The differentiation antigen Ly-6E.1 is expressed in mouse metastatic tumor cell lines

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Abstract We report the cloning of the mouse surface GPI-anchored Ly-6E.1 protein from a highly metastatic mouse adenocarcinoma cell line CSML-100 by differential display. The expression is specific for the metastatic cell line as the closely related, non-metastatic mouse adenocarcinoma cell line CSML-0 does not express Ly-6E.1. Northern blot analysis reveals expression in a number of mouse tumour cell lines, exclusively metastatic ones. To date, active Ly-6A/E has only been described in lymphoid cells. The correlation between Ly-6E.1 expression, and the ability to metastasize, is discussed.

Key words: Tumor progression; Ly-6E.1; Metastasis; Adhesion; Differential display

1. Introduction

The mouse differentiation antigen Ly-6E.1 is a member of the multigene family *Ly-6* and allelic to Ly-6A.2 [1]. The family that consists of 8 members is located on chromosome 15 [2]. Since the cDNA of Ly-6E.1 was cloned in 1986, extensive work has been done to reveal the physiological role of this gene. The protein has been shown to be involved in T-cell activation and has also been suggested to contribute in cell–cell adhesion, but no ligand has so far been identified [2–5]. To date, functional Ly-6A/E has been restricted to lymphoid cells, although its expression in brain, kidney, heart, liver and spleen, as well as in polyoma virus-transformed BALB/c 3T3 cells has been reported [6–9].

Ly-6E.1 is a GPI-anchored protein and thus does not have a cytoplasmic domain. Nevertheless, it is well established that cross-linking of mAbs against Ly-6A/E can contribute to, or even alone induce, activation of T-lymphocytes [2,3,10]. Different models for such a signal transduction through GPI-anchored proteins have been proposed [10]. The cellular responses are broad and include increased protein tyrosine phosphorylation, raised intracytoplasmic Ca^{2+} concentration, increased IL-2 secretion, elevated NF-κB and AP-1 binding activities, as well as a general T-cell stimulation as seen by a prominent increase in incorporation of radiolabelled thymidine [11,12]. However, the function of the Ly-6A/E protein in cells other than lymphoid, e.g. epithelial or fibroblast cells, has not yet been investigated at the molecular level.

In the present work, we describe the detection of Ly-6E.1 expression in a highly metastatic cell line as well as its expression pattern in other mouse tumour cell lines. The polymorphism of the gene is confirmed (*Ly-6A/E*), and no rearrangement of the genes appears to determine the expression level. The protein is shown to be anchored to the cell surface through a GPI moiety. Finally, we discuss the relevance of the protein in the metastatic process.

2. Materials and methods

2.1. Cell culture

All cell lines were grown at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium (DME) containing 10% fetal bovine serum (Gibco-BRL, Life Technologies), penicillin (225 IU/ml, kindly provided by Leo Pharmaceuticals, Denmark) and streptomycin sulphate (30 μg/ml).

2.2. Differential display

Total RNA was isolated by the guanidine thiocyanate method [13] and subsequently treated with DNAse I (Boehringer-Mannheim). Differential display (RT-PCR) was performed as described [14,15] with the 3′-primer 5′-T_{11}CG-3′ and the 5′-primer 5′-GGTACTAAGG-3′ (DNA technology, Denmark). Cycling parameters were 94°C for 30 s, 40°C for 2 min, 72°C for 30 s for 40 cycles followed by 72°C for 7 min.

2.3. cDNA cloning

Total RNA from CSML-100 cells was reverse transcribed at 37°C for 1 h using the primer 5′-T_{11}CG-3′ (DNA technology, Denmark) and SuperScript reverse transcriptase (Gibco-BRL). The cDNA was PCR amplified using primers complementary to the ultimate 5′- and 3′-sequences of the Ly-6E.1 mRNA (5′-GAATTCCCTG-CAACCTTGTC-3′ and 5′-GGGAGAACAAAGGGTTTATT-3′ (DNA technology, Denmark)). Cycling parameters were 94°C for 30 s, 55°C for 2 min, 72°C for 30 s for 25 cycles followed by 72°C for 7 min. The amplified cDNA was purified through gel electrophoresis, and the excised 861 bp fragment was subsequently cloned into pCRII, using the TA Cloning Kit (Invitrogen, USA) as described by the manufacturer.

2.4. Northern hybridisation

Fifteen micrograms of total RNA from CSML-0 and CSML-100 cells was electrophoresed on a 1% agarose formaldehyde gel and blotted onto Hybond-N membranes (Amersham, UK). The 861 bp cDNA fragment of Ly-6E.1 was labelled by random priming using Megaprime RPN 1607 (Amersham, UK) with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham, UK). The prehybridization and hybridization was carried out at 37°C in buffer containing 10× Denhardt's solution, 4× standard saline citrate (SSC), 0.4% sodium dodecyl sulfate (SDS), 0.1 mg/ml single-stranded sperm DNA and 50% formamide. Filters were washed at 65°C in 0.5×SSC and 0.2% SDS for 1 h. Autoradiographs were done on X-OMAT (Kodak) films for 1–5 days in Hypercassettes (Amersham, UK) at −80°C or on Phoshor Screens (Molecular Dynamics) and quantitated using a Phosphor Imager (Molecular Dynamics).

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Abbreviations: GPI, glycosyl phoshatidyl inositol; RT-PCR, reverse transcription-polymerase chain reaction; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); PMSF, phenylmethylsulfonyl fluoride; DTT, DL-dithiothreitol; mAb, monoclonal antibody; pAb, polyclonal antibody

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2.5. Southern hybridisation

Cells were harvested by trypsination and washed in PBS. Chromosomal DNA was isolated as described [16] except that a chloroform extraction was included and that the DNA was precipitated twice. Ten micrograms of DNA was digested for 12 h, electrophoresed in a 0.8% agarose gel, and blotted onto Hybond-N membranes (Amersham, UK). Hybridisations were performed as described in Section 2.4.

2.6. DNA sequencing

Sequencing reactions of plasmid DNA were performed using the dideoxynucleotide chain-termination method (Plasmid Sequencing Kit, Amersham, USA). The 534 bp Ly-6E.1 fragment was sequenced using primers 5′-AACAGCTATGACCATG-3′, 5′-GTAAAACGACGGCCAGT-3′, 5′-CGCCAGTGTGCTGGAATTCG-3′ and 5′-TGATGGATATCTGCAGAATTCGGC-3′ (DNA technology, Denmark) annealing to pCRII. The 861 bp Ly-6E.1 sequence was sequenced using the same primers as noted in Section 2.3.

2.7. Antibodies and peptides

Polyclonal rat antibodies against Ly-6E.1 were obtained by immunization of Vista rats with recombinant Ly-6E.1 protein. Peroxidase-conjugated rabbit anti-rat IgG (P450) and peroxidase-conjugated goat anti-rabbit IgG (P448) were purchased from DAKO (Denmark). Rabbit polyclonal antibodies against PDGF-R were kindly provided by Dr. Klaus Hansen, Danish Cancer Society [17].

2.8. Western blotting

Cells were lysed in TNT buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 25 mM NaF, 1 mM Na_3VO_4, 1 mM DTT, 200 μg/ml PMSF and 2 μg/ml each of aprotinin, leupeptin and antipain), and protein concentrations were determined using the Bradford assay (Bio-Rad). Samples were subjected to SDS-PAGE as described [16]. After electrophoresis, proteins were transferred to Hybond-C nitrocellulose filters (Amersham) using a semidry blotting system. After blocking the filter for 1 h in blocking buffer (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, 5% non-fat dry milk powder), filters were incubated with primary antibody (anti-Ly-6E.1 pAb, 1:1000; anti-PDGF-R pAb, 1:400) in blocking buffer for 1 h at room temperature or at 4°C overnight. Incubation with secondary antibodies were done in blocking buffer for 1 h at room temperature. An enhanced chemiluminescence detection system (ECL, Amersham) was used for developing filters.

2.9. PI-PLC treatment of cells

Cells were detached using PBS (without Ca^{2+} and Mg^{2+}, supplemented with 2.5 mM EDTA) and washed in PBS. The cells were incubated in DME with or without 40 μg/ml PI-PLC at 37°C for 1.5 h. Cells were washed and lysed as described in Section 2.8. *E. coli* containing the pIC plasmid encoding *B. cereus* PI-PLC was kindly proveded by Dr. Dirk Heinz, University of Freiburg, Germany. Purification of PI-PLC was performed as described [18].

3. Results

3.1. Detection, cloning and expression

Differential display was performed on total RNA from the two cell lines, CSML-0 and CSML-100. This resulted in a number of amplified sequences of which one was 534 bp,

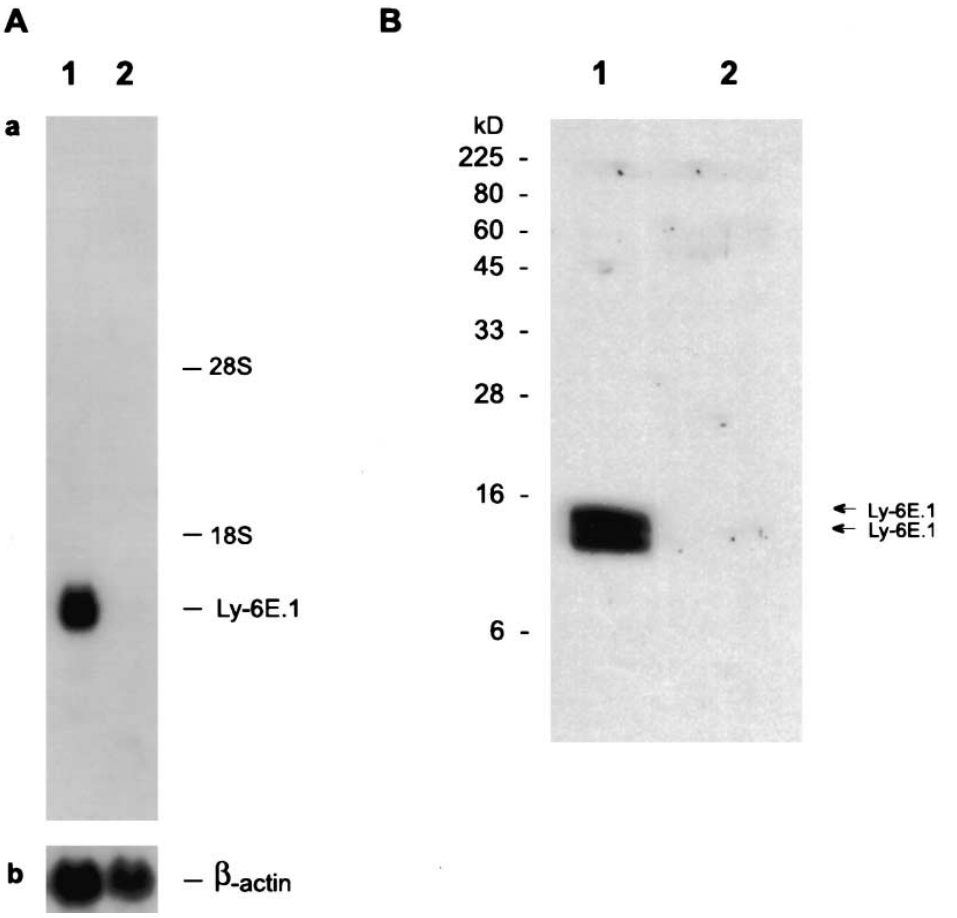

Fig. 1. Expression of Ly-6E.1 mRNA and protein in CSML-100 and CSML-0 cells. A: Northern blot analysis of total RNA (15 μg/lane). The filter was hybridized with the 861 bp of Ly-6E.1 cDNA (a), stripped and reprobed with β-actin (b). Lane 1: CSML-100; lane 2: CSML-0. B: Western blot analysis of total cell lysate from CSML-100 (lane 1) and CSML-0 cells (lane 2) using rat polyclonalclonal antibodies against Ly-6E.1.

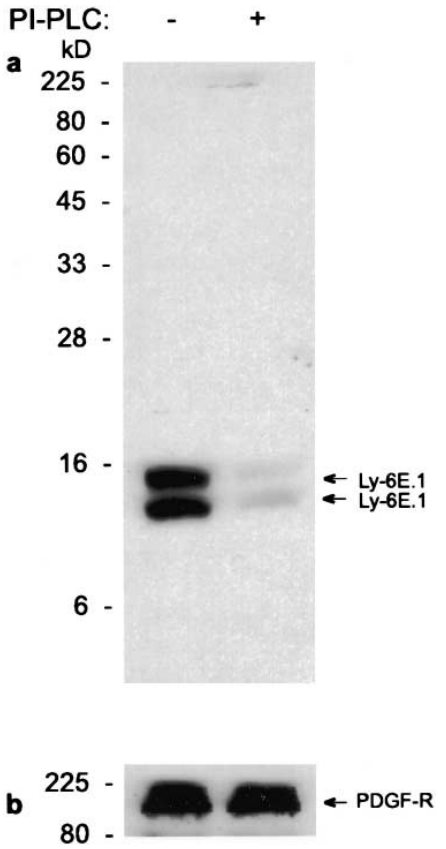

Fig. 2. Sensitivity of the Ly-6E.1 protein to PI-PLC in CSML-100 cells. a: Cells were treated with PI-PLC for 1.5 h as described in Section 2.9. Cell lysates from treated (+) or untreated (−) cells were subjected to Western blot analysis, using rat anti-Ly-6E.1 pAb. b: Western blot analysis using rabbit anti-PDGF-R of the same cell lysates as in (a), as a control for unspecific protease activity in the PI-PLC purification.

which was only detectable in CSML-100 cells. The fragment was cloned and sequenced and shown to be the 534 3′ bp of Ly-6E.1 mRNA.

To perform further investigation of this differential Ly-6E.1 expression, the full-length sequence of Ly-6E.1 mRNA was cloned from CSML-100 cells by RT-PCR. The cloned sequence was 861 bp, and thus did not contain the described 15 bp intron-like sequence from bp 61 to 75 [1].

To reveal the exact level of expression in the metastatic and the non-metastatic cell lines, Northern blot analysis was performed. It was shown that the mRNA level in CSML-100 cells was relatively high (Fig. 1A, lane 1), whereas no expression was detected in CSML-0 cells (Fig. 1A, lane 2).

The cell line-specific Ly-6E.1 expression was also confirmed at the protein level, as determined by Western blot analysis using generated polyclonal antibodies (Fig. 1B). As expected, a high level of protein expression was seen in CSML-100 cells (lane 1) while no protein was observed in CSML-0 cells (lane 2). The protein appeared as a doublet, with sizes between 14 and 15 kDa, which is in good correlation with earlier data from lymphoid cells. The different mobility of the two Ly-6E.1 forms has been explained by different states of glycosylation [19].

To localize the Ly-6E.1 protein in cells, immunocytostaining was performed. These experiments showed that Ly-6E.1 is expressed mainly on the cell surface of CSML-100 cells (data not shown). No staining was seen on CSML-0 cells.

3.2. *Ly-6E.1 is anchored to the membrane via a GPI-anchor on CSML-100 cells*

Ly-6E.1 is described to be GPI-anchored on the cell membrane of lympoid cells. Knowing that the protein is expressed on the cell surface, and that it exists in two forms (Fig. 1B), lead us to investigate the nature of the two variants of the protein. To clarify whether both of the two forms are located on the cell surface, and whether they are both GPI-anchored,

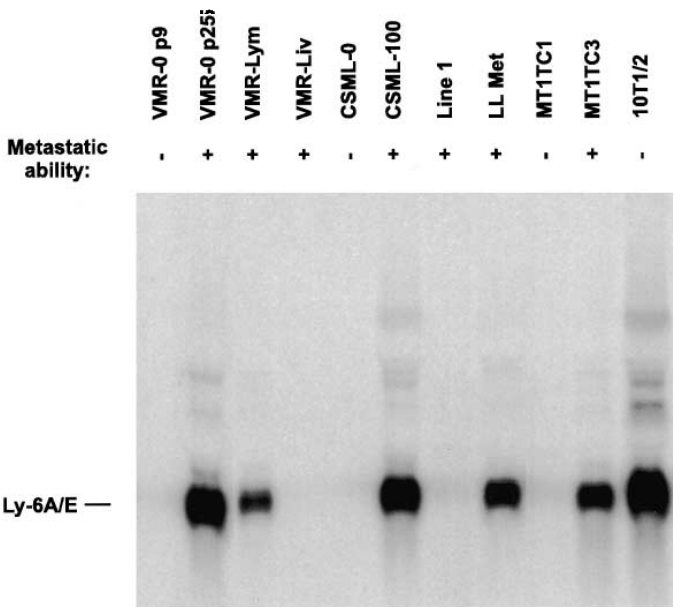

Fig. 3. Expression of Ly-6A/E mRNA in different mouse tumour cell lines and a fibroblast cell line. Northern blot analysis of total RNA (15 μg/lane). The names of the used cell lines is indicated over each lane. The loading is balanced according to ethidium bromide staining of the agarose gel. The Ly-6E.1 probe used is cross-hybridizing with the allelic Ly-6A.2 mRNA. The metastatic ability was determined through subcutaneous injection.

treatment of whole cells with a GPI-specific phospholipase C (PI-PLC) was performed. CSML-100 cells were treated with purified recombinant *B. cereus* PI-PLC (see Section 2.9), and total cell lysate were subjected to Western blot analysis. Upon PI-PLC treatment, both protein bands almost disappeared (Fig. 2a), showing that they both represent GPI-anchored forms of the protein. To exlude that the removal of Ly-6E.1 is caused by contaminating protease activity in the PI-PLC preparation, Western blot against the PDGF receptor from the same lysates was performed. The transmembrane receptor protein remained intact after the treatment (Fig. 2b), verifying the purity of the PI-PLC.

Thus, Ly-6E.1 is GPI-anchored on the cell membrane of CSML-100 adenocarcinoma cells, as on lymphoid cells.

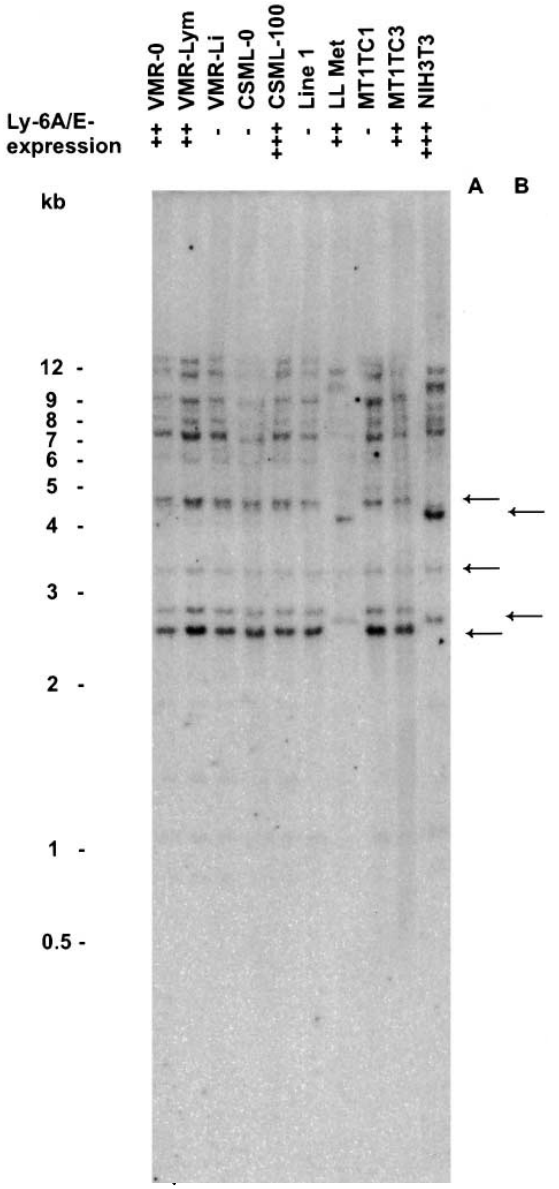

Fig. 4. Southern blot analysis of *Eco*RI-digested chromosomal DNA from various mouse and human tumour cell lines and a mouse fibroblast cell line. The 861 bp Ly-6E.1 cDNA was used as radioactive probe. Approximately 10 μg DNA are loaded in each lane. The cell lines used are indicated over each lane. Molecular sizes (in kb) are indicated at the left side. Arrows in (A) indicate Ly-6E.1-specific bands whereas arrows in (B) indicate Ly-6A.2-specific bands.

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                +1
Ly-6E.1 [M] DTSHTTKSC [LL] I [LL] V [A] LL [CA] ERAQGLE [C] YQ [C] YG
muPAR1  [M] GLPRRL--- [LL] L [LL] L [A] TT [CA] -182aa- [C] YS [C] EG
mEGFR   [M] RPSGTARTT [LL] V [LL] T [A] L- [CA] -164aa- [C] PK [C] DP

Ly-6E.1 VPFETS [C] PS-----IT [C] PYPDGV [C] VTQEAAVIVGSQTRKVK
muPAR1  NNTLG- [C] SSEEASLIN [C] RGPMNQ [C] LVATGLDVLGNRSYTVR
mEGFR   S----- [C] PN-----GS [C] WGGGEN [C] QKLTKII----------

                                                        +79
Ly-6E.1 NNL [C] LPI-- [C] PPNIESMEILGTKVNVKTS [CC] QEDL [C] N*
muPAR1  G-- [C] ATASW [C] QGSHVADSFPTHLNVSV-S [CC] HGSG [C] N*
mEGFR   --- [C] AQQ-- [C] SHRCRGR--------SPSD [CC] HN-- [C] A*
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Fig. 5. Homology between the proteins Ly-6E.1, mouse uPAR and mouse EGFR. It is visible that there is high homology in the signal sequence of all proteins. All of the 10 cysteines in the mature Ly-6E.1 protein that are important for forming structural important intrachain disulfide bonds are conserved in uPAR and EGFR. – indicates the amino acid to which the GPI-anchor is attached on Ly-6E.1 and uPAR. *Only the sequence N-terminal of amino acid number 79 in Ly-6E.1 (or homologous sequences in muPAR1 and mEGFR) onto which the GPI-moiety is attached are shown.

3.3. *Ly-6A/E mRNA expression in various mouse tumour cell lines*

To investigate whether Ly-6A/E is commonly expressed in mouse tumour cell lines, Northern blot analysis of a number of cell lines was performed. Eight cell lines, in addition to CSML-0 and CSML-100, were selected. All cell lines are mouse adenocarcinomas, except Line 1 and LL Met which are lung carcinomas. Of the 10 cell lines, Ly-6A/E was exclusively expressed in metastatic ones (Fig. 3). Expression was also detected in 10T1/2, which is an immortalized fibroblast cell line.

Thus, Ly-6A/E seems to be overrepresented in metastatic cell lines versus non-metastatic ones.

3.4. *Southern blot analysis excludes genomic rearrangement or gene duplication of Ly-6A/E*

To study the origin of this cell-specific expression, Southern blot analysis of the *Ly-6A/E* gene, in CSML-0 and CSML-100 cells, as well as in other tumor cell lines, was performed. Ly-6E.1 cDNA was used as radioactive probe, knowing that it cross-reacts with the sequences of other members of the *Ly-6* gene family. In Fig. 4, a Southern blot of *Eco*RI-digested DNA from 11 cell lines is shown. The restriction pattern for CSML-0 and CSML-100 seems to be identical. There also seems to be no major differences in the banding pattern among the other Ly-6^a haplotype (Ly-6E.1) cell lines, irrespective of Ly-6E.1 expression (all Ly-6^a cell lines originate from either A/Sn or BALB/c mice). Two cell lines, LL Met and NIH 3T3 (originating from C57BL/6 and Swiss mice, respectively), were shown to be of the Ly-6^b haplotype (Ly-6A.2 allele), both expressing the gene. The intensity of the Ly-6A/E-specific bands is well correlated with the amount of DNA in each lane, as determined by ethidium bromide staining of the agarose gel before blotting (data not shown). It is therefore unlikely that substantial rearrangement of the *Ly-6A/E* gene, as well as gene duplication, is causing the differential expression. More likely, is a specific transcriptional regulation of the *Ly-6A/E* gene in the investigated tumor cell lines.

4. Discussion

In the presented experiments, the *Ly-6E.1* gene is identified

as highly expressed in a highly metastatic mouse adenocarcinoma cell line, CSML-100. In contrast, the gene is not expressed in a non-metastatic mouse adenocarcinoma cell line, CSML-0.

In order to get a picture of the Ly-6A/E expression in mouse tumor cell lines in general, eight additional cell lines were checked. Five cell lines expressed Ly-6A/E at various levels. All of these cell lines are metastatic, no expression was seen in non-metastatic ones.

The described expression pattern in different mouse tumor cell lines, motivates speculation of this genes involvement in tumorigenesis and tumor progression. However, the cell lines 10T1/2 (from C3H mice) and NIH 3T3 (from Swiss mice), which both are immortalized fibroblasts, does express Ly-6E.1 and Ly-6A.2, respectively, at high levels (Fig. 3, data not shown). Both of these cell lines are non-transformed and thus non-tumorigenic. This shows that Ly-6A/E not per se is a transforming protein, oncogene, but that its expression in metastatic cell lines might contribute to the progression towards a more aggressive phenotype. The data are in good agreement with the recent report, in wich the malignant phenotype of Ly-6A/E-expressing polyoma virus-transformed BALB/c 3T3 cells, was described [5]. The cells were transformed, and sorted in populations expressing Ly-6A/E at high versus low levels. In vivo experiments showed that the population expressing Ly-6A/E at a high level simultaneously displayed a considerably more malignant phenotype. The question whether the Ly-6E.1 expression itself is persuading the increased metastatic ability, or if it simply is a sign and a secondary effect of increased malignancy, has not yet been answered.

The involvement of Ly-6A/E in tumor progression can with the present data be hypothesized in two ways.

First, is its ability to mediate signal transduction in T-cells a possible explanation. It has earlier been shown that cross-linking of Ly-6E.1 can mediate increased binding activities of nuclear factor κB (p65-p50) and AP-1 (Fos/Jun) [11]. The increased activity of these transcription factors may promote the tumour progression. Nonetheless, it should be noted that since no ligand for Ly-6E.1 has been identified, all results obtained through cross-linking experiments using mAbs against this protein may be irrelevant.

A second theory is based on the fact that Ly-6A/E can confer homotypic aggregation of transgenic thymocytes overexpressing Ly-6A/E as well as aggregation with non-transgenic thymocytes, T-lymphocytes and B-lymphocytes [5]. This shows that Ly-6A/E is involved in cell–cell adhesion and that there exists a naturally occurring ligand expressed on at least lymphoid cells. The data are obtained by in vitro experiments; thus it is still unknown whether the shown adhesion has a physiological role. Nevertheless, it is not difficult to speculate a possible role in metastasis. Metastasis is a multistep cascade where adhesion of the tumor cells to endothelial cells in the distant organ is a crucial step [20]. The proposed involvement of Ly-6A/E in this process has to be investigated closer.

When interpreting the above considerations, one should know that the Ly-6E.1 protein has high homology with structural important amino acids in both uPAR and mouse EGFR. The scheme in Fig. 5 shows that in addition to homology in the signal sequence, all 10 cysteines in the mature Ly-6E.1 protein can be aligned to the two other proteins. This suggests a related tertiary structure of the proteins and can indicate a similar or related function among them. This might help in the study of the function of Ly-6E.1 in cancer cells, as well as in the search for a ligand. Recently two human genes, *E48* [21] and *RIG-E* [22], were cloned. Both proteins have high homology to members of the Ly-6 family. This might indicate the presence of a human '*Ly-6*' gene family.

To investigate the speculated roles and importance of Ly-6E.1 in the metastatic cascade, transfection experiments conducting gain and loss of function of this protein, involving different tumour cell lines, followed by in vivo investigations need to be done.

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