

A potential role for the plasmin(ogen) system in the posttranslational cleavage of the neural cell adhesion molecule L1

Naushaba Nayeem^{1,*}, Steve Silletti^{1,*}, Xiu-Ming Yang², Vance P. Lemmon³, Ralph A. Reisfeld¹, William B. Stallcup⁴ and Anthony M. P. Montgomery^{1,‡}

¹Department of Immunology, The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, CA 92037, USA

²Signal Pharmaceuticals, 5555 Oberlin Drive, San Diego, CA 92121, USA

³Neuroscience Department, Case Western Reserve University, Cleveland, OH 44106, USA

⁴The Burnham Institute, 10901 N. Torrey Pines Road, La Jolla, CA 92037, USA

*These two authors have contributed equally to this work

‡Author for correspondence

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SUMMARY

L1 is a neural recognition molecule that promotes neural developmental and regenerative processes. Posttranslational cleavage of L1 is believed to be important for regulating its function *in vivo*, but little is known of the proteolytic systems responsible. In this study we present evidence that plasmin can regulate both L1 expression and function. The addition of plasmin to cell lines results in a dose-dependent loss of surface L1 expression, with the simultaneous appearance of soluble L1 species. The addition of plasminogen to primary neurons and melanoma cells also resulted in the generation of plasmin and the concomitant release of L1. One product of plasmin-mediated cleavage is an amino-terminal fragment of approximately 140 kDa that has been previously described

as a natural posttranslational cleavage product *in vivo*. This fragment was confirmed to result from cleavage at two sites in the middle of the third fibronectin-like domain of L1. Cleavage at a further site, proximal to the transmembrane domain of L1, was also observed at higher plasmin concentrations. Plasmin was further confirmed to abrogate homophilic L1 interactions required for cellular aggregation. Based on these findings we propose that plasmin is likely to be an important regulator of L1-mediated processes including those documented in the nervous system.

Key words: L1, Cleavage, Plasmin

INTRODUCTION

Human L1 is a member of a subfamily of phylogenetically conserved neural recognition molecules that share a common complex multidomain structure, restricted expression patterns, and multiple functional specificities (Brümmendorf and Rathjen, 1993; Hortsch, 1996). Differential and dynamic expression of L1-type molecules has been described in the developing nervous system, wherein such expression has been linked to a variety of active processes including myelination, and axonal pathfinding (Cohen et al., 1997; Dahme et al., 1997). Despite its designation as a neural CAM (cell adhesion molecule), L1 has been described on non-neural cell types, including normal and transformed cells of epithelial and hematopoietic origin (Mujoo et al., 1986; Thor et al., 1987; Probstmeier et al., 1990; Nolte and Martini, 1992; Kowitz et al., 1993; Kujat et al., 1995; Ebeling et al., 1996; Pancook et al., 1997).

The common domain organization that unifies members of the L1-subfamily includes an extracellular region of six immunoglobulin-like (Ig-like) domains and five fibronectin type III (FN-III) domains (Moos et al., 1988; Brümmendorf

and Rathjen, 1993; Bateman et al., 1996). Structure-function studies have shown that these multiple interactive domains facilitate either homophilic or heterophilic interactions (Hortsch, 1996). A region in the second Ig-like domain of L1 has recently been shown to be essential for homophilic L1-L1 binding (Zhao and Siu, 1995). A single RGD motif in the sixth Ig-like domain of human L1 has been shown to support interactions with multiple RGD-dependent integrins (Ebeling et al., 1996; Montgomery et al., 1996; Felding-Habermann et al., 1997). Additional heterophilic ligands also include the axon-associated proteins TAG/axonin-1 (Kuhn et al., 1991; Felsenfeld et al., 1994) and F3/11 (Olive et al., 1995), and chondroitin sulfate proteoglycan (Grumet et al., 1993; Friedlander et al., 1994). L1 has also been reported to undergo multiple cis-type interactions with molecules such as NCAM (Feizi, 1994), CD9 (Schmidt et al., 1996) and CD24 (Kadmon et al., 1995).

Another important and unifying property of the L1-subfamily is a susceptibility to posttranslational cleavage *in vivo*. Thus, both human and mouse L1 as well as related molecules in the rat (NILE) and chick (Ng-CAM and Nr-CAM), have been shown to be sensitive to cleavage within the

third fibronectin-like domain (Faissner et al., 1985; Sadoul et al., 1988; Wolff et al., 1988; Nybroe et al., 1990; Kayyem et al., 1992; Burgoon et al., 1995). Such cleavage of L1 results in an extracellular amino-terminal fragment of approximately 140 kDa (L1-140) and a transmembrane fragment of approximately 80 kDa (L1-80). Both of these fragments have been detected in developing and adult mouse brain (Rathjen and Schachner, 1984; Faissner et al., 1985; Sadoul et al., 1988), rat brain (NILE; Liljelund et al., 1994) and human cerebellum (Wolff et al., 1988). Treatment of cultured mouse cerebellar cells with trypsin is known to yield the L1-140 cleavage product, indicative of a serine protease-sensitive site in the third fibronectin like repeat (Faissner et al., 1985; Sadoul et al., 1988).

While the functional significance of L1 cleavage remains to be determined it is likely to have a profound effect on L1-mediated processes. Evidently, the release of L1 from the cell surface will abrogate its role in cell-cell interaction. Furthermore, the affinity of L1 for certain extracellular matrix (ECM) components (Martini and Schachner, 1986; Poltorak et al., 1990; Montgomery et al., 1996) may result in the deposition of cleaved L1 fragments in occluding matrix and in this context L1 may function as a substrate adhesion molecule. Despite the potential significance of posttranslational L1 cleavage, little is known of the proteolytic mechanisms responsible. In this study we demonstrate that plasmin, a serine protease of fundamental importance in many biological processes (Carmeliet and Collen, 1995), can efficiently cleave L1, affecting both its expression and its function.

MATERIALS AND METHODS

Reagents

Anti-L1 antibodies used include the 5G3 monoclonal antibody (mAb) which was produced in our laboratory (Mujoo et al., 1986), and two anti-L1 polyclonal antibodies (6096 and anti-L1-ECD). The anti-L1 mAb 5G3 used in this study recognizes an epitope close to the amino terminus of L1 within residues 56-175. The mapping of the 5G3 epitope to this sequence is based on transfection studies with L1 deletion mutants (Dahlin-Huppe et al., 1997). The polyclonal antibody 6096 was kindly provided by Dr J. Hemperly (Becton Dickinson, Research Triangle Park, NC). A mAb (clone GG-7) specific for the Fc-region of human IgG was obtained from Sigma (St Louis, MO). Additional mAbs specific for poly-histidine tags and glutathione S-transferase were obtained from Qiagen (Valencia, CA) and Pierce Chemical Company (Rockford IL), respectively. Human plasmin was purchased from CalBiochem (La Jolla, CA), and human plasminogen from Hematologic Technologies (Essex Jct., VT).

Construction of L1-fusion proteins

A recombinant L1-Fc fusion protein consisting of the entire extracellular domain (ECD) of human L1 fused with the Fc region of human IgG (hinge-CH2-CH3) was constructed using the pIg-tail expression system (Novagen, Madison, WI) as described by Wong et al. (1995). COS-7 cells were transiently transfected with the pIg vector using the Lipofectamine reagent (Gibco, Gaithersburg, MD) and serum-free conditioned medium was used as a source of the soluble chimeric protein.

Production of a GST-fusion protein encoding the third fibronectin-like repeat of L1 followed by a 6-histidine tag (GST-FN3-His) was as follows. The region of L1 encoding amino acids 792-903 was amplified using an upstream primer encoding an L1 start at amino acid 792 and an internal *EcoRI* site (5'-GGA GAA TTC TAC CCC CAG

GCA-3') in conjunction with a downstream primer which encodes an L1 stop at amino acid 903 as well as a 5-histidine overhang (5'-ATG GTG GTG GTG GTG CTC GGG GTG GCC AG-3'). The resulting product was subjected to a second round of amplification using the original upstream primer and a 6-histidine-encoding downstream primer containing an internal *EcoRI* site (5'-CGA ATT CAT GAT GGT GGT GGT GGT G-3') prior to digestion with *EcoRI* and ligation into a pGEX-6P-1 vector (Pharmacia Biotech, Uppsala, Sweden). Recombinant fusion proteins were purified from IPTG-induced log-phase cultures on a Sepharose 4B-coupled glutathione affinity matrix essentially as described by the manufacturer (Pharmacia Biotech).

Cell lines

M21 human melanoma cells were derived from the UCLA-SO-M21 cell line which was provided by Dr D. L. Morton (University of California, Los Angeles, CA). M24met cells and the M24met subclone M24met/cl used in this study were derived in our laboratory and have been characterized as highly metastatic human melanoma cell lines (Mueller et al., 1995). The FG human pancreatic carcinoma cell line (Leavesley et al., 1992) was originally obtained from Dr Vito Quaranta (Scripps Research Institute, CA). Mouse myeloma J558L cells were purchased from the American Type Culture Collection (Rockville, MO) and were stably transfected to express full length human L1 (J558L-L1 cells) as described (Montgomery et al., 1996). M24met, FG and J558L-L1 cells were maintained in RPMI containing 10% FBS. The M21 cells were maintained in RPMI supplemented with 1% Neutrodoma-SP (Boehringer, Mannheim).

Membrane preparation

M21 cells (1×10^8) were harvested, washed, and resuspended in ice-cold hypotonic lysis buffer containing 20 mM Hepes (pH 7.4), 1 mM EDTA, 0.02% sodium azide and protease inhibitors. The cell lysate was subsequently sonicated and centrifuged three times at 1000 g. After each centrifugation the supernatants were collected and pooled. The membrane fraction was pelleted by centrifugation of the pooled supernatant at 100,000 g for 1 hour. Pelleted material was resuspended in 2 ml of Hepes-buffer (pH 7.4), containing 1 mM EDTA, and protease inhibitors.

Plasmin and plasminogen treatment of cell cultures

Cell lines expressing L1 were maintained in serum-free medium in the presence of plasmin at range of concentrations (0.15-0.6 U/ml) or plasminogen at a final concentration of 0.9 μ M. Cell cultures were incubated with or without plasmin at 37°C for 2-18 hours as specified. To abrogate plasmin activity some cultures were supplemented with α 2-antiplasmin. After treatment, adherent cell monolayers (M21, M24met, FG) were harvested with versene. No significant loss of viability of the harvested cells was observed and all cells were subsequently washed and processed for flow cytometric analysis, or for western blot analysis (see below). Culture supernatant from treated and control cultures was also collected, and insoluble material removed by centrifugation at 100,000 g for 30 minutes. Protein in the supernatants (200 μ l aliquots) was precipitated using cold acetone. Pelleted material was air-dried and resuspended in SDS-PAGE sample buffer for western blot analysis. Plasmin activity in the conditioned medium derived from M24met cell cultures treated with plasminogen alone or in combination with α 2-antiplasmin was assayed directly using the plasmin substrate S-2403 as described (Reinartz et al., 1993).

Plasmin treatment of recombinant L1-fusion proteins

Serum-free culture supernatant (200 μ l aliquots) containing L1-Fc chimera was treated with plasmin (0.045-2.8 U/ml) for 2 hours at 37°C and the reaction stopped by the addition of aprotinin (5 μ g/ml). The fusion protein present in control or treated supernatants was then precipitated using acetone and the pelleted material resuspended in SDS-PAGE sample buffer for western blot analysis.

The GST-FN3-His fusion protein was treated with plasmin on glutathione Sepharose 4B beads. Coated beads were washed and resuspended in a 50 mM Hepes buffer (pH 8.0) and 100 μ l aliquots of the bead slurry were treated with plasmin at 0.05 U/ml for 37°C for 2 hours. Control beads were incubated in the absence of plasmin. After treatment the beads were pelleted by centrifugation at 15,000 *g*, the supernatant collected and the beads resuspended in SDS-PAGE sample buffer. The beads were boiled for 15 minutes to release untreated or plasmin digested fusion protein. Released fusion protein and collected supernatant was subjected to western blot analysis with an anti-His tag mAb or used for protein sequencing.

Flow cytometric analysis

Untreated cells or cells treated with plasmin or plasminogen were washed and resuspended in ice-cold PBS containing 0.5% BSA and 0.02% sodium azide (pH 7.4). The cells were then incubated with anti-L1 mAb 5G3 (20 μ g/ml) for 1 hour, washed and treated with a secondary phycoerythrin-conjugated goat anti-mouse antibody (Southern Biotechnology). Stained cells were subsequently analyzed with a Becton-Dickinson FACScan flow cytometer. Control cells were either stained with an isotype matched control antibody or received no primary antibody.

Western and slot blot analysis

Western-blot analysis was performed on acetone-precipitated culture supernatants derived from plasmin-treated cell cultures or on plasmin treated L1-Fc or GST-FN3-His constructs. Additional analysis was performed on plasmin treated M21 cell membrane preparations. All samples were heated to 80°C prior to SDS-PAGE using 8% or 8-16% Tris-glycine precast gels (Novex, San Diego, CA). Following electrophoresis, proteins were transferred onto PVDF membranes (Novex, San Diego, CA) and blocked membranes were incubated with anti-L1 mAb 5G3 (1.4 μ g/ml) or rabbit anti-L1 antisera diluted 1:2000. In the case of the L1-Fc, or the GST-FN3-His constructs, membranes were also incubated with an anti-Fc mAb (ascites 1:500) or with an anti-6 \times His mAb (0.2 μ g/ml). Membranes were then incubated with goat anti-rabbit IgG or goat anti-mouse IgG antibodies conjugated to horseradish peroxidase (1:5000). For slot-blot analysis, 0.5 ml aliquots of culture supernatants, derived from M24met cell cultures treated with plasmin or with plasminogen alone or in combination with α 2-antiplasmin, were transferred onto PVDF membranes using a 24-slot vacuum-blot apparatus (Hybri-Slot; Bethesda Research Laboratories). Membranes were probed with rabbit anti-L1 antisera (6096) diluted 1:2000, followed by goat anti-rabbit IgG antibodies conjugated to horseradish peroxidase (1:5000). Antibody labeling was visualized with ECL chemiluminescent detection reagents (Amersham Life Sciences).

Isolation and treatment of primary neurons

Mass cultures of pure sympathetic neurons from the superior cervical ganglion of postnatal day 1 rats (Fisher 344; Harlan Sprague Dawley, San Diego) were prepared as described by Yang et al. (1998) and were cultured in a defined medium (UltraCulture; Biowhittaker, Walkersville, MD) supplemented with 2 mM glutamine and 1% penicillin and streptomycin. Neurons were plated in 4-well chamber slides coated with poly-ornidine and laminin in the presence of nerve growth factor (10 ng/ml). Cultures were treated for 4 days with 5-fluoro-2'-deoxyuridine and uridine (Sigma) in order to generate a glia-free sympathetic neuron culture. Plasminogen was added to the culture medium at 0.1-0.5 μ M for 24 hours. Medium was collected after 24 hours, concentrated and subject to western blot analysis with an affinity purified anti-L1 polyclonal antibody (anti-L1-ECD). Plasmin activity in the medium was assayed directly using the plasmin substrate S-2403 (Reinartz et al., 1993). Adherent neurons were stained for L1 with the affinity purified anti-L1 polyclonal; antibody (anti-L1-ECD) and a secondary Cy3-conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR). Treated and untreated

neurons were analyzed under constant settings using a Bio-Rad MRC 1024 laser confocal microscope.

Generation of tumors overexpressing plasminogen-activator inhibitor-2 (PAI-2)

Wild-type M24met melanoma cells or M24met cells stably transfected to overexpress PAI-2 (Mueller et al., 1995) were used to generate tumours *in vivo* using chick embryos as host animals. This animal model has previously been used to define the role of plasminogen activators in tumor growth and invasion and is described in full by Ossowski and Reich (1980). Transfected and wild-type tumor cells (4×10^6) were inoculated onto the chorioallantoic membranes of 10-day-old chick embryos (Leghorn; AA-Lab eggs, Westminster, CA). Small vascularized tumor nodules were evident after six days and were resected, trimmed to remove host tissue and used to generate lysates. Samples were equalized for protein content and were assessed for levels of intact transmembrane L1 by western blot analysis using an affinity purified anti-L1 polyclonal antibody (anti-L1-ECD). Levels of L1 evident on western blots were quantified by scanning densitometry using a Personal Densitometer SI (Molecular dynamics).

Protein sequencing

The plasmin cleavage sites within the third fibronectin-like domain of L1 were determined by amino-terminal sequencing of two carboxy-terminal cleavage products resulting from plasmin digestion of the GST-FN3-His fusion protein. The cleavage products were separated by SDS-PAGE and transferred onto a Problot membrane (Promega, Maddison, WI). The sequence of the excised cleavage products was obtained by automated Edman degradation sequencing using a Applied Biosystems gas-phase sequencer.

Cell aggregation assays

The effect of plasmin-treatment on L1-dependent cell aggregation was studied using mouse myeloma cells transfected to express full length human L1 (J558L-L1 cells). The myeloma cells were maintained in serum-free medium overnight, washed and resuspended in serum free-RPMI (0.5% BSA, pH 7.4), and added at 1.5×10^5 cells/well to a 24-well cell culture plate. The cells were then allowed to form aggregates by gentle rotation at 37°C for 30 minutes. Some cells were pretreated and rotated in the presence of anti-L1 mAb 5G3 at 100 μ g/ml. Plasmin was then added to wells containing the cell aggregates at specified concentrations. Some wells received both plasmin and α 2-antiplasmin. The cellular aggregates were then incubated for 90 minutes at 37°C. All treatments were performed in duplicate, and at the end of incubation, cells were gently resuspended and the number of single cells in each well counted with a hemacytometer.

RESULTS

Exogenous plasmin or plasminogen releases L1 from the cell surface

In order to evaluate the contribution of plasmin to L1 processing, we determined whether the treatment of cells with exogenous enzyme would result in the loss of surface L1-expression and the concomitant appearance of L1-cleavage products in cell culture supernatant. Expression of L1 at the cell surface was assessed using anti-L1 mAb 5G3 which recognizes an epitope close to the amino terminus of human L1 within residues 56-175 (data not shown).

Flow cytometric analysis confirmed that the addition of exogenous plasmin to L1⁺ cell lines (M21, M24met and FG) results in a significant loss of surface L1 expression (Fig. 1A,B,C). A clear dose-dependent decrease in the amount of L1

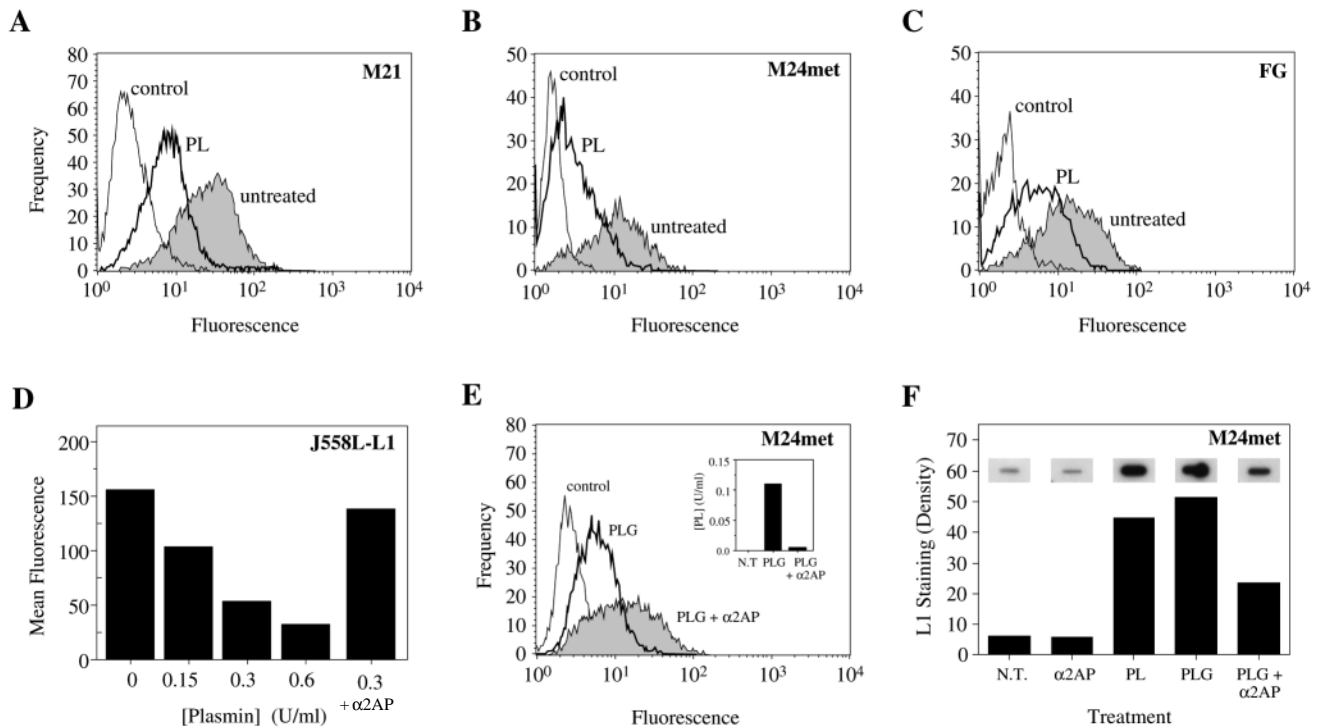


Fig. 1. The effect of plasmin and plasminogen treatment on L1 expression in different cell lines. M24met, M21 and FG tumour cell lines were incubated in the presence or absence of plasmin (0.6 U/ml) for 18 hours in serum free medium and surface L1 expression determined by FACS analysis using the anti-L1 mAb 5G3 (A-C). Serum-starved myeloma cells transfected to express full length human L1 (J558L-L1) were treated with plasmin (0.15-0.6 U/ml) for 4 hours and surface L1 expression assessed by FACS analysis (D). Some cells were treated with a combination of plasmin and α 2-antiplasmin (D). M24met cells in serum free medium were incubated for 18 hours with plasminogen alone (0.9 μ M) or in combination with α 2-antiplasmin and surface L1 expression determined by FACS analysis using the anti-L1 mAb 5G3 (E). Levels of plasmin activity generated were determined using the chromogenic substrate S-2403 (E inset). Levels of soluble L1 released into culture supernatant in response to the treatment of M24met cells with plasmin or plasminogen was assessed by slot blot analysis with anti-L1 polyclonal antibody 6096 (F). Control cells received phycoerythrin-conjugated secondary stage antibody alone. Abbreviations used: PL, plasmin; PLG, plasminogen; α 2AP, α 2-antiplasmin.

present was also observed when an L1-transfected mouse myeloma line (J558L-L1) was treated with a range of plasmin concentrations (Fig. 1D). Addition of α 2-antiplasmin together with the plasmin effectively prevented any significant loss of L1-immunoreactivity (Fig. 1D).

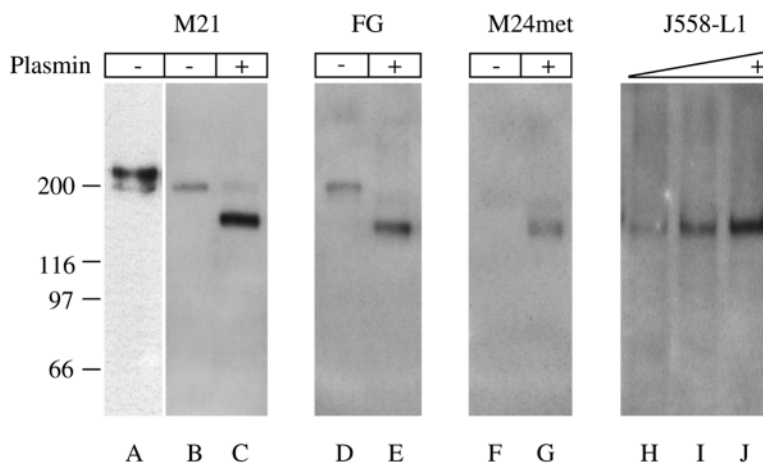
The M24met cells used in this study express significant levels of urokinase-type plasminogen activator (uPA) and its receptor (uPAR) (Stahl and Mueller, 1994). Since the uPA/uPAR complex is instrumental in the generation of plasmin from plasminogen, we tested whether the addition of exogenous plasminogen to M24met cells would also result in a loss of L1 expression. Importantly, addition of this ubiquitous zymogen (0.9 μ M) to serum-free M24met cell cultures did indeed result in the generation of plasmin (Fig. 1E, inset) and a concomitant loss of L1(5G3)-immunoreactivity (Fig. 1E). No significant loss of expression was detected when plasminogen was added in the presence of α 2-antiplasmin (Fig. 1E). These findings are important since they demonstrate that in the presence of physiological levels of plasminogen the cells themselves can generate sufficient plasmin activity to reduce the levels of surface L1 detected. In this regard, the localization of plasminogen and thus plasmin activity at the cell surface may be important since a significant amount of L1 was lost from the cell surface despite relatively low levels of plasmin activity detected in the culture supernatant (Fig. 1E, inset). In

further studies, we also observed that levels of L1-expression were higher on M24met cells cultured in the presence of plasminogen-depleted human serum (American Diagnostics) than on these cells maintained in the same plasma reconstituted with exogenous plasminogen (data not shown).

To confirm that the loss of reactivity with mAb 5G3 was due to the release of L1 fragments from the cell surface, we looked for evidence of increased L1-immunoreactivity in culture supernatants. To this end, M24met cells were treated with plasmin or plasminogen in the presence or absence of α 2-antiplasmin and the amount of soluble L1 in the culture supernatant determined by slot blot analysis with an anti-L1 polyclonal antibody (6096). Slot blots, and the quantification of these blots by densitometry, are shown in Fig. 1F. Minimal levels of soluble L1 were detected in medium derived from untreated M24met cells, and these levels were unchanged by the addition of α 2-antiplasmin alone (Fig. 1F). However, the addition of either plasmin or plasminogen resulted in a significant increase in the amount of soluble L1 detected (Fig. 1F). Importantly, the dramatic increase in soluble L1 seen with plasminogen treatment was reduced by the simultaneous addition of α 2-antiplasmin (Fig. 1F).

Together these data suggest that plasmin can efficiently cleave and release L1 from the surface of a variety of cell types in culture. Importantly, we observed that plasmin could still

Fig. 2. Western-blot analysis of L1 fragments released in response to plasmin treatment of cultured cell lines. The molecular mass of membrane-associated L1 was determined by western blot analysis of an M21 cell membrane preparation using mAb 5G3 (A). Soluble L1 species present in plasmin treated or untreated cell culture supernatants were determined by western blot analysis with mAb 5G3 (B-J). M21, FG M24met cell cultures in serum free medium were treated with 0.6 U/ml of plasmin for 18 hours (C,E,G). Untreated cell cultures were maintained under the same conditions in the absence of plasmin (B,D,F). Myeloma cells transfected to express full length human L1 (J558L-L1) were treated with a range of plasmin concentrations (0.15, 0.6 and 2.4 U/ml) for 2 hours prior to harvesting supernatant (H-J). All samples were subjected to SDS-PAGE under non-reducing conditions. Molecular mass is indicated in kilodaltons.



reduce surface L1-expression by the J558L-L1 myeloma cells in absence of cations and in the presence of 0.5 mM ethylenediaminetetraacetic acid (EDTA; data not shown). This result suggests that plasmin is acting directly and argues against cleavage due to plasmin-dependent activation of matrix metalloproteinases.

Plasmin-mediated cleavage results in the release of a large amino-terminal fragment of L1

We have demonstrated that plasmin cleaves L1 at the cell surface, resulting in a significant increase in soluble L1-immunoreactivity. However, the structural origin and character of the released L1 fragments remained to be determined. To address this we performed western blot analysis of culture supernatant derived from plasmin treated cell cultures using mAb 5G3.

As a first step the relative molecular mass of integral membrane associated L1 was determined. Using membranes prepared from M21 cells, full length L1 was primarily detected as a band of ~215 kDa, with a minor product at 200 kDa (Fig. 2A). This molecular mass profile is consistent with that observed in human neuroblastoma cells (Mujoo et al., 1986). The remaining cell lines used in this study also expressed integral L1 as a primary product of greater than 200 kDa (data not shown). In the absence of any enzyme treatment we detected a minor soluble species of 200 kDa in both M21 and FG cell culture supernatants (Fig. 2B and D). The presence of a constitutively shed species of this molecular mass has also been described in other L1 expressing tumour cell lines (Mujoo et al., 1986) and may result from membrane proximal cleavage by a membrane associated metalloproteinase as recently described by Beer et al. (1999). Significantly, the addition of plasmin resulted in the detection of a single soluble L1-fragment of 145-155 kDa depending on the cell line tested (Fig. 2C,E,G). In this regard, it is important to note that the amount of soluble L1 detected following treatment with plasmin was, in all cases, greater than that constitutively released. Furthermore, the constitutively released 200 kDa fragment is reduced or absent in plasmin treated cultures, suggesting that this fragment is susceptible to further plasmin degradation. Consistent with our previous observation of a dose-dependent cleavage of L1 from the surface of J558L-L1 cells (Fig. 1D), we found a corresponding dose-dependent increase of the soluble L1 fragment when these cells were treated with a range of plasmin concentrations (Fig. 2H-J).

Primary neurons can generate plasmin from plasminogen resulting in the release of L1

Given the central role of L1 in neural development and regeneration it was further determined whether the results obtained with the cell lines could be reproduced with primary neurons. For this purpose purified rat sympathetic neurons derived from the superior cervical ganglion (Yang et al., 1998) were incubated with plasminogen and levels of plasmin activity and L1 solubilization determined. Importantly, the addition of plasminogen to the cultured neurons resulted in the detection of plasmin activity within 24 hours (Fig. 3A) and the concomitant appearance of a L1 fragment of approximately 140 kDa in the medium (Fig. 3B). The ability of these cells to generate plasmin is consistent with a previous report documenting the expression of plasminogen-activators (tPA and uPA) by rat sympathetic neurons *in vivo* and *in vitro* (Wang et al., 1998). It is important to note that L1 was released even following the addition of plasminogen at concentrations 7-14-fold lower than that present in the serum and at a detectable plasmin activity as low as 0.1 U/ml (Fig. 3A and B). The primary neurons, like M21 and FG cells (Fig. 2B and D), also constitutively released L1 as a large ectodomain fragment of 200 kDa (Fig. 3A). The fact that plasminogen resulted in the release of L1 from the cell surface as a 140 kDa fragment while only marginally degrading the constitutively released L1 ectodomain fragment present in the medium (Fig. 3B) is consistent with previous findings that membrane-associated plasminogen activators generate and localize plasmin activity at the cell surface. In accordance with the release of L1, it was also observed that the addition of plasminogen resulted in a significant reduction in the expression of L1 on the surface of the neurites (Fig. 3C). The detection of the large L1 ectodomain fragment in untreated neuron conditioned medium (Fig. 3B) is important since it suggests that the metalloproteinase-dependent L1-shedding mechanism recently described in a variety of tumor cell lines by Beer et al. (1999) can also occur in primary neurons.

Tumor cells transfected to express high levels of plasminogen-activator inhibitor-2 (PAI-2) express higher levels of intact transmembrane L1 *in vivo*

A subclone of the M24met cell line (M24met/cl cells) was successfully transfected with a full length cDNA encoding human PAI-2 (Mueller et al., 1995). Stable overexpression of PAI-2 in these cells (PAI-2-4 cells) was subsequently shown to

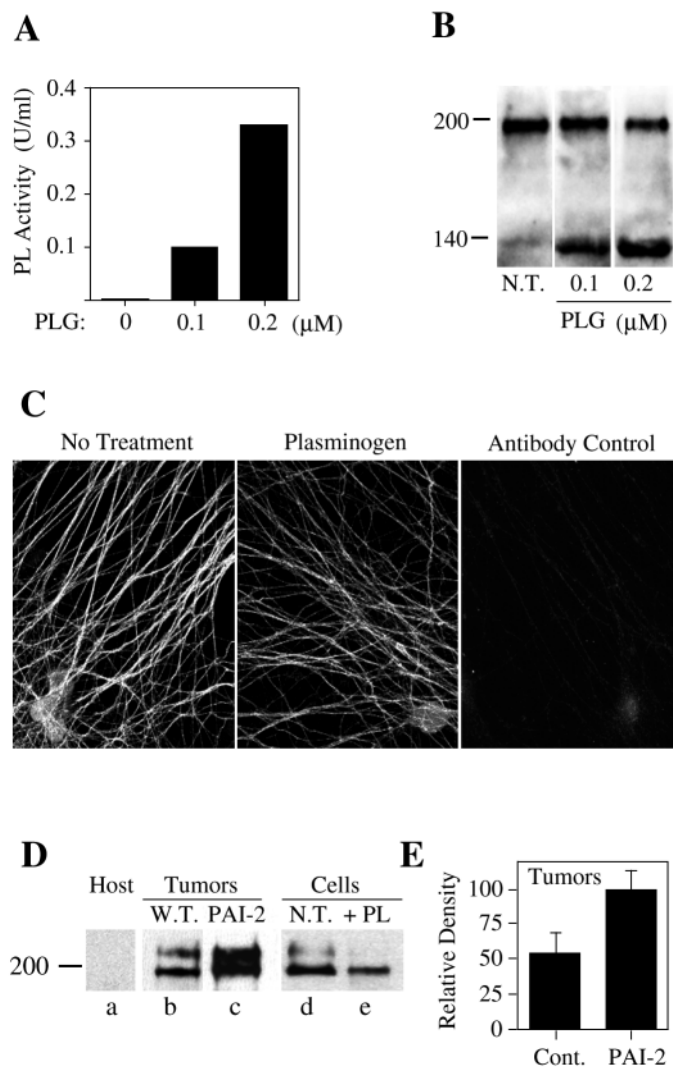


Fig. 3 Effect of plasminogen on L1-release by primary neurons and impact of PAI-2 overexpression on L1 levels in vivo. Plasminogen (PLG) at 0.1–0.2 μM was added to cultured rat sympathetic neurons and the plasmin (PL) activity generated after 24 hours determined using the chromogenic substrate S-2403 (A). Soluble L1-fragments released into the medium after 24 hours were detected by western blot analysis using anti-L1-ECD polyclonal antibody (B). Note the appearance of L1-140 with plasminogen treatment. Levels of L1 expression remaining after treatment with plasminogen (0.5 μM) were determined by immunostaining with anti-L1 polyclonal antibody L1-ECD (C). Background staining using rabbit control IgG is shown (right panel). Images were obtained using a 63 \times objective and a Bio-Rad MRC 1024 confocal microscope using identical settings. The effect of PAI-2 overexpression on levels of intact L1 expression in vivo was determined by western blot analysis and scanning densitometry (D and E). Cell lysates derived from chick chorioallantoic membrane (Host, lane a), wild-type tumors (W.T., lane b) or PAI-2 overexpressing tumors (lane c) were equalized for protein and immunoblotted with anti-L1-ECD polyclonal antibody (D). For quantification, scanning densitometry was performed on western blots obtained from wild-type tumors ($n=6$) or tumors overexpressing PAI-2 ($n=6$) (E). Results are expressed as relative density after scanning both species of intact L1. The effect of plasmin (PL) treatment on surface L1 expression by M24met cells in vitro is shown for comparison (D; lanes d and e).

prevent plasmin-mediated degradation of underlying ECM (Mueller et al., 1995).

Exploiting this cell system we tested the hypothesis that tumours formed by M24met cells overexpressing PAI-2 (PAI-2-4 cells) would express higher levels of intact L1 due to protection from plasmin-mediated cleavage in vivo. To assess the suitability of this system, we first confirmed that the addition of plasminogen to the PAI-2-4 cells did not result in a loss of surface L1 expression (not shown) as was previously observed in wild-type M24met cells (Fig. 1E). Secondly, levels of L1 expression by the PAI-2-4 cells were not significantly greater than those of the wild-type cells when both cell lines were maintained in the absence of plasminogen (i.e. serum free medium; not shown).

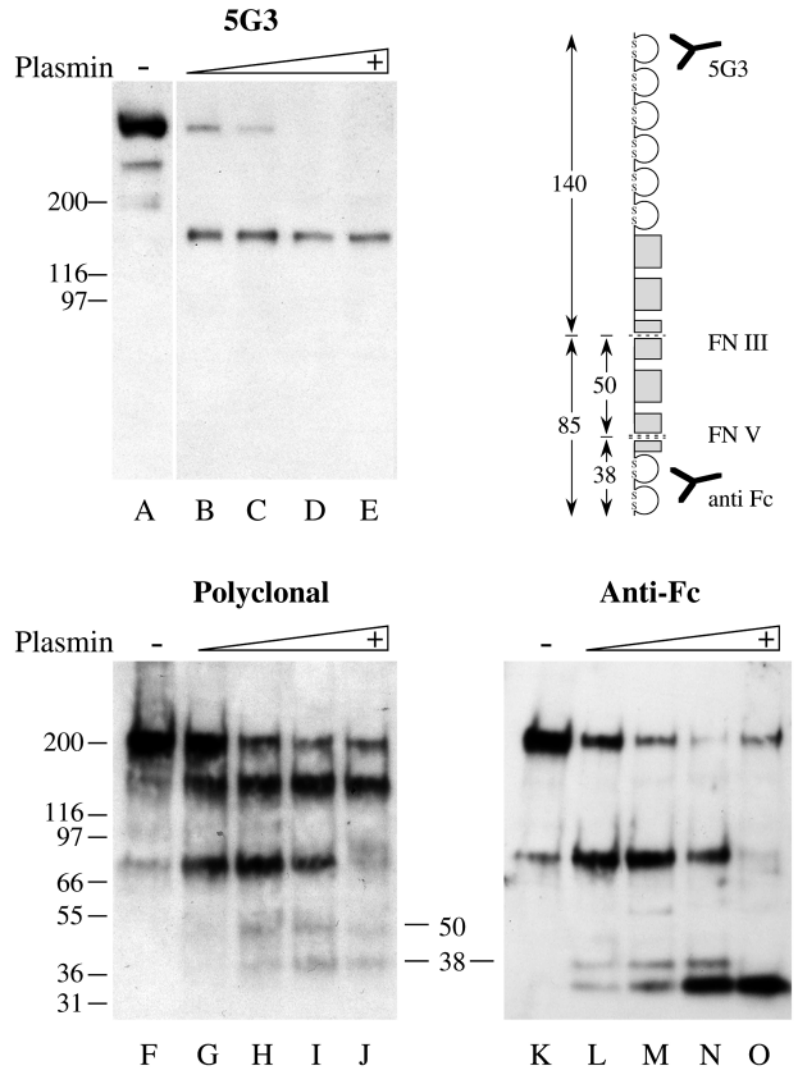
Tumours were generated using chick embryos as hosts (Ossowski and Reich, 1980) and small vascularized tumor nodules were successfully derived from both PAI-2-4 cells ($n=6$) and wild-type M24met cells ($n=6$). Viable tumours of comparable size were resected and lysed for western blot analysis. Consistent with a previous report (Mujoo et al., 1986), the resected tumors expressed full length L1 as two species of approximately 215 and 200 kDa (Fig. 3D, lanes b and c). Host tissue (chorioallantoic membrane) failed to stain for L1 (Fig. 3D, lane a). After equalizing for protein content, the levels of intact L1 detected in the tumors overexpressing PAI-2 ($n=6$) were found to be significantly higher than in the wild-type tumors ($n=6$). This differential expression was confirmed by scanning densitometry of both the L1 species (Fig. 3E) and an example of this differential staining is shown in Fig. 3D (lane b versus c). The disparity in L1 levels observed between the wild-type and PAI-2 transfected tumors is consistent with the ability of PAI-2 to specifically prevent the generation of plasmin in vivo and in this context prevent the release of L1. In order to confirm that plasmin will directly reduce the surface expression of both of the L1 species detected (200 and 215 kDa) the M24met/cl cells were also treated with plasmin in vitro. Consistent with the ability of plasmin to release L1 from the cell surface we did observe a reduction in the expression of these species (Fig. 3D, lanes d and e). Confirming true solubilization of L1, the smaller products of plasmin cleavage evident in culture supernatant (e.g. L1-140, Fig. 2), were not detected in the cell lysates (not shown).

Plasmin cleaves L1 within its third fibronectin-like repeat and proximal to the transmembrane domain

Thus far, we have demonstrated that plasmin can cleave L1 in a manner which releases a 145–155 kDa fragment. However, in order to determine whether plasmin cleaves at one or more sites, and to define the structural relationship of individual cleavage products to each other and the full length molecule, we utilized a recombinant human L1-fusion protein chimera consisting of the L1 extracellular domain (ECD) fused at its carboxy terminus to the Fc region of human IgG1 (hinge-CH2-CH3). Using a combination of mAb 5G3 and an anti-human Fc antibody we could then detect the presence of either amino-terminal (5G3-reactive) or carboxy-terminal (anti-Fc-reactive) L1-fragments.

Dimerization of the L1-Fc chimera, due to linkage at the Fc-hinge, results in a product with an apparent molecular mass of ~400 kDa under non-reducing conditions (Doherty et al., 1995). On western blot analysis with mAb 5G3 we were able

Fig. 4. Western-blot analysis of L1 cleavage products generated by plasmin treatment of a L1-Fc chimera. The Fc-fusion protein containing the entire ECD of human L1 was incubated in the presence or absence of plasmin and the intact or cleaved products identified with anti-L1 mAb 5G3 (A-E), with an anti-L1 polyclonal antibody (anti-L1-ECD)(F-J) or with an anti-human Fc mAb (K-O). mAb 5G3 was used to detect samples that were treated with plasmin at 0.36, 0.7, 1.4 and 2.8 U/ml and which were subsequently subjected to SDS-PAGE under non-reducing conditions only. The anti-L1 polyclonal antibody and the anti-human Fc mAb were used to detect samples that were treated with plasmin at 0.04, 0.08, 0.16 and 0.32 U/ml and which were subsequently subjected to SDS-PAGE under reducing conditions. Molecular mass is indicated in kilodaltons.



to confirm the presence of this unreduced dimerized L1-Fc product (Fig. 4A). Importantly, digestion of this dimerized chimera with a range of plasmin concentrations results in the generation of a single 140 kDa cleavage product detectable with mAb 5G3 (Fig. 4B-E). Given that mAb 5G3 detects an epitope close to the amino terminus of L1 (data not shown), the 140 kDa cleavage product must contain the amino-terminal Ig-like domains.

To look for evidence of other L1 cleavage products, additional experiments were performed with an anti-L1 polyclonal antibody (anti-L1-ECD). These experiments were done under reducing conditions which prevent dimerization of the L1-Fc construct (Doherty et al., 1995). As expected, our anti-L1 polyclonal antibody detected the reduced L1-Fc chimera as a major band of 200 kDa (Fig. 4F). Consistent with our previous results, the addition of plasmin in the range of 0.04-0.32 U/ml resulted in the progressive digestion of the 200 kDa L1-Fc construct, with the concomitant appearance of the 140 kDa species (Fig. 4G-J). The smaller product of this cleavage was also detected by the polyclonal antibody as an 85 kDa fragment (Fig. 4G-I). This fragment (but not the 140 kDa fragment) was susceptible to further degradation at higher plasmin concentrations (Fig. 4H-J), resulting in the near total loss of the 85 kDa fragment (Fig. 4J) and the appearance of 50 kDa and 38 kDa fragments that were only weakly recognized by the polyclonal antibody (Fig. 4H-J). It is important to note that mAb 5G3, which binds to an epitope close to the amino terminus of L1, only recognized the 140 kDa fragment and none of the smaller degradation products (Fig. 4B-E).

In order to further define the origin and structural relationship of these cleavage products, we performed further experiments using an anti-Fc mAb which detects those fragments that include the carboxy terminus of the L1-Fc chimera. As expected, this mAb detected the reduced form of the L1-Fc chimera as a major band of 200 kDa (Fig. 4K). However, following digestion with plasmin only the 85 kDa species and a smaller 38 kDa fragment were detected (Fig. 4L-O). At higher plasmin concentrations the 38 kDa fragment is further degraded to a 32 kDa product that is clearly evident with the anti-Fc mAb (Fig. 4N and O) but was not detected by the polyclonal anti-L1 antibody, possibly due to a loss of

available L1 epitopes. It is important to note that the anti-Fc antibody did not recognize either the 140 kDa species or the 50 kDa fragment.

Together these findings are consistent with a pattern of cleavage that is depicted schematically in Fig. 4. At relatively low plasmin concentrations there is cleavage of the L1-Fc chimera, resulting in the appearance of an amino-terminal 140 kDa fragment that is detected by mAb 5G3 (Fig. 4B-E) but not by the anti-Fc mAb (Fig. 4L-O). Such cleavage simultaneously gives rise to a 85 kDa fragment that includes the Fc domain, but is not recognized by mAb 5G3 (Fig. 4B-E). At higher plasmin concentrations the 85 kDa fragment is further cleaved, generating an internal 50 kDa fragment which is neither recognized by 5G3 or the anti-Fc mAb, and additional 38 and 32 kDa fragments that contain the Fc-domain (Fig. 4L-O). Given that the Fc domain alone has a molecular mass of 26 kDa, the 32 and 38 kDa fragments can be explained by cleavage in the fifth FN-like repeat of the L1-ECD (Fig. 4 schematic). The 140 kDa fragment detected upon proteolysis of the L1-Fc chimera is consistent with the 145-155 kDa amino-terminal fragments detected after plasmin-treatment of the cultured cell lines (Fig. 2). The small discrepancy in size is expected, since the form of L1 expressed by the cell lines is

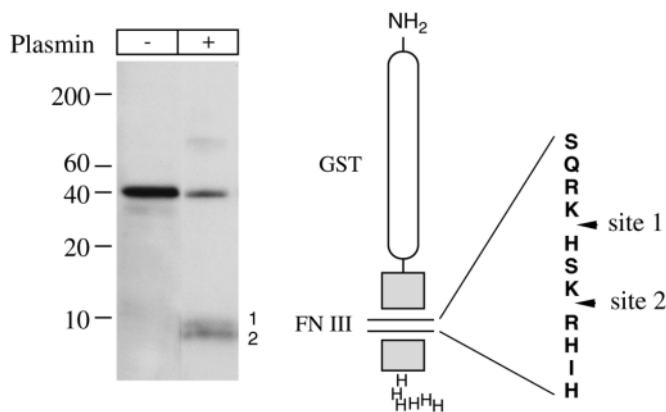


Fig. 5. Demonstration of plasmin cleavage within the third fibronectin-like domain of L1. A GST-fusion protein consisting of the third fibronectin-like domain of L1 and a 6 \times histidine tag (GST-FN3-His) was immobilised on glutathione Sepharose 4B beads and the beads treated with plasmin at 0.05 U/ml at 37°C for 2 hours. The resulting fragments were detected by western blotting with an anti-His tag mAb. Amino-terminal sequencing of the two small carboxy-terminal fragments indicated two plasmin cleavage sites indicated schematically. Specific cleavage sites are numbered and arrowed and amino acids are shown using the abbreviations: S, Ser; Q, Gln; R, Arg; K, Lys; H, His; I, Iso. Molecular mass is indicated in kilodaltons

greater than 200 kDa, presumably due to differential glycosylation.

The molecular mass of the amino-terminal L1 fragment detected upon proteolysis of the L1-Fc chimera (i.e. 140 kDa) is consistent with cleavage in the third FN-like domain of L1 (Fig. 4 schematic). In order to confirm this and to determine the cleavage site(s), we generated an additional L1-construct consisting of just the third FN-like domain of L1. In this construct, the amino terminus of the FN domain was linked to glutathione-S-transferase (GST), and the carboxy terminus to a 6 \times histidine (His) tag. The GST-FN3-His fusion protein was purified and immobilized on glutathione Sepharose beads and was then treated with plasmin. Carboxy-terminal fragments resulting from plasmin cleavage were detected using an anti-His-tag antibody. The untreated GST-FN3-His fusion protein was detected as a single species of 42 kDa while treatment with plasmin resulted in cleavage in the middle of the FN3 domain and the generation of two small carboxy-terminal fragments (Fig. 5). Sequence analysis of the carboxy-terminal fragments confirmed two plasmin cleavage sites in close proximity to each other with one site centered on a dibasic sequence (SKR) which is conserved in a variety of L1-type proteins (Burgoon et al., 1995; Kayyem et al., 1992). Together, these findings support the contention that the large amino-terminal fragment generated upon plasmin treatment of the L1-Fc chimera (140 kDa) or as a result of treating cells in culture (145–155 kDa) is a result of cleavage in the third FN-like repeat of L1.

Plasmin abrogates homophilic L1-L1-mediated aggregation

Cell-cell interactions mediated via a homophilic L1-L1 binding mechanism have been shown to be important in a variety of biological processes, including axonal elongation and tumour

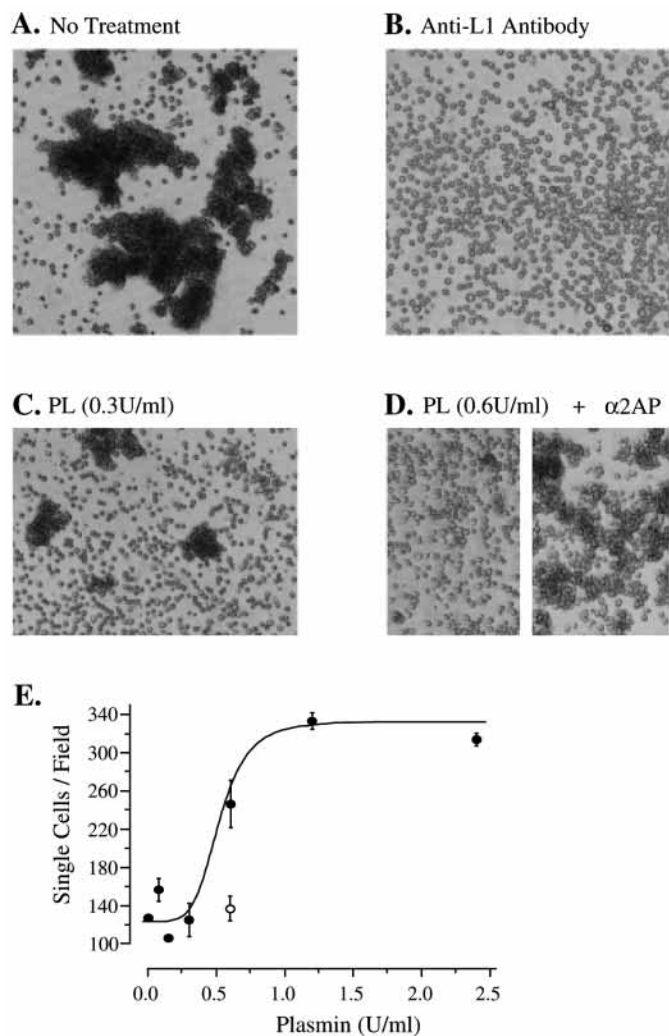


Fig. 6. Plasmin disrupts cellular aggregation mediated by homophilic L1-L1 binding. Dispersed L1+ myeloma cells (J558L-L1 cells) were rotated to promote L1-dependent aggregation and were subsequently incubated with plasmin (C and D) or remained untreated (A). Further cells were treated with a combination of plasmin and α 2-antiplasmin (D). To demonstrate specificity some cells were rotated in the presence of mAb 5G3 (B). Cells were photographed using an Olympus IX70 inverted stereo microscope and a 10 \times objective. To quantify the effect of plasmin on L1-mediated aggregation, the cells were gently resuspended and the number of single cells counted using a hemocytometer (E).

cell aggregation. In order to assess the functional consequences of plasmin cleavage on L1-L1 binding we tested the effect of plasmin on the L1-transfected J558L cells (J558L-L1) which spontaneously aggregate via a homophilic L1-L1 binding mechanism (Lemmon et al., 1989; Montgomery et al., 1996).

Upon rotation, single cell suspensions of J558L-L1 cells were observed to form large multi-cellular aggregates (Fig. 6A). Confirming the role of L1 in this process, the addition of anti-L1 mAb 5G3 prior to, and during rotation completely prevented aggregate formation (Fig. 6B). We have previously shown that this antibody can block L1-L1 binding (Montgomery et al., 1996). The addition of plasmin to preformed J558L-L1 cell aggregates resulted in significant

disaggregation (Fig. 6C and D). This response was further confirmed by quantifying the proportion of single cells, which increased in number with increasing cell dispersal (Fig. 6E). Importantly, the process of disaggregation was blocked by the simultaneous addition of α 2-antiplasmin (Fig. 6D and E). No significant loss of viability was observed during any of the treatments.

DISCUSSION

Plasmin is the end product of a tightly regulated enzymatic cascade that is initiated by the limited proteolysis and conversion of plasminogen by the aptly named tissue- or urokinase-type plasminogen activators (tPA and uPA). The plasmin/PA system has been implicated in a plethora of normal and pathological processes. Thus, recent gene targeting and transfer studies have confirmed the quintessential role of the PA/plasmin system in fibrinolysis, wound healing, tissue remodeling, and normal brain function (Carmeliet and Collen, 1995). In this study, we have implicated this important and broadly acting proteolytic pathway in the regulation of L1 expression and function resulting from the ability of plasmin to cleave and release L1 from the cell surface. Conversion of plasminogen by both melanoma cells and primary neurons is shown to result in the release of L1. Conversely, overexpression of a specific inhibitor of plasminogen activators (PAI-2) prevents this process in melanoma cells and appears to protect L1 from degradation *in vivo*.

We have demonstrated that one product of plasmin cleavage is a large amino-terminal fragment of 140-155 kDa (L1-140) that results from cleavage within the third FN-like repeat of L1 (L1-FN3). Importantly, similar amino-terminal fragments resulting from cleavage within L1-FN3 have been described in mouse, rat and human brain tissue (Sadoul et al., 1988; Wolff et al., 1988; Hlavin and Lemmon, 1991; Poltorak et al., 1993; Liljelund et al., 1994) and in human cerebrospinal fluid (Nybroe et al., 1990; Poltorak et al., 1995). Results presented in this study are comparable with those described following mild trypsinization of L1 (Faissner et al., 1985; Sadoul et al., 1988). Thus, trypsin has been shown to generate both L1-140 and L1-80 fragments, and although the trypsin cleavage site(s) was not identified in these studies, L1-FN3 is also likely to be the target domain for trypsin mediated cleavage (Faissner et al., 1985; Sadoul et al., 1988).

It is important to note that studies performed with trypsin have indicated that the amino-terminal L1-140 fragment is not released after cleavage, possibly because it can remain in a non-covalent association with its complimentary 80 kDa cleavage partner (L1-80) (Faissner et al., 1985; Sadoul et al., 1988). In our studies plasmin or plasminogen treatment clearly led to the release of soluble L1-140 and analysis of treated cells did not show evidence for retention of this fragment on the cell surface. Release of L1-140 in response to plasmin may reflect the ability of this serine protease to cleave at two sites in L1-FN3 and at an additional site more proximal to the transmembrane domain of L1. Given our findings it is important to note that soluble forms of L1-140 have been described in cerebrospinal fluid and in brain supernatant (Nybroe et al., 1990; Poltorak et al., 1995).

While our findings point to a potentially important role for

plasmin in regulating L1-expression and function, a role for other serine proteases, particularly in the generation of L1-140, needs to be addressed. In this regard, however, we found no evidence that urokinase, thrombin or cathepsin-G could release L1 from the surface of our cell lines or cleave L1 present in membrane preparations (not shown). These serine proteases were tested under the same conditions as plasmin and at equivalent or higher specific activities. Interestingly, we did observe some loss of L1 expression after treating M21 melanoma cells with α -chymotrypsin. However, since the presence of this potent serine protease is primarily restricted to the pancreas, stomach and small intestine its relevance in regulating the expression and function of L1 is less apparent than plasmin.

While the pathophysiological significance of our findings remain to be determined in an *in vivo* setting, we have clearly demonstrated that primary neurons can convert plasminogen to plasmin and concomitantly release L1. Furthermore, we have shown that such plasmin-mediated cleavage effectively abrogates homophilic L1-L1 binding; an interaction vital to most L1-mediated processes including neurite outgrowth (Lagenaur and Lemmon, 1987; Lemmon et al., 1989). The significance of our findings is further underscored by the extensive overlap of neurological functions that involve both plasmin and L1, including neurite extension (Lagenaur and Lemmon, 1987; Monard, 1988; Seeds et al., 1997), granule cell migration (Krystosek and Seeds, 1981; Moos et al., 1988), nerve regeneration (Salles et al., 1990; Jung et al., 1997) and even long term potentiation in the hippocampus (Mizutani et al., 1996; Luthl et al., 1994). It is also of interest that excessive plasmin-catalyzed degradation in response to neural stimulation has been linked to the pathogenesis of excitotoxic-induced neurodegeneration in the hippocampus (Chen and Strickland, 1997). Our findings raise the possibility that L1 expressed in the hippocampus might be a target molecule in this neuropathology.

The generation of plasmin is evidently dependent upon a cellular source of plasminogen-activators (tPA and/or uPA) and upon the availability of plasminogen. In the context of the nervous system it is significant that both L1 and plasminogen-activators have been localized on the axonal growth cones of mammalian sensory and sympathetic neurons (Krystosek and Seeds, 1981; Monard, 1988; van den Pol and Kim, 1993; Seeds et al., 1997; Wang et al., 1998). Plasminogen activators have also been described in association with astrocytes and microglia (Tranque et al., 1992). While plasminogen is a ubiquitous zymogen expressed at high levels in the serum it is also produced by microglia (Kohsaka et al., 1994) and can be expressed by hippocampal neurons in response to injury (Matsuoka et al., 1998). Plasminogen activators localized to axonal growth cones are proposed to play a role in axonal pathfinding, in part by promoting the generation of plasmin which can then cleave cell-cell and cell-matrix contacts (Monard, 1988; Seeds et al., 1997). Since homophilic L1 binding at the level of the growth cone has been implicated in axonal elongation, it is conceivable that the local generation of plasmin could provide an important means for disrupting such L1-L1-interactions. This may be important mechanistically since motility requires both the formation and break down of cell-cell or cell-matrix contacts.

The ability of melanoma cells to generate plasmin and

concomitantly cleave L1 could have important ramifications for tumor progression. By breaking down L1-mediated cell-cell contact plasmin may reduce tumour cohesion, concomitantly allowing the detachment of metastatic cells from the primary tumour mass. In this regard, L1 has been shown to promote the aggregation of a variety of tumour lines (Rathjen and Schachner, 1984; Kowitz et al., 1993). The affinity of soluble L1 for certain extracellular matrix (ECM) components including laminin (Poltorak et al., 1990; Montgomery et al., 1996) may result in the deposition of cleaved L1 fragments in occluding ECM. Since the L1-140 fragment contains an RGD motif that interacts with a variety of integrin heterodimers, it is conceivable that deposition of this fragment will result in the modification of the tumor microenvironment to favor adhesion and concomitantly invasion. In this regard, we have previously demonstrated that L1-fragments containing the RGD motif promote melanoma cell migration via integrin $\alpha v \beta 3$ (Montgomery et al., 1996). The potential role of L1 in tumor progression is further underscored by the fact that L1 is expressed by a diverse array of tumour cell types, including those of neuroectodermal, epithelial, and myelomonocytic origin (Mujoo et al., 1986; Linnemann et al., 1989; Kowitz et al., 1993).

Posttranslational cleavage is a characteristic of the L1-subfamily that is likely to have important pathophysiological consequences. However, the relevant proteolytic pathways have remained undefined. In this study we demonstrate that plasmin, the end product of the plasminogen/PA cascade, is able to cleave and release L1 from the cell surface, effectively abrogating L1-mediated cell-cell adhesion. Importantly, the primary product of plasmin cleavage (i.e. L1-140) has also been described in tissues. Based on our findings we propose that the plasmin(ogen) system is likely to be an important regulator of L1-mediated processes in vivo.

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