor cell proliferation. Notably, binding as well as proliferative activity of D5.7A$^+$ cells is strengthened by proteolytic cleavage of the molecule.

Discussion

We here describe cloning, sequencing and functional characterization of a rat molecule named D5.7A. The

Table 1

A. Metastasis formation by D5.7A cDNA transfected low metastasizing tumor line

Tumor[a] *line*	*Tumor takes*	*Excision (days)*	*Survival rate* [P *values*]	*Survival*[b] *time*	*LN metastases* [P *values*] *draining*	*distant*	*Lung Metastases (No of nodules)* [P *values*]
BSp73AS-mock	5/5	28	4/5	54	1/1	0/1	0/1 (na[d])
BSp73AS-D5.7A	5/5	28	1/5 [0.067]	69.3 (54–89)	4/4	3/4 [0.004]	4/4 [0.004] (3–15) [0.057]
BSp6S-mock	5/5	12	4/5	58	1/1	0/1	0/1 (na[d])
BSp6S-D5.7A	5/5	12	1/5 [0.067]	50.5 (45–53)	0/4	0/4 [n.s.][c]	4/4 [0.004] (6–10) [0.006]
BSp3A-mock	5/5	28	4/5	55	1/1	1/1	1/1 (3)
BSp3A-mock	5/5	21	5/5	na[d]	na[d]	na[d]	na[d] (na[d])
BSp3A-D5-7A	5/5	28	2/5 [0.054]	80.7 (80–82)	3/3	3/3 [0.025]	3/3 [0.054] (5–10) [0.029]
BSp3A-D5.7A	5/5	21	3/5	78.3 (75–81)	2/2	2/2	2/2 (3;8)

B. Influence of D5.7 on metastasis formation

Tumor[a] *line*	*Antibody*[e] *application*	*Tumor takes*	*Mean tumor*[f] *diameter (cm)*	*LN metastasis*[f] [P *values*] *draining*	*diameter (cm)*	*Lung*[f] *metastasis*	*No of nodules*	*Mean diameter (cm)*	[P *values*]
BSp73AS-mock	control IgG1	10/10	0.92	2/10	0.25	0/10	na[d]	na[d]	
BSp73AS-mock	D5.7	10/10	0.91	1/10	0.20 [n.s.]	0/10	na[d]	na[d]	
BSp73AS-D5.7A	control IgG1	10/10	0.90	8/10	0.46	5/10	(1–2)	0.23	
BSp73AS-D5.7A	D5.7	10/10	0.92	3/10	0.20 [<0.001]	2/10	(1)	0.10	[0.068]

[a]Rats received 5×10^5 tumor cells (i.f.p.). At the time the primary tumor reached a mean diameter of 0.5 cm the primary tumor and the draining lymph node were excised, surviving animals were killed after 120 days; [b]Survival time of rats which developed metastases, i.e. have not been cured by excision of the primary tumor; [c]n.s.: non significant; [d]na: not applicable; [e]Rats received 200 μg antibody, i.v., twice per week; [f]Rats were sacrificed 28 days after tumor cell application

molecule has originally been identified by the mAB D5.7, which recognizes metastasizing but not non-metastasizing tumor cells. Nonetheless the molecule has been detected on non-transformed cells and the expression pattern resembled the one of the panepithelial glycoprotein EGP314 (Claas *et al*., 1996). D5.7A, indeed, is the rat ortholog of EGP314.

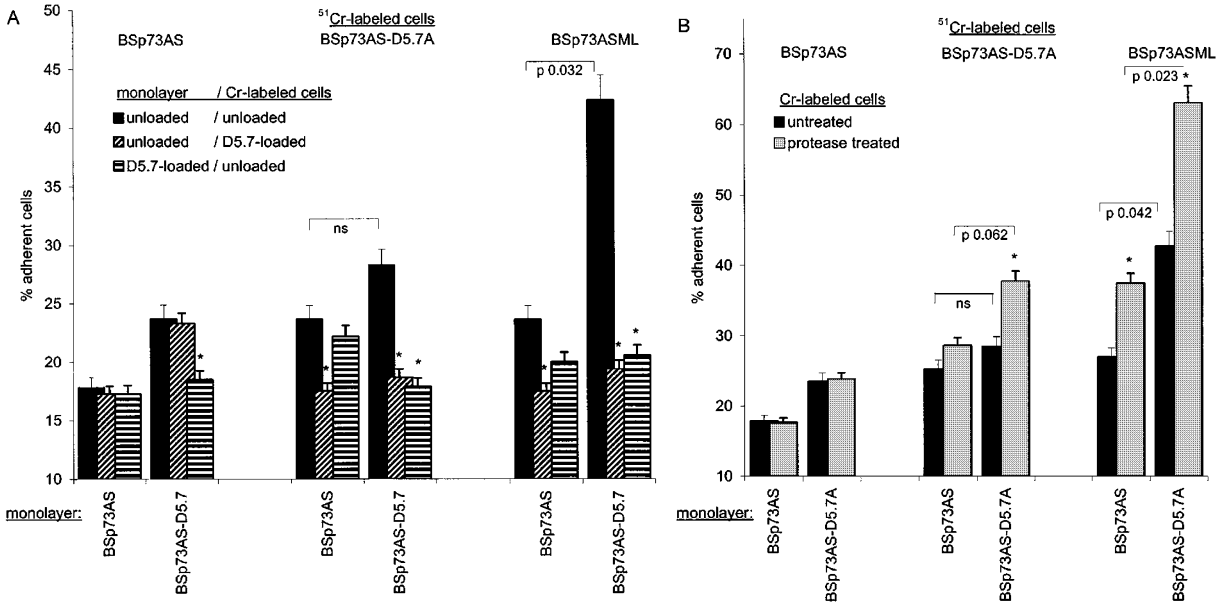
Cloning and sequencing of the molecule recognized by D5.7 revealed a high degree of similarity to EGP314 in human and mouse. Like EGP314 (Bergsagel *et al*., 1992; Bjork *et al*., 1993; Linnenbach *et al*., 1993; Perez and Walker, 1989; Strnad *et al*., 1989; Szala *et al*., 1990b) it is a type 1 transmembrane molecule with 12 conserved cystein residues and two N-glycosylation sites. Furthermore, an EGF-like domain and a thyroglobin-like repeat are conserved (Fornaro *et al*., 1995; Kiefer *et al*., 1991; Malthiery and Lissitzky, 1987). Also similar to EGP314 (Cooper *et al*., 1984; Fornaro *et al*., 1995), D5.7A has potential tyrosine phosphorylation sites. D5.7A has a molecular weight of 42 kD, which is reduced by tunicamycin treatment to 32 kD (Claas *et al*., 1996). After biotinylation and precipitation of D5.7A a second molecular weight molecule was detected when dissolved under reducing conditions. This lower molecular weight molecule has also been detected in the human system and results from a dibasic proteolytic cleavage (Perez and Walker, 1989; Schön *et al*., 1993). Interestingly, Schön *et al*. (1993) further characterized this cleavage product and found that a wide variety of enzymes specifically cleaves membrane bound EGP314 in a 6 kD and a 32 kD product.

EGP314 is used as a tumor marker for the detection of residual tumor cells e.g. in the bone marrow and in ascitic fluid (Bjork *et al*., 1993; de Leij *et al*., 1994; Momburg *et al*., 1987; Shetye *et al*., 1988; Stein *et al*., 1994). Yet, it has not been known, whether the molecule may be actively involved in tumor progression. To answer this question we transfected three weakly metastasizing tumor lines, a pancreatic adenocarcinoma, a pheochromoblastoma and a fibrosarcoma with D5.7A cDNA. The potential for metastatic growth was evaluated after intrafootpad application of the transfected tumor cells and excision of the primary tumor. Although not all rats developed metastases, the incidence of tumor growth in distant lymph nodes and the lung was significantly increased in comparison to the control tumor lines. Furthermore, as shown for the D5.7A cDNA transfected adenocarcinoma, settlement or growth in the lung could be retarded by treatment with D5.7. Interestingly, only the D5.7A cDNA transfected pancreatic adenocarcinoma and the pheochromoblastoma settled in lymphatic tissue, whereas the transfected fibrosarcoma grew only in the lung. Thus, there appear to be additional factors, which dictate the route of tumor progression. The finding that D5.7A supported metastasis formation may appear to contrast the finding by Basak *et al*. (1998) who described that a metastatic mouse colon carcinoma line transfected with mouse Ep-CAM displayed reduced invasion through matrigel and inhibited metastasis formation in the liver when injected into the spleen. Yet, the experimental systems cannot be evaluated in a comparative manner, because we transfected low or non-metastatic lines, which did not penetrate through matrigel. Beside of the differences in the experimental system we suggest and want to discuss that the function of the molecule may be dictated or at least be significantly influenced by

Table 2 Aggregation of D5.7A-positive tumor cells

Tumor line	*Single cells: aggregates (single cells as % of total number of cells)*[a] *Freshly harvested*		*Rotated 30 min, 37°C*		*Trypsinized/rotated 30 min, 37°C*	
BSp73AS	150:0	(100)	150:0	(100)	150:0	(100)
BSp73ASML	150:0	(100)	98:22	(65.3)	37:1	(24.7)
BSp6S	150:0	(100)	105:21	(70.0)	147:1	(98.0)
BSp6S-D5.7A	150:0	(100)	79:26	(53.7)	97:24	(64.7)

[a]The aggregation assay was performed in triplicates. From each sample 150 cells were counted. Standard deviations between triplicates were <3%. Cell viability (exclusion of trypan blue) was >98%



neighboring molecules. The interpretation is strengthened by our observation that (i) the pattern of metastasis formation differed in dependence on the origin of the transfected tumor line and (ii) D5.7A cDNA transfected tumor lines showed differences in their metastatic capacity, metastasis formation being weakest in BSp3A-D5.7A cells. The elegant study by Litvinov *et al.* (1997) which demonstrates that Ep-

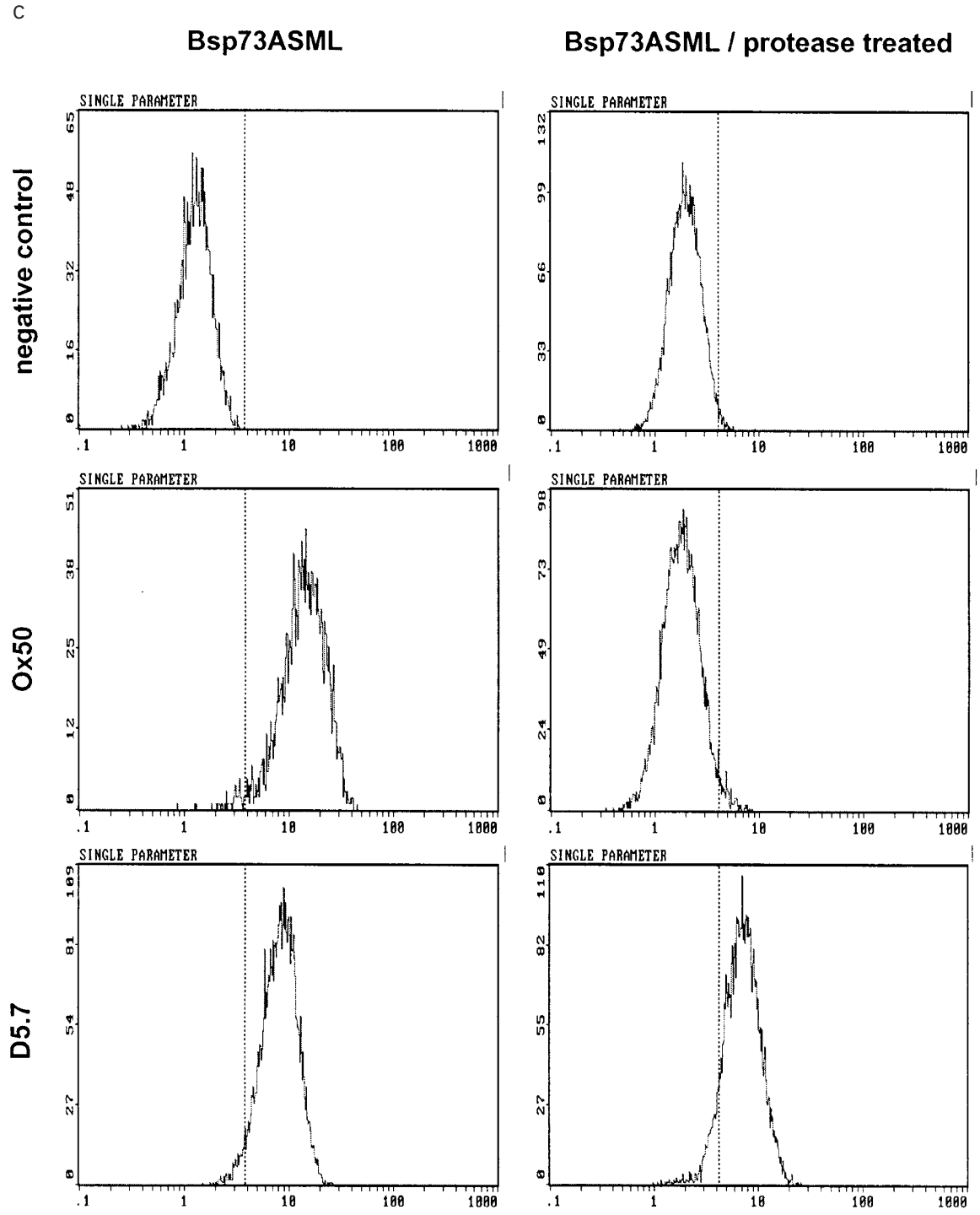


Figure 5 D5.7A in cell-cell adhesion. BSp73AS-mock and BSp73AS-D5.7A were seeded in Ca^{2+} free medium on 96 well flat bottom plates and were grown to confluence (**a**) The tumor lines BSp73AS-mock, BSp73AS-D5.7A and BSp73ASML were labeled with ^{51}Cr and were seeded (5×10^4 cells/well) on the monolayer. Where indicated either the monolayer or the added cells were preincubated for 30 min with D5.7 (10 μg/ml control IgG1 or D5.7). Non-bound antibody was washed off before labeled cells were added to the monolayer. (**b**) BSp73AS. BSp73AS-D5.7A and BSp73ASML cells were labeled with ^{51}Cr. Where indicated, cells were treated thereafter for 30 min at 37°C with 0.5% trypsine. After washing 5×10^4 cells were seeded on a monolayer of either BSp73AS or BSp73AS-D5.7A cells. (**a** and **b**) Cells were incubated for 60 min. Non-adherent cells were washed off and, adherent cells were lysed in 2% SDS. Radioactivity was determined in a γ-counter. Results are derived from three independently performed experiments. The mean percentage (+s.d.) of cells (triplicate cultures); which had bound to the monolayer is shown. Significance of differences ($P<0.1$) are indicated by asterisk. Similar binding profiles were observed after 30 and 120 min. (**c**) BSP73ASML cells were stained with 0×50 (anti-CD44) and D5.7 before and after 0.5% trypsine treatment (30 min at 37°C). Whereas expression of CD44 was completely lost by trypsine treatment, expression of D5.7A was unaltered

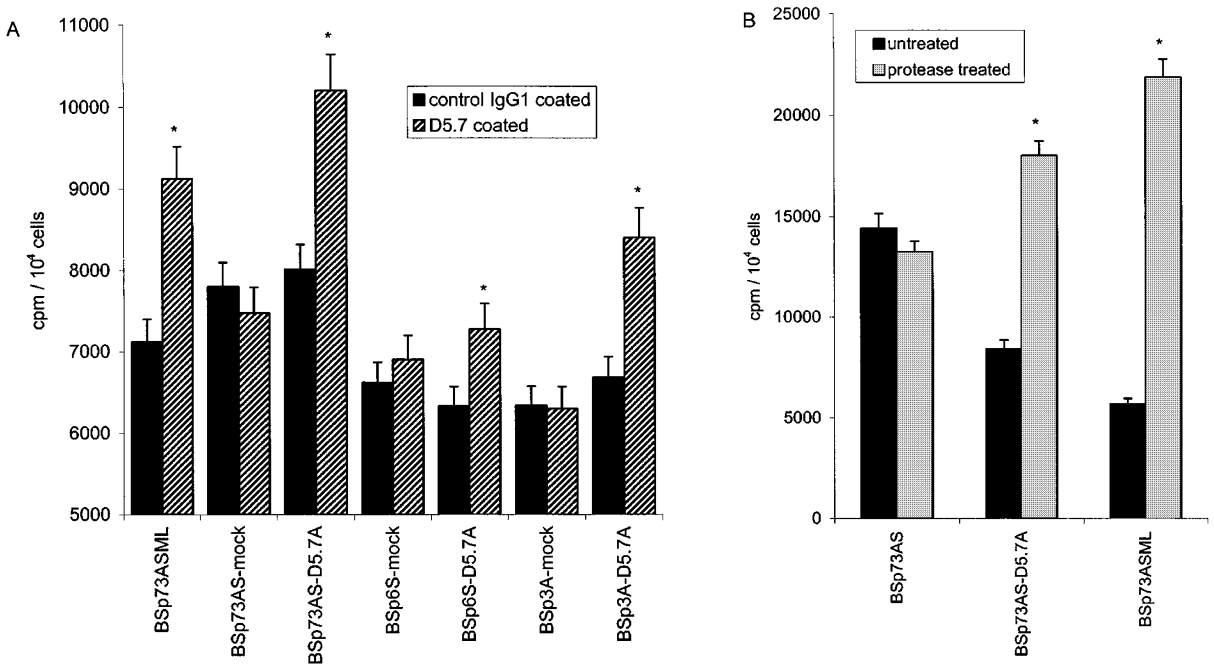


Figure 6 Influence of D5.7A cross-linking on proliferative activity. (**a**) The constitutively D5.7A$^+$ cell line BSp73ASML as well as BSp73AS-mock, BSp6S-mock, BSp3A-mock, BSp73AS-D5.7A, BSp6S-D5.7A and BSp3A-D5.7A (1×10^4 cells/well) were seeded on microtiter plates coated with control IgG1 or D5.7 (10 μg/ml). [^{3}H]thymidine was added after 16 h of incubation and plates were incubated for an additional 8 h. (**b**) BSp73AS, BSp73AS-D5.7A and BSp73ASML cells were seeded in microtiter plates (10^4 cells/well). Cells were incubated for 30 min at 37°C in PBS or in 0.5% trypsine. After washing, medium containing [^{3}H]thymidine was added. Cells were incubated for 16 h. Thereafter (**a** and **b**), cells were trypsinized, harvested and [^{3}H]thymidine uptake was determined in a β-counter. The mean c.p.m./10^4 cells (+s.d.) of triplicate cultures is shown. Significance of differences ($P<0.1$) are indicated by asterisk

CAM despite of its function as a homophilic cell adhesion molecule may loosen cell–cell adhesion by a gradual abrogation of adherens junctions, provides an additional hint on mechanisms by which a cell–cell adhesion molecule can contribute to the development of the malignant phenotype.

Trying to explore further by which mechanism the rat ortholog of EGP, D5.7A, facilitates tumor progression, we evaluated in a first set of experiments the adhesive and migratory potential of D5.7A. There was no evidence for an involvement of D5.7A in cell matrix adhesion. This has been tested for D5.7A cDNA transfected tumor lines of different origin and BSp73ASML. Adhesiveness to a variety of components of the extracellular matrix was also not influenced by protease pretreatment of D5.7A$^+$ cells (data not shown). Furthermore, D5.7A cDNA transfected tumor lines did not penetrate through matrigel. Migration on substrate coated plates was evaluated *in vitro* by wounding of a monolayer. D5.7A and mock-transfected lines showed similar migratory capacity and wound healing was not influenced by D5.7. Accordingly, healing of wounded skin in the rat could not be influenced by application of D5.7, although epithelial cells express D5.7A at high density (M Zöller, unpublished observations). Thus, we have no apparent evidence that D5.7A facilitates tumor progression via interaction with the extracellular matrix.

However, D5.7A is engaged in cell–cell adhesion. Transfection with D5.7A cDNA increased the adhesive capacity of cells, even if the binding partner did not express D5.7A, which points towards heterophilic binding. The heterophilic nature of the D5.7A binding is in discordance with reports by other groups on homophilic binding of human Ep-CAM (=EGP314, GA733-2) (Nelson *et al*., 1996; Litvinov *et al*., 1994). Litvinov *et al*. (1994) describe homophilic, Ca^{2+}-independent adhesion of murine cell lines transfected with the human EGP40 cDNA, the adhesive interaction being of the strength to allow sorting of EGP-transfected from parental non-transfected lines. Instead, Fornaro *et al*. (1995) found no evidence for homophilic binding of Trop-2$^+$ (GA733-1) or Trop-1$^+$ (GA733-2) cells. Furthermore, EGP has been described to be, albeit weakly, expressed on plasma cells (Bergsagel *et al*., 1992) and dendritic cells (Borkowski *et al*., 1996). EGP as well as D5.7A are strongly expressed on thymic epithelial cells (Claas *et al*., 1996; Nelson *et al*. 1996), which predominantly are in contact with lymphoid cells and not with epithelial cells. Nelson *et al*. (1996), indeed, noted expression on maturing thymocytes as well as on activated T cells, which finding was interpreted in the sense that Ep-CAM via homophilic interactions between thymic epithelium/dendritic cells and lymphocytes may facilitate T cell maturation and migration. Yet, D5.7A was neither expressed on thymocytes, nor on activated lymphocytes of the rat. The apparent discrepancy considering homophilic versus heterophilic binding of D5.7A/EGP314 tempted us to explore D5.7A mediated cell adhesion in a modified version of the aggregation assay described by Litvinov *et al*. (1994) as well as in dependence on the conformation of the molecule. The results of the aggregation assay, i.e. aggregation of

BSp73ASML, but not of BSp73AS, and aggregation of Sp6S-D5.7A after trypsine treatment, which abolished aggregation of BSp6S, do not definitively exclude heterophilic binding. Yet, in view of the fact that the majority of cell surface molecules will be removed by the protease treatment, it becomes likely that homophilic binding contributes to D5.7A-mediated cell-cell adhesion. Furthermore, the adhesive capacity of D5.7A$^+$ cells to a monolayer of cells was also augmented after protease pretreatment of the D5.7A$^+$ cells and this phenomenon was significantly more pronounced when both interacting cells were D5.7A$^+$. We want to mention that Helfrich *et al*. (1994) described a cellular ligand of 30 kD on Ep-CAM$^+$ tumor cells, which has not yet been defined, but theoretically could well be proteolytically cleaved Ep-CAM. Since metastasizing tumor cells frequently express either proteolytic enzymes or their receptors, the significantly increased adhesive properties of cleaved D5.7A could well contribute to the process of metastasis formation by D5.7A$^+$ tumor cells. We should finally comment on the differences between BSp73AS cells versus BSp73ASML cells as compared to the three non-transfected versus the D5.7A transfected lines. Two phenomena appear particularly noteworthy. BSp73ASML cells adhered more strongly to D5.7A$^+$ cells and adhesion was significantly augmented by trypsine treatment. Notably, BSp73ASML cells are known to display on a completely disorganized cytoskeleton and are missing vinculin (Matzku *et al*., 1985; Rodriguez-Fernandez *et al*., 1992). Next to mention, BSp73ASML cells express a whole set of surface molecules, which are not detected on the non-metastatic lines (Matzku *et al*., 1989; Claas *et al*., 1996). Thus, it could well be that in the absence of an organized cytoskeleton the adhesive functions of D5.7A take over a more dominanting role. Considering the additional metastasis-associated molecules expressed on BSp73ASML cells, but not on the D5.7A cDNA transfected lines, there is evidence that these molecules may directly interact or mutually influence functional activity (Claas *et al*., 1998; M Zoeller, unpublished finding). Both phenomena could contribute to the differences in adhesiveness between BSp73ASML and the D5.7A cDNA transfected lines. Taken together, we interpret the findings in the sense that (i) there exists more than one ligand for D5.7A, (ii) homophilic binding of D5.7A may depend on conformational changes as brought about by protease treatment and (iii) functional activity is influenced by neighboring molecules, probably in the cell membrane as well as in the cytoplasma.

Ep-CAM expression is associated with poor prognosis in cancer patients (Takes *et al*., 1997; Tandon *et al*., 1990; Zorzos *et al*., 1995). It has been hypothesized that this association may be linked to the proliferative activity of Ep-CAM$^+$ cells (Litvinov *et al*., 1996), which could arise as a consequence of the Ep-CAM-mediated downregulation of E-cadherin mediated cell–cell adhesion (Litvinov *et al*., 1997). Alternatively, though not mutually exclusive, Ep-CAM may function as a signal transducing molecule (Fornaro *et al*., 1995) or may become part of a signal transducing complex (Schön *et al*., 1995). In view of these findings and the metastasis association of D5.7A, we finally asked, whether D5.7A can exert an influence on cell proliferation. Indeed, crosslinking of D5.7A provided a growth promoting stimulus and the proliferative activity was significantly increased after protease treatment. It has been suggested (Perez and Walker, 1989; Schön *et al*., 1993) that the particular cleavage site of EGP314 may be of relevance for the function of the molecule, possibly as a growth factor receptor with proteolytically regulated activity. Furthermore, it has been described for colon carcinoma cells (Remacle-Bonnet *et al*., 1997) that surface-bound plasmin induces selective proteolysis of IGF-binding protein-4 and thus, promotes autocrine IGF-II bio-availability. Taking into account that D5.7A/GA733 contains an IGF-binding domain homologous region (Fornaro *et al*., 1995; Kiefer *et al*., 1991; Malthiery and Lissitzky, 1987), a similar pathway of proteolytically induced accessibility is well conceivable. Thus, functional activity of D5.7A appears to depend largely on proteolytic cleavage of the molecule, which according to our findings not only strengthens cell–cell adhesion, but also provides a proliferative stimulus.

Taken together, D5.7A, the rat ortholog of EGP314 facilitates tumor progression. By the differences in the metastatic settlement of D5.7A cDNA transfected tumor cells in dependence on the origin of the transfected line, it is tempting to speculate that D5.7A functions in concert with additional molecules. It may do so particularly after enzymatic cleavage, which appears to be an essential trigger for functional activity of the molecule. Besides of downmodulating cadherin-mediated binding (Litvinov *et al*., 1997), our data suggest that D5.7A, mainly after proteolytic cleavage, allows for homophilic as well as heterophilic attachment, for aggregate formation and, likely in concert with surrounding elements, for tumor cell proliferation. These elements, although by themselves not sufficient for proceeding through the metastatic cascade could well contribute to metastasis formation in the appropriate environment. Whether signals are transduced by cleaved D5.7A or by molecules, which associate with the cleaved form of D5.7A, remains to be explored. Extended efforts to unravel the question on functional activity of EGP314, which is used as a therapeutic target, with the D5.7A rat ortholog may greatly facilitate the establishment of therapeutic concepts.

Material and methods

Animals and tumors

BDX rats were obtained from Charles River, (Sulzfeld, Germany). They were kept under specific pathogen free conditions, fed with conventional diet and water ad libitum. They were used for experiments at the age of 8–10 weeks.

The following tumor lines were used: BSP73ASML (metastasizing pancreatic adenocarcinoma), BSp73AS (non/low-metastasizing pancreatic adenocarcinoma) (Matzku *et al*., 1989), BSp6S (subline of a non/low-metastasizing fibrosarcoma) and BSp3A (subline of a non/low-metastasizing pheochromoblastoma) (Zöller *et al*., 1978). BSp73AS-D5.7A, BSp6S-D5.7A, BSp3A-D5.7A (BSp73AS, BSp6S and BSp3A transfected with cDNA coding for D5.7A) and BSp73AS-mock, BSp6S-mock, BSp3A-mock (BSp73AS, BSp6S, BSp3A transfected with the pCDNA3 vector) are described below. All these tumor lines were derived from the BDX rat strain. The tumor lines regressor (RG) and progressor (PROG), sublines of a metastasizing colon

carcinoma of the BDIX strain, have been kindly provided by F Martin (Reisser *et al*., 1993). The adherently growing tumor lines were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS). Confluent cultures were either trypsinized (BSp73ASML, PROG and RG) or treated with EDTA (BSp73AS, BSp6S, BSp3A) and split. COS-7 cells were obtained from the ATCC. They were cultured in DMEM supplemented with 10% fetal calf serum.

Antibodies

The mAB D5.7 (mouse IgG1) and an isotype matched control antibody, 3–9, of irrelevant specificity have been described previously (Matzku *et al*., 1989; Zöller *et al*., 1992). Polyclonal rabbit anti-mouse IgG1 and fluorescence dye labeled derivatives were obtained from Southern Biotechnology (Birmingham, AL, USA). For FACS analysis cells were harvested, washed and resuspended at 5×10^5 cells/50 μl PBS containing 2% FCS. Cells were incubated with the first antibody (10 μg/ml) for 30 min at 4°C and after washing with the second, dye-labeled antibody (30 min at 4°C) at appropriate dilutions. After washing again, fluorescence staining was evaluated using an EPICS XL (Coulter Hialeah, FL, USA).

cDNA library and selection of clones coding for the surface molecule D5.7A

mRNA was prepared from the colon carcinoma cell line RG and oligo(dT)/Not primed cDNA was prepared using the Librarian Kit (Invitrogen, San Diego CA, USA). The cDNA was inserted unidirectional into the mammalian expression vector pcDNA3 after ligation of BstXI adaptors and cleavage with *Not*I. The ligated DNA was transformed into *E. coli*/TOP10F′. After cultivation, isolation of the plasmid DNA and plating of aliquots, the size of the library was estimated to about 2×10^7 clones.

For the isolation of clones coding for the surface molecule D5.7A, 15 μg of the cDNA was transfected into COS-7 cells by electroporation. After 3 days cells were incubated with D5.7 and panned on anti-mouse IgG coated plates. cDNA of adherent cells was extracted and transfected into COS-7 cells. The transfected COS-7 cells were stained with the mAB D5.7 and a PE-coupled goat anti-mouse IgG (Seed and Aruffo, 1987). Strongly stained cells were isolated by fluorescence activated cell sorting using a FACStar (Becton Dickinson, Mountain View, CA, USA). Plasmid DNA of the stained cells was isolated by lysis according to the method described by Hirt (1967). Genomic DNA and proteins were separated by centrifugation. The plasmid DNA/RNA mixture was digested with RNase A (20 μg/ml) and Proteinase K (50 μg/ml), followed by a phenol-chloroform extraction to remove residual protein. The remaining plasmid DNA was precipitated and used for the transformation of *E. coli* by electroporation. Three thousand colonies were picked and grown in 96 well plates. Five pools of 600 colonies were used for the transfection of COS-7 cells. Expression of D5.7A on transfected COS-7 cells was quantified and the pool of colonies which gave the highest expression was used again for transfection making ten pools each consisting of 60 colonies. The procedure was repeated making ten pools from six colonies each. Thereafter the plasmid DNA was isolated from a single bacterial colony and was used for DEAE transfection of COS-7 cells. After 3 days cells were analysed for antigen expression by FACS. The cDNA of this positive clone was sequenced according to the method of Sanger *et al*. (1977).

Transfection of D5.7A-negative tumor lines with D5.7A-cDNA

The tumor lines BSp73AS, BSp6S and BSp3A were harvested during growth in log phase. Cells (1×10^7/ml) were resuspended in RPMI 1640 containing 10% FCS and were mixed with 15 μg of the pcDNA3-D5.7A plasmid in a sterile cuvette. Electroporation was performed after 5 min incubation at room temperature at 260 V and 1050 μF using an Eurogentec Genepulser with capacitance extender. Transfected cells were selected by growth in RPMI 1640 containing 10% FCS and 500 μg/ml G418. G418 resistant cells were analysed for D5.7A expression by flow cytometry and were recloned.

Immunoprecipitation of biotinylated membrane proteins

For immunoprecipitation, cells were detached by trypsinization and surface membrane proteins were labeled with NHS-Biotin (Boehringer Mannheim) according to Meier *et al*. (1992). Cells were lysed in immunoprecipitation buffer (RIPA buffer: 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% Natriumdeoxycholate, 0.1% SDS, 2 mM PMSF, 5 mM Iodoacetamide) for 30 min at 4°C and cleared by centrifugation (13 000 r.p.m., 4°C, 30 min). Lysates were precleared overnight by addition of 1/10 volume Protein G Sepharose (1 : 1 in PBS). Afterwards lysates corresponding to approximately $1-5 \times 10^6$ cells were incubated with D5.7 (5 μg/ml) for 2 h at 4°C. Protein G Sepharose (1 : 1 in PBS, $1/10^{th}$ of the total volume) was added for 60 min. Immune complexes were washed four times with immunoprecipitation buffer and eluted from Protein G Sepharose with Laemmli buffer. SDS–PAGE (Laemmli, 1970) was performed under non-reducing and reducing conditions (5% 2-mercaptoethanol) using a 4–20% gradient gel. Separated proteins were transferred to PVDF membranes (Towbin *et al*., 1979) and bands were visualized after incubation with Streptavidin-HRP using the ECL detection system (Amersham, Braunschweig).

Adhesion, aggregation, migration and proliferation assays

Adhesion, aggregation and proliferation assays were performed either with untreated cells or with cells treated for 30 min at 37°C with 0.5% trypsine. In adhesion studies cells were labeled with ^{51}Cr, washed and resuspended in Ca^{2+} free medium. They were seeded either on a monolayer of adherent cells or on plates coated with components of the extracellular matrix, i.e. hyaluronic acid, collagen type I, type III and type IV, laminin, fibronectin and vitronectin. Components of the extracellular matrix were coated at 10 μg/ml, except for hyaluronic acid which was coated at a concentration of 100 μg/ml. Where indicated, the mAB D5.7 (10 μg/ml) was added to the culture medium. Cells were incubated for 20 min to 2 h and were washed repeatedly. The adherent cells were lysed by 2% SDS, transferred into counting vials and counted in a γ-counter. The percentage of adherent cells is shown.

To evaluate aggregate formation, cells were suspended in Ca^{2+} free medium containing 1% bovine serum albumin at a density of 1×10^6 cells/ml. Cells were incubated in microtubes on a rotating platform for 30 min at 37°C. Before and after incubation cells were counted and the ratio of single cells to attached cells was evaluated.

The migratory activity of $D5.7A^+$ cells was evaluated in a matrigel assay. Membranes with a pore size of 8 μm were coated with 50 μl matrigel (Sigma, St Louis, MO, USA). The membranes were inserted into 24 well plates which contained medium without supplements. Tumor cells (1×10^4) were suspended in RPMI 1640 supplemented with 10% FCS and were seeded on the matrigel. Plates were incubated for 48 h at 37°C. Thereafter cells at the bottom of the chamber were counted using an inverted microscope.

Proliferation of D5.7A transfected cells was tested by [^{3}H]thymidine incorporation. Cells were seeded on control IgG or D5.7 coated plates. The supernatant contained either a control antibody or D5.7 [^{3}H]thymidine was added immediately or after 16–72 h. After 8 – 16 h of incubation

with [^{3}H]thymidine, cells were trypsinized and transferred to a β-counter to evaluate thymidine incorporation.

Metastasis assay

BDX rats received 5×10^5 tumor cells, intrafootpad (i.f.p.). The tumor and the draining lymph node were excised by amputation in the knee as soon as the primary tumor reached a diameter of 0.5 cm. Where indicated rats received twice per week an intravenous injection of 200 μg/ml D5.7 or of a control IgG1 antibody, starting at the day of tumor cell inoculation. Animals were observed for local tumor growth, weight loss, anemia and shortage of breathing. They were sacrificed at a defined stage or before reaching a moribund stage. Metastasis formation was controlled macroscopically and by histology.

Statistics

Significance of differences was evaluated by the two tailed student's *t*-test.

Abbreviations

BSA: bovine serum albumin, FCS: fetal calf serum, i.f.p.: intrafootpad, mAB: monoclonal antibody, PROG: progressor, RG: regressor.

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