The leukocyte specific actin-binding protein L-plastin is aberrantly expressed in several nonhematopoietic malignant tumors. However, little is known about the functional consequences of L-plastin expression. Here, we investigated the function of L-plastin in human malignant melanoma cells. Knock-down of endogenous L-plastin by siRNA treatment reduced migration of the melanoma cell line IF6. However, in melanoma patients, no correlation existed between L-plastin expression and tumor stages. This implied that additional factors such as phosphorylation of L-plastin may influence its function in tumor cells. To investigate this further, EGFP-tagged wild-type L-plastin (wt-LPL-EGFP) and a mutated, nonphosphorylatable L-plastin protein (5A7A-LPL-EGFP), were expressed in the L-plastin negative melanoma cell line MV3. Biochemical analysis revealed that wt-LPL-EGFP is phosphorylated in MV3 cells while 5A7A-LPL-EGFP is not. Although both wt-LPL-EGFP and 5A7A-LPL-EGFP were targeted to, and promote the formation of, vinculin-containing adhesion sites, static adhesion to either Matrigel or isolated extracellular matrix molecules was neither influenced by expression of wt-LPL-EGFP nor by expression of 5A7A-LPL-EGFP when compared with EGFP expressing control cells. In contrast, haptotactic, but not chemotactic, migration of melanoma cells towards either Matrigel or isolated extracellular matrix molecules was similarly enhanced, if either wt-LPL-EGFP or 5A7A-LPL-EGFP were expressed in MV3 cells. Interestingly, only cells expressing the phosphorylatable wt-LPL-EGFP protein showed enhanced invasion into Matrigel. In line with these findings the \textit{in vivo} metastatic capacity of mouse B16 melanoma cells correlates with expression and phosphorylation of L-plastin. These data show that an increase in melanoma cell invasiveness requires not only expression but also phosphorylation of L-plastin.

**Material and methods**

**Antibodies**

Function blocking monoclonal antibodies directed against different integrin subunits were from Chemicon (Tencula, USA). The mouse monoclonal L-plastin antibody (LPL4A.1) was obtained from Lab Vision. The rabbit polyclonal antiserum (RecPla) against recombinant L-plastin was produced in our own laboratory. The mouse monoclonal S100 antibody was purchased from Novoceastra, and the mouse monoclonal CD45 antibody was obtained from Serotec. The rabbit polyclonal EGFP antiserum was from BD Clontech. TRITC-conjugated phallolidin, TRITC-conjugated wheat germ agglutinin (WGA) and the mouse monoclonal vinculin antibody were obtained from Sigma (Taufkirchen, Germany).

**Immunohistochemistry**

Human malignant melanoma specimens from patients were routinely fixed, dehydrated and paraffin embedded. Sections of 5 \mu m thickness were used for immunohistochemistry. For detection of L-plastin expression, the monoclonal L-plastin antibody (clone LPL4A.1) and the universal DAKO APAAP (alkaline phosphatase

*Correspondence to:* Department of Immunology and Serology, University of Heidelberg, Im Neuenheimer Feld 305, D-69120 Heidelberg, Germany. E-mail: yvonne.samstag@urz.uni-heidelberg.de

Received 10 August 2006; Accepted after revision 15 December 2006

DOI 10.1002/j.22589
Published online 8 February 2007 in Wiley InterScience (www.interscience.wiley.com).
anti-alkaline phosphatase) kit were used. Sections were deparaffinized, treated in a microwave oven for 15 min at 600 W in 10 mM citrate buffer pH 6.1, washed in Tris-buffer (0.05 M Tris pH 7.6 and 0.15 M NaCl) and incubated with 5 μg/ml of the L-plastin antibody in Tris-buffer for 1 hr at room temperature. Adjacent sections were stained for S100 and CD45 to identify tumor cells and lymphocytes, respectively. The alkaline phosphatase anti-alkaline phosphatase (APAAP) staining technique was performed according to the instructions of the manufacturer. Cell nuclei were counterstained with hematoxylin. Specimens were analyzed by light microscopy.

**Statistical analysis**

For statistical analysis, penetration depth (Clark level), tumor stage (TMN classification, UICC 1987) and L-plastin expression were taken into account. The χ² test was used to analyze the statistical significance of the relationship between L-plastin expression and penetration depth or tumor stage. p Values were considered significant when p < 0.05.

**Generation of cell lysates**

Cells were washed once in ice-cold Tris-buffered saline (TBS) and lysed for 30 min on ice in TKM-lysis buffer as described. Postnuclear lysates were either used directly or frozen in liquid nitrogen and stored at −80°C until further use.

**SDS-Polyacrylamide gel electrophoresis and two-dimensional gel electrophoresis**

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to standard procedures. For two-dimensional (2D) gel electrophoresis, ReadyStrip™ IPG (immobilized pH gradient) 17 cm strips pH 4–7 (BioRad) and the BioRad Protein IEF cell system were used according to the instructions supplied by the manufacturer. Following isoelectric focussing, equilibrated IPG strips were placed on top of an SDS-PAGE gel and sealed with 1% LowMelt agarose (w/v) prepared in SDS-PAGE running buffer. Gels were run overnight until the bromphenol blue front reached the end of the gel.

**Immunoblotting**

Proteins were transferred onto PVDF membranes (0.45 μm pore size) according to standard procedures. Membranes were blocked and proteins were detected by incubation with the respective primary antibodies at the indicated concentrations overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (Jackson ImmunoResearch). Bound antibodies were visualized by enhanced chemiluminescence detection (ECL, Amersham).

**Plasmid constructs and site directed mutagenesis**

The plasmid containing the wild-type (wt) full-length L-plastin cDNA was a generous gift from P. Matsudaira (Whitehead Institute for Biomedical Research, Cambridge, MA) and was used for construction of expression plasmid vectors for transfection studies. Point mutations of Ser5 and Ser7 [5SASTA7 (TCA → GCA), (TCC → GCC)] were created using the QuickChange site-directed Mutagenesis XL Kit (Stratagene, La Jolla) according to the instructions of the manufacturer.

To generate full-length L-plastin-EGFP fusion proteins, the entire coding region of L-plastin was amplified from the wt- or S5A7ST-A-L-plastin cDNA by polymerase chain reaction (PCR) introducing a 5′ BamHI and a 3′ EcoRI restriction site and inserted into the BglIII/EcoRI digested pEGFP-N1 vector (BD Clontech) upstream and in-frame with the EGFP cDNA. All constructs were controlled by standard DNA sequencing.

**Cell culture, transfections and generation of stable expressing cell populations**

The human melanoma cell lines S30, IF6, BLM and MV3 (a generous gift of Dr. van Muijen, University Hospital Nijmegen, The Netherlands), and the mouse melanoma cell lines B16F1 and B16F10 were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (PCS), nonessential amino acids, 2 mM glutamine, 25 mM HEPES and 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂.

MV3 cells were transfected using the Lipofectamine 2000 (LF2000) reagent (Invitrogen) following the manufacturer’s instructions. Briefly, cells were seeded in a 24-well plate at 2 × 10⁵ cells/well and transfected with 0.8 μg of the respective cDNA. Cells were incubated for 48 hr until they were analyzed for expression of the transgene.

To establish stable transfectants, MV3 cells were selected for resistance to the appropriate antibiotic (1 mg/ml G418). Transfectants were bulk sorted at least 2 times by EGFP fluorescence (FACSVantage cell sorter, Beckton Dickinson) to obtain stable expressing cell populations (more than 90% expressing cells). Sorted cells were kept at 1 mg/ml G418 and were monitored routinely for expression of the EGFP fusion protein by flow cytometry.

**Generation of 3T3 conditioned medium**

Conditioned medium was prepared by incubating confluent 3T3 cell cultures grown in 10 cm dishes with 6 ml of serum-free RPMI1640 medium for 2–3 days. The medium was then collected, clarified by centrifugation and stored in aliquots at −20°C until use.

**Preparation of esiRNA**

 esiRNA was prepared from ssRNA as described. In brief, ssRNA was obtained from a polymerase chain reaction (PCR)-derived L-plastin DNA template (obtained from the esiWay Silencing Resource, RZPD Heidelberg/Berlin, RZPDp3000F087D) carrying T7 promoters, and was digested with Dicer (cloned human Dicer, Ambion) for 16 hr at 37°C. The Dicer generated dsRNA of 21 bp (esiRNA) was purified, and transfections of human melanoma cells were done as mentioned above using 100 ng esiRNA (or a scrambled control siRNA (obtained from Ambion)) and 1 μl lipofectamine 2000 for each well of a 24-well plate containing 500 μl of growth medium. Cells were analyzed 72 hr after transfection.

**Flow cytometry**

Cells (2 × 10⁵) expressing either EGFP or EGFP-fusion proteins were pelleted, resuspended in 300 μl PBS and analyzed directly for EGFP-fluorescence with a FACSCalibur flow cytometer using the CellQuest Pro software (Becton Dickinson).

**Immunofluorescence staining and confocal laser scanning fluorescence microscopy**

For immunofluorescence staining, cells were plated on collagen I- or Matrigel-coated glass coverslips for the indicated time at 37°C. Coverslips were washed in PBS (PBS containing 1 mM CaCl₂ and 0.5 mM MgCl₂) and adherent cells were fixed in 3% paraformaldehyde, quenched in 50 mM NH₄Cl and permeabilized with 0.3% Triton X-100. Cells were blocked in PBS−G (PBS containing 0.2% gelatine and 0.02% sodium azide) and stained with phallolidin-TRITC, WGA-TRITC or a monoclonal vinculin antibody. Cells were then washed twice in PBS−G, twice in PBS−G, twice in dH₂O and mounted in Moviol. Samples were examined by confocal laser scanning microscopy (Leica TCS NT). Images were processed using Adobe Photoshop 6.0.
Adhesion assay

For the analysis of tumor cell adhesion, 96-well flat bottom plates (Nunc, Maxisorp Immunoplate) were coated with either Matrigel in PBS, human fibronectin (BD Biosciences) in carbonate buffer (15 mM Na2CO3 and 35 mM NaHCO3, pH 9.4) or with rat collagen I (BD Biosciences) in 0.02 N acetic acid at the indicated concentrations for 1 hr at 37°C. Wells were washed once with PBS+C and blocked with 0.5% BSA (w/v) in PBS+C for 30 min at room temperature. After washing the wells with PBS+C, 50 µl of PBS+C containing 0.1% BSA (w/v) were added to each well.

Cells grown in culture were released from the cell culture dishes by incubation with PBS containing 4 mM EDTA for 10 min at 37°C, pelleted and resuspended in PBS containing 1% BSA (w/v) at a concentration of 10^6 cells/ml. To label the cells, Calcein AM (Vybrant® Cell Adhesion Assay Kit, Molecular Probes) was added (1 µM final concentration) and cells were incubated for 20 min at 37°C/5% CO2. Then, cells were pelleted and resuspended in PBS containing 0.1% BSA at a concentration of 4 x 10^5 cells/ml. Of this cell suspension 50 µl were added to each well of the coated 96-well plate and the plate was centrifuged at 80 g for 1 min. Cells were allowed to adhere at 37°C/5% CO2 for 15 min. The wells were filled to capacity with PBS+C containing 0.1% BSA, and the plate was sealed and centrifuged at an inverted position at 20 g for 2 min. The liquid, containing nonadherent cells, was removed completely and wells were filled with 50 µl of PBS+C. Fluorescence intensity of adherent cells was determined with a fluorescence microplate reader equipped with standard fluorescein filters. For comparison, 50 µl of the original cell suspension were measured directly and taken as the 100% value. In addition, PBS+C filled wells were measured to obtain the background fluorescence. The percentage of adhesion was determined by dividing the background subtracted fluorescence of adherent cells by the total corrected fluorescence of cells (100% value) and multiplying by 100. This assay was repeated at least 3 times with 2 independently generated stable cell populations.

Haptotactic and chemotactic cell migration assay

Haptotactic cell migration was analyzed essentially as described,28 with minor modifications. Briefly, the underside of transwell migration inserts (PET membrane, 8 µm pore size, 6.4 mm diameter, BD Biosciences) was coated with the indicated concentrations of Matrigel, fibronectin, collagen I or BSA as mentioned above. After washing with migration medium (RPMI 1640 medium supplemented with 0.1% (w/v) BSA), filters were placed into the chambers with the coated membrane side facing the lower compartment and 600 µl of migration medium were added to the lower compartment. For chemotaxis experiments, 600 µl of either 3T3 conditioned medium or RPMI 1640 medium supplemented with 10% FCS were added to the lower compartment instead of migration medium. Cells were labeled with CFDA-SE for an additional 30 min at 37°C/5% CO2 with 1 µM CFDA-SE (Molecular Probes) in PBS+. The labeling solution was removed and the cells were incubated in culture medium for an additional 30 min at 37°C/5% CO2. Afterwards, cells were detached from the cell culture dish by incubation with PBS containing 4 mM EDTA for 10 min at 37°C, pelleted, washed and resuspended in migration medium at a concentration of 2.5 x 10^5 cells/ml. Of this cell suspension 200 µl were added to the top of the filter. Chambers were subsequently incubated at 37°C/5% CO2. Cells were allowed to migrate for 3 hr, then filters were removed and cells from the upper membrane surface were wiped off with a cotton swab. Filters were then washed, fixed and mounted on glass slides. Cells that had migrated to the coated side of the filter were detected by confocal laser scan microscopy and cells in 4 defined optical fields were counted for each filter. This assay was repeated at least 3 times with 2 independently generated stable cell populations.

Invasion assay

The invasiveness of tumor cells was determined by using BD BioCoat Matrigel invasion chambers. The Matrigel invasion chambers consist of cell culture inserts containing an 8 µm pore size PET membrane that had been treated with Matrigel basement membrane matrix. Matrigel was rehydrated and 750 µl of either 3T3 conditioned medium or RPMI 1640 medium containing 10% FCS were added to the lower compartment as a chemoattractant. Note that in the absence of FCS in the lower compartment, almost no invasion occurred (data not shown). Cells were labeled with 1 µM CFDA-SE in PBS+ as mentioned above and resuspended in migration medium at a concentration of 1.25 x 10^5 cells/ml. Of this cell suspension 400 µl were added to the top of the membrane and subsequently incubated at 37°C/5% CO2 for 20 hr to allow tumor cell invasion. Numbers of invasive cells on the underside of the membrane were determined as mentioned above. The invasion assay was repeated at least 3 times with 2 independently generated stable cell populations.

**Figure 1** – siRNA mediated knock-down of L-plastin leads to reduced haptotactic migration. (a) Immunoblot analysis: Lysates from the L-plastin positive human melanoma cell line 116, transfected with either no siRNA (mock), a scrambled control siRNA (control siRNA), or the L-plastin specific esiRNA (L-plastin siRNA). Blots were stained with a monoclonal L-plastin antibody (upper panel, L-plastin), the same membrane was then stripped and reprobed with a polyclonal T-plastin antiserum (middle panel, T-plastin) and a monoclonal β-actin antibody (lower panel, β-actin) as controls. The graphs show the normalized results of the densitometric scanning for L-plastin and T-plastin. The expression level in mock-transfected cells was set as 100%. Bars indicate the means of 2 independent experiments and the standard errors of the mean values. (b) Haptotactic migration: IF6 cells transfected with either no siRNA (mock), a scrambled control siRNA (control siRNA) or the L-plastin specific esiRNA (L-plastin siRNA) were allowed to migrate for 6 hr at 37°C across cell culture inserts with 8 µm pore size, which were coated on their lower surface with 20 µg/ml collagen I. The numbers of cells migrated to the lower surface of the cell culture inserts were determined. The number of migrated mock-transfected cells was set as 100. Cells in 4 defined optical fields were counted for each cell culture transwell insert. Bars indicate the means and the standard errors of the mean values. One representative experiment is shown out of 2.
Results

siRNA mediated knock-down of L-plastin leads to reduced haptotactic migration

To investigate, whether expression of L-plastin influences the migratory behavior of human melanoma cells, we transfected the melanoma cell line IF6, which expresses L-plastin, with an L-plastin specific esiRNA (L-plastin siRNA). The L-plastin-specific esiRNA was obtained by reverse transcription of a PCR-derived L-plastin dsDNA template (corresponding to nt 3290–3631 (3′-UTR) of the L-plastin cDNA) into dsRNA, which was then digested with the RNase Dicer. The Dicer generated mix of dsRNAs of 21 bp was purified, and used for lipofections. This led to a reduction of L-plastin protein levels by around 60%, whereas expression levels of T-plastin did not change (Fig. 1a). Indeed, the L-plastin knock-down strongly reduced haptotactic migration of the melanoma cells (Fig. 1b). As expected, transfection of the L-plastin siRNA in an L-plastin negative cell line had no influence on migration (data not shown). Note that static adhesion to extracellular matrix molecules was not altered by the L-plastin siRNA (data not shown).

Expression of L-plastin in human melanoma does not correlate with penetration depths or tumor stages

As the siRNA experiment suggested a role for L-plastin in melanoma cell migration, we investigated, whether L-plastin is expressed in primary human melanomas, and whether the expression of L-plastin correlates with penetration depths or tumor stages. To this end, melanoma specimens derived from patients with different tumor stages were analyzed by immunohistochemistry using the monoclonal L-plastin antibody LPL4A.1 (Fig. 2). This antibody does not cross-react with T- or I-plastin and recognizes both nonphosphorylated as well as phosphorylated L-plastin (compare Fig. 3a). Adjacent sections were stained with the leukocyte marker CD45 as well as with the melanoma tumor marker S100 (Fig. 3f). Surprisingly, the numbers of L-plastin positive tumors in the groups with bad prognosis were not significantly higher compared to the groups with good prognosis. Accordingly, no correlation was found between L-plastin expression and the pene-
but not in 5A7A-LPL-EGFP expressing cells. Henning more acidic spot was detected in wt-LPL-EGFP expressing cells et al. Lin

Phosphorylated L-plastin in MV3 cells makes up monoclonal L-plastin antibody. As shown in Figure 3 2D-gelelectrophoresis followed by immunoblotting using the grown on collagen I-coated cell culture dishes were analyzed by expressing wt-LPL-EGFP or 5A7A-LPL-EGFP cell populations expression in MV3 cells. Lysates obtained from the stably expressing cell populations contained at least 90% of EGFP-fusion-protein fluorescence. Flow cytometric analysis showed that sorted cell an EGFP tag allowed us to bulk sort transfected cells for EGFP from sorted cell populations demonstrated proper expression of the respective fusion proteins (Fig. 3a). No cleavage or degradation of the expressed fusion proteins was observed. Next, we investigated whether wt-L-plastin becomes phosphorylated upon expression in MV3 cells. Lysates obtained from the stably expressing wt-LPL-EGFP or 5A7A-LPL-EGFP cell populations grown on collagen I-coated cell culture dishes were analyzed by 2D-gellectrophoresis followed by immunoblotting using the monoclonal L-plastin antibody. As shown in Figure 3d, a second more acidic spot was detected in wt-LPL-EGFP expressing cells but not in 5A7A-LPL-EGFP expressing cells. Henning et al. and Lin et al. demonstrated earlier by 3P labeling that this second more acidic spot corresponds to phosphorylated L-plastin. Phosphorylated L-plastin in MV3 cells makes up ~15% of total L-plastin as determined by densitometric scanning.

L-plastin promotes the formation of vinculin-containing adhesive structures (podosomes) independent of its phosphorylation state

As it was reported that prolonged exogenous expression of L-plastin changes the shape of fibroblast-like cells, we first characterized the overall morphology of the stably L-plastin-EGFP transfected MV3 cells. To this end, cells were grown at high cell densities on collagen I-coated glass coverslips for 18 hr and analyzed by confocal laser scanning microscopy (Fig. 4a). Under these conditions, MV3 cells were adherent and show only little motility. Counterstaining of the actin cytoskeleton with phalloidin (Fig. 4a, F-actin) and with the membrane binding lectin wheat germ agglutinin (data not shown) demonstrated that no changes in the overall cell morphology occurred upon expression of either EGFP, or wt-LPL-EGFP, or 5A7A-LPL-EGFP fusion proteins (Fig. 4a).

In migrating macrophages, a portion of L-plastin is phosphorylated and found in adhesive structures called podosomes. Therefore, we determined whether L-plastin localizes to podosomes in migrating melanoma cells. To this end, stably transfected MV3 cells were allowed to adhere to and spread on Matrigel-coated glass coverslips at low cell densities for 1 hr in serum-free medium. Under these conditions (in contrast to Fig. 4a), most of the cells are motile and display cell extensions. The cells were processed for immunofluorescence staining using phalloidin to visualize F-actin, and a monoclonal vinculin antibody to visualize podosomes (Fig. 4b). Colocalization of the different L-plastin fusion proteins with vinculin was then analyzed by confocal laser scanning microscopy. EGFP-transfected control cells only rarely formed vinculin-containing podosomes at the tips of cell extensions (Fig. 4b; i and v). EGFP alone was absent from the tips of cell extensions (Fig. 4b; i and v), whereas F-actin was nevertheless found there (Fig. 4b; ii). In contrast, both wt-LPL-EGFP and 5A7A-LPL-EGFP transfected cells frequently formed vinculin-containing podosomes at the tips of cell extensions (Fig. 4b, vii and xiii). Moreover, both wt-LPL-EGFP (Fig. 4b; vi and x) and 5A7A-LPL-EGFP (Fig. 4b; vii and xvi) fusion proteins colocalized with vinculin and F-actin in these structures. We conclude that L-plastin promotes formation of, and is targeted to, vinculin-containing podosomes at the tips of cell extensions independent of its phosphorylation state.

Adhesion of human melanoma cells to extracellular matrix molecules is not influenced by L-plastin expression

The ability of tumor cells to adhere to and interact with different components of the extracellular matrix (ECM), an integrin dependent process, is a prerequisite for migration on and invasion into the basement membrane. Therefore, we studied the effect of L-plastin expression on MV3 cell adhesion to components of the

| TABLE I – RELATIONSHIP BETWEEN L-PLASTIN EXPRESSION AND TUMOR STAGES |
|--------------------------|------------------|----------------|
| Number of specimens analyzed | L-plastin positive (%) | p value |
| Clark level | I | II | III | IV | V | TMN classification |
| Mis | 10 | 20 | 20 | 19 | 2 | Mis |
| I | 10 | 20 | 8 | 19 | 2 | I |
| II | 10 | 20 | 8 | 19 | 2 | II |
| III | 10 | 20 | 8 | 19 | 2 | III |
| IV | 10 | 20 | 8 | 19 | 2 | IV |
| TMN classification | Mis | Mis | Mis | Mis | Mis | Mis |
| I | 10 | 20 | 8 | 19 | 2 | I |
| II | 10 | 20 | 8 | 19 | 2 | II |
| III | 10 | 20 | 8 | 19 | 2 | III |
| IV | 10 | 20 | 8 | 19 | 2 | IV |
| Clark level | I | II | III | IV | V | TMN classification |
| Mis | 10 | 20 | 8 | 19 | 2 | Mis |
| I | 10 | 20 | 8 | 19 | 2 | I |
| II | 10 | 20 | 8 | 19 | 2 | II |
| III | 10 | 20 | 8 | 19 | 2 | III |
| IV | 10 | 20 | 8 | 19 | 2 | IV |

Clark level: Level I: melanoma in situ (Mis); Level II: tumor thickness < 0.75 mm; Level III: tumor thickness 0.75–1.5 mm; Level IV: tumor thickness 1.51–4.0 mm; Level V: tumor thickness > 4.0 mm. TMN classification (UICC 1987): Stage I: pT1N0M0, pT2N0M0; Stage II: pT3N0M0; Stage III: pT4N0M0, pTxN1M0 and pTnxN2M0; Stage IV: pTxNxM1. Lower part of the table: Clark levels and tumor stages were grouped for good (Clark Level I and II; TMN classification Mis-II) or bad (Clark Level III–V; TMN classification III–IV) prognosis. The number of L-plastin expressing tumors in the groups with poor prognosis (Clark level III–V; TMN classification III–IV) was not significantly (n.s.) higher compared to the group with good prognosis. p values were calculated using the χ² test.
**Phosphorylation of L-plastin enhances invasiveness of human melanoma cells into Matrigel**

Invasion into the basement membrane is a complex task for a cell. It involves adhesive, migratory and proteolytic processes. To analyze the involvement of L-plastin in tumor cell invasion, MV3 cells stably expressing either EGFP, wt-LPL-EGFP or 5A7A-LPL-EGFP were seeded onto Matrigel invasion chambers. As for the chemotaxis assay (compare Fig. 5c, Chemotaxis), the lower compartment of the invasion chamber was either filled with 3T3 conditioned medium or with culture medium containing 10% FCS, whereas the upper compartment was filled with culture medium containing 0.1% BSA. After 20 hr, the numbers of cells that had successfully invaded the Matrigel layer and migrated to the underside of the cell culture insert were determined (Fig. 5d, Invasion). Only expression of phosphorylatable wt-LPL-EGFP, but not of nonphosphorylatable 5A7A-LPL, significantly increased the invasiveness of human melanoma cells when compared with EGFP expressing control cells and nontransfected MV3 cells. The process of invasion was β1-integrin dependent, since the addition of a function blocking β1-integrin antibody completely abolished invasion (data not shown). These data imply that phosphorylation of L-plastin is required to promote tumor cell invasion.

**L-plastin expression and phosphorylation correlates with in vivo metastasis in the mouse B16 melanoma model**

To test whether expression and phosphorylation of L-plastin correlate with tumor cell metastasis in vivo, we took advantage of a well-established mouse melanoma model of 2 isogenic variants of the mouse melanoma cell line B16 with different metastatic capabilities: The incidence of pulmonary metastases after i.v. injection into C57BL/6 mice differs significantly between B16F1 (low metastasis) and B16F10 (high metastasis) cells. Immunoblot analysis of cell lysates obtained from these cell lines revealed that the highly metastatic B16F10 cells expressed higher amounts of L-plastin (Figs. 6a, and 6b) than the low metastatic B16F1 cells. Moreover, as determined by 2D-gel electrophoresis, only the highly metastatic B16F10 cells contained substantial amounts of respective integrins (data not shown). These findings indicate that neither expression nor phosphorylation of L-plastin influences integrin mediated MV3 cell adhesion.

**L-plastin expressing human melanoma cells show enhanced haptotactic, but not chemotactic, migration**

For the analysis of the migratory properties of L-plastin expressing MV3 cell populations, we performed haptotactic migration assays (Fig. 5b). To this end, the underside of transwell inserts (8 μm pore size) was coated with Matrigel or isolated ECM components. Cells were placed on top of the transwell inserts and allowed to migrate across the transwell inserts for 3 hr at 37°C. This analysis revealed that higher numbers of wt-LPL-EGFP protein expressing cells migrated to the underside of the transwell inserts when compared with EGFP expressing control cells and nontransfected MV3 cells. Moreover, the enhancement of haptotactic migration through expression of L-plastin was independent of its phosphorylation state, since wt-LPL-EGFP and 5A7A-LPL-EGFP expressing cells showed equal effects (Fig. 5b). This finding holds true for haptotaxis towards Matrigel, collagen I and fibronectin (Fig. 5b, Haptotaxis). Haptotactic migration represents an integrin mediated, chemotaxis-independent form of migration. Tumor cells can, in addition, be attracted by chemotactic gradients. Therefore, we performed similar migration assays as mentioned above, but in the presence of either 3T3 conditioned medium or 10% FCS as a chemoattractant in the lower compartment (Fig. 5c, Chemotaxis). All cell populations showed comparable levels of chemotactic migration (Fig. 5c, Chemotaxis). Thus, L-plastin expression specifically enhances haptotactic but not chemotactic migration.
FIGURE 5 – Influence of L-plastin expression in melanoma cells on adhesion, migration and invasion. (a) Adhesion of human melanoma cells to extracellular matrix molecules is not influenced by L-plastin expression. MV3 cells stably expressing either EGFP, wt-LPL-EGFP or 5A7A-LPL-EGFP fusion proteins were allowed to adhere for 15 min at 37°C to either Matrigel (upper panel), collagen I (middle panel) or fibronectin (lower panel) coated to the plate in the indicated concentrations. Nonadherent cells were removed and the percentages of adherent cells were determined in triplicate. Bars indicate the means and the standard errors of the mean values. One representative experiment is shown out of at least 3 independent ones. (b) and (c) L-plastin expressing human melanoma cells show enhanced haptotactic, but not chemotactic, migration. (b) Haptotaxis. MV3 cells stably expressing either EGFP, wt-LPL-EGFP or 5A7A-LPL-EGFP fusion proteins were allowed to migrate for 3 hr at 37°C across cell culture inserts with 8 μm pore size, which were coated on their lower surface with either 50 μg/ml Matrigel (upper panel), 20 μg/ml collagen I (middle panel) or 20 μg/ml fibronectin (lower panel). Note that coating of both sides of the transwell insert or coating with 1% BSA abolishes directed migration in the haptotaxis assay (data not shown). The numbers of cells migrated to the lower surface of the cell culture inserts were determined. Cells in 4 defined optical fields were counted for each cell culture transwell insert. Bars indicate the standard errors of the mean values. One representative experiment is shown out of at least 3 independent ones. (c) Chemotaxis. Cells were allowed to migrate for 3 hr at 37°C across cell culture inserts with 8 μm pore size in the presence of either 3T3 conditioned medium (upper panel) or 10% FCS (lower panel) in the lower compartment. The numbers of cells migrated to the lower surface of the cell culture inserts were determined. Cells in 4 defined optical fields were counted for each cell culture transwell insert. Bars indicate the standard errors of the mean values. One representative experiment is shown out of at least 3 independent ones. (d) Phosphorylation of L-plastin is required to enhance invasion of human melanoma cells. MV3 cells stably expressing either EGFP, wt-LPL-EGFP or 5A7A-LPL-EGFP fusion proteins were allowed to migrate for 20 hr at 37°C across Matrigel cell culture invasion inserts with 8 μm pore size in the presence of either 3T3 conditioned medium (left panel) or 10% FCS (right panel) in the lower compartment. The numbers of cells having invaded the Matrigel matrix and migrated to the lower surface of the cell culture invasion inserts were determined. Cells in 4 defined optical fields were counted for each cell culture transwell insert. Bars indicate the means and the standard errors of the mean values. One representative experiment is shown out of at least 3 independent ones.
the functional importance of L-plastin expression for tumor metastasis. In the mouse B16 melanoma model, B16F1 melanoma cells were stained with a polyclonal L-plastin antiserum (upper panel, Fig. 6a). The position of phosphorylated L-plastin is indicated by an open arrowhead. Note that because of lower L-plastin expression levels, the blot of the B16F10 cells needed to be exposed for a longer time than the B16F1 blot. (b) Normalized results of the densitometric scanning of the L-plastin immunoblot. The L-plastin expression level in B16F1 cells was set as 1. (c) Phosphorylation status of L-plastin. Lysates of B16F1 (low metastasis) and B16F10 (high metastasis) cells were subjected to 2D-electrophoresis. L-plastin was detected by immunoblot analysis using a polyclonal L-plastin antiserum. The position of phosphorylated L-plastin is indicated by an open arrowhead. Note that because of lower L-plastin expression levels, the blot of the B16F1 cells needed to be exposed for a longer time than the B16F10 blot. (d) Phosphorylated L-plastin corresponds to 1.4% of total L-plastin in B16F1 cells, and to 10.8% of total L-plastin in B16F10 cells as determined by densitometric scanning.

Discussion

L-plastin, the hematopoietic isoform of the plastin/fimbrin family, is frequently expressed in human tumors. However, the functional importance of L-plastin expression for tumor cell growth and metastasis was so far unclear. In colon cancer, a correlation of L-plastin expression with advanced tumor stages was observed whereas in breast cancer, no such correlation could be seen.

Here, we used human malignant melanoma as a model to systematically investigate the functional consequences of L-plastin expression in human tumor cells. We demonstrate by histological examination that L-plastin is expressed in around 25% of human malignant melanomas. However, despite the fact that siRNA-mediated knock-down of L-plastin in human melanoma cells negatively influenced cell migration in vitro, L-plastin expression in tumor specimens from patients did not correlate with tumor stages or penetration depths. One possibility to explain this finding was that, besides expression of L-plastin, other factors are needed in addition to promote metastasis.

To investigate systematically whether phosphorylation of L-plastin may influence tumor cell metastasis, we analyzed the effects of expression of cDNA-encoded phosphorylatable (wt-LPL-EGFP) versus nonphosphorylatable (5A7A-LPL-EGFP) L-plastin on key features of metastasis such as adhesion, migration, and invasion. MV3 cell populations stably expressing wt-LPL-EGFP (which undergoes spontaneous phosphorylation in MV3 cells) or nonphosphorylatable 5A7A-LPL-EGFP showed no change in static adhesion to Matrigel, or the ECM components collagen I and fibronectin, both being substrates for β1-integrins. In line with this finding, a siRNA mediated knock-down of L-plastin did not influence the adhesiveness of the L-plastin positive melanoma cell line IF6.

In contrast, with regard to haptotactic migration towards Matrigel, collagen I or fibronectin, both wt-LPL-EGFP and 5A7A-LPL-EGFP expressing cells migrated faster than EGFP expressing control cells. Thus, L-plastin enhances haptotactic migration independent of its phosphorylation state. This is in line with our finding that siRNA mediated knock-down of endogenous L-plastin in IF6 melanoma cells reduced haptotactic migration. Given the lack of changes in the adhesiveness to β1-integrins, it is likely that expression of wt-LPL-EGFP and 5A7A-LPL-EGFP influences postadhesive-integrin-induced signaling events leading to rearrangements of the actin cytoskeleton and enhanced migration. Indeed, we could demonstrate that expression of L-plastin in MV3 cells promotes the formation of adhesion sites (podosomes) at cell extensions independent of its phosphorylation state. It is tempting to speculate that L-plastin may serve as a platform to target proteins that are involved in actin cytoskeleton remodeling—e.g., vinculin—to adhesive sites, resulting in enhanced migration. In line with this, in PMN derived from an L-plastin knock-out mouse, phosphorylation of paxillin and Syk upon adhesion to a polyRGD-coated surface were reduced. Both proteins are known to be involved in actin cytoskeleton remodeling at adhesion sites.

Differences in the migratory behavior of wt-LPL-EGFP, 5A7A-LPL-EGFP or EGFP-expressing cells were not observed in a chemotactic migration assay. Given the fact that wt-LPL-EGFP and 5A7A-LPL-EGFP-expressing cells reached their maximal migration speed already during haptotaxis without the need for additional chemotactic signals, especially in the absence of chemotactic stimuli the expression of L-plastin may decide whether tumor cells rapidly colonize distant tissues. In this regard, it has been shown that in the absence of chemokine signaling, either because of the lack of chemokine receptor expression on the tumor cell itself or because of the lack of chemokine secretion by the tissue, tumor cells can be attracted by the extracellular matrix itself (haptotaxis).

Importantly, for tumor cell invasion, cells need to penetrate basement membranes. Here, we have shown that expression of wt-LPL-EGFP, however not of the nonphosphorylatable 5A7A-LPL-EGFP mutant, enhances the invasive capacity of MV3 melanoma cells. A possible involvement of L-plastin in tumor cell invasion was also found for prostate cancer cells. Thus, expression of antisense L-plastin constructs reduced the invasiveness of the cells in vitro. However, data regarding the functional importance of L-plastin phosphorylation were not analyzed in this study. Our data clearly demonstrate that not only expression but also phosphorylation of L-plastin is required to enhance melanoma cell invasion. In line with this notion, B16F10 melanoma cells, which show high metastasis in vivo, expressed high amounts of L-plastin, which undergoes phosphorylation. This is not the case in the low metastatic B16F1 melanoma cells.

The fact that both nonphosphorylatable 5A7A-LPL-EGFP as well as phosphorylatable wt-LPL-EGFP are similarly targeted to adhesion sites does not rule out the possibility that, in contrast to 5A7A-LPL-EGFP, wt-L-plastin-EGFP is phosphorylated at these adhesion sites. This could explain why only wt-LPL-EGFP promotes invasion, because phosphorylation of L-plastin at adhesion sites may allow the recruitment of further proteins necessary for enhanced invasion. Both phosphorylated L-plastin as well as matrix MMPs were found in podosomes. This opened up the possibility that expression of phosphorylated L-plastin in tumor...
cells enhances the production and secretion of matrix MMPs. However, expression of MT1-MMP as well as secretion of MMP2, the matrix MMPs known to be expressed in MV3 cells, 

was not different between cells expressing wt-LPL-EGFP or the nonphosphorylatable S5A7A-LPL-EGFP fusion protein (data not shown).

Summing up the presented data, we propose the following model of L-plastin function in human melanoma cells: expression of L-plastin leads to enhanced cell migration towards extracellular matrix components. However, mere expression of L-plastin does not promote melanoma cell invasion into basement membranes. Only, if at least part of the L-plastin is phosphorylated, either by proper signaling events from the tumor cell environment or by constant activation of signal transduction pathways in the course of cell transformation, melanoma cell invasion is promoted. As invasion is crucial for metastasis to occur, it is likely that only phosphorylated L-plastin promotes metastasis in vivo. This would explain the controversial data concerning the correlation between L-plastin expression and tumor progression in tumor patients, since none of these studies considered the phosphorylation state of L-plastin. 

Acknowledgements

We thank Dieter Stefan for cell sorting employing the BD FACS Vantage™ cell sorter and Finola Kirstein for contributions to the cDNA cloning.

References