Metalloproteinase-mediated release of the ectodomain of L1 adhesion molecule

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Accepted 4 June; published on WWW 21 July 1999

SUMMARY

The L1 adhesion molecule is an approx. 200-220 kDa type I membrane glycoprotein belonging to the immunoglobulin (Ig) superfamily. L1 can bind in a homotypic fashion and was shown to support integrin-mediated binding via RGDs in the 6th Ig-like domain. In addition to its cell-surface expression, L1 can occur in the extracellular matrix (ECM). Here we demonstrate that L1 is constitutively released from the cell surface by membrane-proximal cleavage. L1 shed from B16F10 melanoma cells remains intact and can serve as substrate for integrin-mediated cell adhesion and migration. The release of L1 occurs in mouse and human cells and is blocked by the metalloproteinase inhibitor TAPI (Immunex compound 3). This compound has been shown previously to block release of L-selectin and TNF-α which is mediated by the membrane-bound metalloproteinase TNF-α converting enzyme (TACE). Using CHO cells that are low in TACE expression and do not release L-selectin we demonstrate that L1 release is distinct from L-selectin shedding. We propose that cell-surface release may be necessary for the conversion of L1 from a membrane into an ECM protein.

Key words: L1, L-selectin, Adhesion, Shedding, Metalloproteinase, TAPI, Integrin

INTRODUCTION

The L1 cell adhesion molecule is a 200-220 kDa transmembrane glycoprotein which belongs to the immunoglobulin superfamily (Kadmon and Altevogt, 1997; Rathjen and Schachner, 1984; Faissner et al., 1985; Moos et al., 1988). Homologous molecules exist in several species including mouse (L1) (Faissner et al., 1985), rat (NILE) (McGuire et al., 1978), chick (NgCAM) (Burgoon et al., 1991), Drosophila (neuroglian) (Bieber et al., 1989), and human (L1) (Wolff et al., 1988; Kobayashi et al., 1995). The molecule consists of 6 Ig-like domains and five fibronectin-type III repeats followed by a transmembrane region and a highly conserved cytoplasmic tail of approx. 114 amino acids. L1 was originally recognized as a neural adhesion molecule shown to be involved in granule neuron migration in the developing mouse cerebellar cortex (Lindner et al., 1983), the fasciculation of neurites (Fischer et al., 1986) and neurite outgrowth on other neurites and Schwann cells (Chang et al., 1990; Seilheimer and Schachner, 1987). Recent studies on L1-knockout mice have confirmed that L1 is an important molecule for the development of the nervous system (Dahme et al., 1997; Cohen et al., 1997). L1 expression was also found on normal and transformed cells of hematopoietic origin in both mouse and humans (Kowitz et al., 1992, 1993) and on certain epithelial and endothelial cells (Thor et al., 1987; Pancock et al., 1997; Debiec et al., 1998).

Studies in the nervous system have shown that L1 can mediate binding by several mechanisms: (i) homotypic binding involving L1-L1 interactions (Kadmon et al., 1990a,b), (ii) assisted homophilic binding between L1 and L1/NCAM complexes at the surface of adjacent cells (Kadmon et al., 1990a,b) and (iii) heterotypic binding of which the interaction with the axon-associated CAM axonin-1 (Kuhn et al., 1991) and the GPI anchored molecule CD24 (Kadmon et al., 1995) are well characterized. L1 was also identified as a ligand for several RGD-binding integrins, i.e. α5β1, αvβ1, αvβ3 as well as the platelet integrin αIIbβ3 (Ruppert et al., 1995; Montgomery et al., 1996; Ebeling et al., 1996; Felding-Habermann et al., 1997; Blaess et al., 1998). Integrin-mediated cell binding and migration is supported by RGDs in the 6th Ig-like domain of L1 (Ruppert et al., 1995; Felding-Habermann et al., 1997; Montgomery et al., 1996; Ebeling et al., 1996; Duczmal et al., 1997). In addition to its function as a cell surface adhesion molecule L1 can be shed from the membrane (Richter-Landsberg et al., 1984; Montgomery et al., 1996) and be deposited in the ECM (Montgomery et al., 1996; Martini and Schachner et al., 1986) suggesting a potential role for L1 as a matrix constituent. A fundamental question is therefore how L1 is released from the cell surface. However, despite the fact that L1 shedding has been known for a long time the underlying molecular mechanism mediating this process has not been evaluated.

A large group of transmembrane proteins can undergo cleavage and release of their ectodomain into the medium (for review see Hooper et al., 1997; Turner and Hooper, 1999).
These proteins are diverse in structure and function and comprise molecules like tumor necrosis factor (TNF-\(\alpha\); Kriegler et al., 1988; Mohler et al., 1994; Gearing et al., 1994; McGehee et al., 1994), P-selectin (Tanzaka et al., 1998), IL-6 receptor (Müllberg et al., 1993), L-selectin (Kahn et al., 1994; Preece et al., 1996), pro-TGF-\(\alpha\) and \(\alpha\)-amyloid precursor protein (Arribas and Massague, 1995; Arribas et al., 1997). Since metalloproteinase inhibitors, in particular hydroxamate-based compounds, can effectively prevent the shedding, it is thought that a metalloproteinase(s) is responsible for the cleavage of the membrane into a soluble form. Recently, a metalloproteinase (TNF-\(\alpha\)-converting enzyme or TACE) that cleaves TNF-\(\alpha\) has been identified as a member of the ADAM metalloproteinase family (membrane proteins with \(\alpha\) disintegrin and metalloproteinase domain) (Black et al., 1997; Moss et al., 1997). TACE/ADAM17 also appears to be involved in the membrane release of L-selectin and TGF-\(\alpha\) (Peschon et al., 1998). Whether TACE can cleave additional molecules and whether other ‘shedding-proteases’ exist is presently unknown. It is also not known how the protease(s) select their substrate, since consensus cleavage sites have not been identified.

In the present report we have addressed two aspects of L1: (i) are metalloproteinases involved in the release of L1 from the cell surface, and (ii) can the released L1 serve as an integrin substrate. We find that L1 in both mouse and human cells is released from the cell surface following membrane-proximal cleavage in a phorbolmyristate acetate (PMA)-independent manner. This process is blocked by the metalloproteinase inhibitor TAPI (Immunex compound 3). The released L1 is functionally intact and can support integrin-mediated cell adhesion. We also present evidence that the mechanism of L1 release is distinct from L-selectin shedding.

**MATERIALS AND METHODS**

**Cell culture**

ESb-MP is an adherent variant of the metastatic murine cell line ESb which has monocyte features (Lang et al., 1987). ULMC cells were obtained from the Tumorbank of the DKFZ Heidelberg. B16F10 melanoma cells and N2A neuroblastoma cells were provided by Claudine Kieda, INSERM, Orléans, France. Mouse J558 myeloma cells transfected with human L1 were obtained from Margot Zöller, DKFZ Heidelberg and Melitta Schachner, ETH Zürich, respectively. The mouse T cell hybridoma H3D-1 was obtained from Bruno Kyewski of our department. Human Mel 63 melanoma cells were a gift from Ulrich Moebius, DKFZ Heidelberg, ECV 304 cells were obtained from Claudine Kieda, INSERM, Orléans, France. Mouse 1558 myeloma cells transfected with human L1 were obtained from Vance Lemmon, University of Cleveland, USA. All cells were kept at 37°C, 5% CO\(_2\) and 100% humidity and were cultivated in RPMI 1640 supplemented with 10% FBS, 2-mercaptoethanol, Hepes and glutamine except for N2A cells that were kept in DMEM and ECV 304 cells that were grown in Opti-MEM (Gibco-BRL, Eggenstein, Germany). Spleen cells were collected from six- to eight-week-old DBA/2 mice. Residual erythrocytes were lysed by brief incubation in 155 mM NH\(_4\)Cl, 0.1 mM EDTA, 10 mM KHCO\(_3\) solution followed by washing of the cells. CHO cells stably expressing mouse L1 or human L-selectin were obtained by lipofectamin transfection (Gibco-BRL) using L1-pCDM8 or L-selectin-pCDM8 (gift of K. Kishimoto, Ridgefield, USA). Transfectants were enriched by cell sorting as described below.

**Antibodies and peptides**

The following monoclonal antibodies (mAbs) were used and have been described in previous publications (Kowitz et al., 1992; Ruppert et al., 1995): mAb 324 against the mouse L1 adhesion molecule, mAb 79 against mouse CD24 (heat stable antigen, HSA), mAb HMa5-1 against mouse \(\alpha\)5 integrin, mAb RMV-7 against mouse \(\alpha\)4-integrins, mAbs HMB-1 and HMB-3 against integrin mouse \(\beta\)1 and \(\beta\)3 chains, respectively. The mAb to human L1 UI 127.11 (Ebeling et al., 1996) and mAb 74-SH7 against the cytoplasmic tail of L1 (Leimon et al., 1989) have been described. mAb DREG 56 against human L-selectin and mAb Mel-14 against mouse L-selectin were obtained from Eugene C. Butcher, Stanford University, USA. mAbs were used in a purified form or as hybridoma supernatants.

Polyclonal antibodies against the cytoplasmic portion of L1 were prepared in rabbits by coupling the L1 C-terminal peptide AGGNDSSGATSPINPAVLE via an N-terminal cysteine to KLH. Following two booster injections the rabbits were bled and the sera tested for L1 reactivity by ELISA with immobilized L1.

**Cytfluorography**

The staining of cells with saturating amounts of mAbs, either hybridoma supernatants or purified antibodies, and phycocerythrin (PE)-conjugated goat antibodies to rat or hamster immunoglobulins has been described elsewhere (Lang et al., 1987; Hubbe et al., 1993). Stained cells were analyzed with a FACScan cell analyzer using Cellquest software. To enrich for stable transfectants, CHO cells were stained with mAbs to mouse L1 or human L-selectin and sorted under sterile conditions using a FACS vantage (Becton & Dickinson, Heidelberg, Germany).

**Northern blot analysis**

Northern blot analysis has been described before (Altevogt et al., 1989). Briefly, Nylon membranes (Hybond-N+, Amersham-Pharmacia, Freiburg, Germany) with UV-fixed total RNA (10 pg/lane) extracted from the various cells using Trizol (Gibco-BRL) were prehybridized for 10 minutes and hybridized overnight at 65°C in the same solution (7% SDS, 1 mM EDTA, 0.5 M NaH\(_2\)PO\(_4\), pH 7.2) containing a \(^{32}\)P-labeled mouse TACE specific probe (gift of Dr R. Black, Immunex, Seattle, USA) or for control a \(\beta\)-actin probe. The blots were washed and exposed to X-ray sensitive films.

**Analysis of L1 shedding**

Two types of assay were used. In the short term shedding assay cells were labelled with \[^{125}\text{I}]\text{Na using lactoperoxidase-catalyzed iodination as described (Lang et al., 1987). Cells were aliquoted and incubated for 1 hour at 37°C in complete RPMI 1640 medium to allow shedding. Shedding proceeded in the presence or absence of 100 ng/ml PMA or inhibitors as indicated in individual experiments. TAPI (Immunex compound 3) was obtained from Immunex, Seattle, USA and kept as a 5 mM stock solution in DMSO. 1,10 phenanthroline was obtained from Sigma (Deisenhofen, Germany). After incubation the cells were pelleted and the supernatants used for further analysis. A control cell pellet was lysed in the presence of NP-40 and served as cell surface control. Supernatants and NP-40 lysates were precleared with Sepharose-rot-IgG followed by mAb 324 coupled to Sepharose. For the precipitation analysis of human L1 or L-selectin the respective mAbs were pre-adsorbed to Protein-A-Sepharose beads (Pharmacia, Uppsala, Sweden) or Protein-G-Sepharose (Pharmacia, Uppsala, Sweden) and mAb Mel-14 against mouse L-selectin were obtained from Eugene C. Butcher, Stanford University, USA. The mAbs were used in a purified form or as hybridoma supernatants.

The second type of assay employed biosynthetic labelling of cells with \[^{35}\text{S}]\text{methionine/cysteine as described (Lang et al., 1987). After labelling for 2 hours with 0.5 mg/ml of \[^{35}\text{S}]\text{methionine/cysteine (Amersham-Pharmacia, Freiburg, Germany) the cells were diluted...
into complete RPMI 1640 medium, seeded into tissue culture plates and cultured as described above. After 6 or 12 hours aliquots of cells and medium were removed and used for immunoprecipitation. The labelling of COS-7 cells was carried out with cells that had been transiently transfected 24 hours before the assay using Superfect (Quiagen, Hilden, Germany).

**Purification of proteins**

An L1-Fc fusion protein containing the Fc portion of human IgG1 and the ectodomain of mouse L1 was constructed by PCR using an L1 cDNA clone as template and the primer TTAAGCTTAGAAGATGCTCTGATGATCGTACCTGTG (containing a HindIII site, a Kozak sequence and the start codon) and the reverse primer GGGAATCCTTACCTTGATGAGAACCCTGACACGGGCCAGT. The latter primer contained an overhang with an artificial splice donor site and an EcoRI restriction site to allow subsequent directional cloning into the HindIII/EcoRI site of the plg vector (Ebeling et al., 1996). For all PCR reactions 2 units of Taq polymerase expand long template mixture (Boehringer Mannheim, Germany) was used. Plasmid-DNA was transfected into COS-7 cells and supernatant containing the Fc-fusion protein was purified by Protein A-Sepharose chromatography. This procedure has been described in detail elsewhere (Ebeling et al., 1996). Shed L1 from the tissue culture supernatant of B16F10 cells was purified using a mAb 324 affinity column as previously described for brain L1 (Ruppert et al., 1995).

**Cell adhesion and migration assays**

For binding of cells to purified L1, the antigen was diluted in TBS (approx. 20 μg/ml) and coated on LABTEK slides for 16 hours at 4°C. Wells were blocked with 3% BSA in PBS (phosphate buffered saline, lacking Ca2+ and Mg2+) for 30 minutes at room temperature, washed with HBSS containing 10 mM Hepes, 2.5 mM Ca2+ and Mg2+ and used for the assay. For binding, cells (5x105/ml) were suspended in HBSS containing 10 mM Hepes, 0.5 mM Mn2+ and 0.2 ml aliquots were added to the coated slides. The binding assay was performed for 30 minutes at room temperature. For antibody blocking studies, cells were preincubated with purified antibody at the indicated concentration for 10 minutes at room temperature and then transferred to the chamber slides. After the assay the slides were dipped in PBS to remove unbound cells, fixed with 2% glutaraldehyde and counted by video microscopy using IMAGE 1.47 software. For the haptotactic cell migration assay the purified L1 was adsorbed overnight to the backside of Transwell chambers (Costar, 6.5 mm diameter, 5 μm pore size). B16F10 cells at 4x105/ml in complete RPMI medium were seeded into the upper chamber and were allowed to transmigrate to the lower compartment of the chamber for 16 hours at 37°C. Transmigrated cells were counted in the lower chamber.

**RESULTS**

**L1 release from B16F10 melanoma and ESb-MP cells**

It was previously reported that human melanoma cells can release the L1 adhesion molecule from the cell surface both in vitro and in vivo (Montgomery et al., 1996). We observed a similar release of mouse L1 in B16F10 melanoma cells. L1 release into the medium was studied in a short term assay following cell surface labelling. For this iodinated cells were incubated for 1 hour at 37°C and the medium collected for immunoprecipitation. Fig. 1A shows that an L1 protein band of approx. 200 kDa, which was slightly smaller than the membrane form, was detected in the culture medium of B16F10 cells and the monocytic cell line ESb-MP (see below). In the mouse, L1 can be precipitated from the cell surface as two bands of 220-200 kDa and 180 kDa, respectively. These two bands, which occur at variable ratios in different cell types, represent the complete molecule and a form that is proteolytically cleaved in the extracellular portion during biosynthesis but is retained at the cell surface. As shown in Fig. 1A, both components were detected in the medium but only the size of the full length L1 was approx. 15-20 kDa smaller than the cell surface form.

To study the release mechanism further, B16F10 melanoma cells were biosynthetically labelled with [35S]methionine/cysteine for 2 hours and aliquots of cells were plated in RPMI 1640 medium for the indicated length of time at 37°C. Cells were harvested and lysed in cell lysis buffer containing NP-40. Postnuclear extracts (cell lysate) and medium were subjected to immunoprecipitation using mAb 324 to mouse L1 coupled to Sepharose. Samples were eluted in SDS-sample buffer and analyzed by SDS-PAGE and autoradiography. (B) B16F10 cells were metabolically labelled with [35S]methionine/cysteine for 2 hours and aliquots of cells were plated in RPMI 1640 medium for 1 hour at 37°C. Cells were lysed in NP-40 and cell lysate and medium were subjected to immunoprecipitation using mAb 324 to mouse L1 coupled to Sepharose. Samples were eluted in SDS-sample buffer and analyzed by SDS-PAGE and autoradiography.

**Released L1 supports cell adhesion and migration**

To analyze whether the shed L1 remained functional or was subject to rapid protease degradation L1 was purified from the tissue culture supernatant of B16F10 cells. The affinity purification protocol has been used before for the isolation of the transmembrane form of L1 from mouse brain (Ruppert et al., 1995). Biochemical analysis of the purified L1 from culture supernatant revealed the presence of an intact 200 kDa L1 molecule of similar size as an L1-Fc protein comprising the ectodomain of L1 (Fig. 2A).
We compared the shed L1 to full-length brain-derived L1 or the L1-Fc fusion protein, respectively, for the ability to support integrin-mediated cell binding. We previously reported that VLA-5 and \( \alpha_5 \beta_3 \) can bind to L1 in the mouse (Ruppert et al., 1995; Oleszewski et al., 1999). Three different cell lines expressing either \( \alpha_5 \)- and \( \alpha_v \)-integrins (B16F10 and ULMC) or \( \alpha_v \)-integrins only (IH3-1) were therefore used to study cell adhesion. Fig. 2B summarizes the cell binding data and shows that in the presence of 0.5 mM Mn\(^{2+} \) ions all three cell lines were able to bind to the L1 forms although to different levels. The binding of B16F10 and ULMC was blocked in the presence of antibodies to \( \alpha_5 \) and \( \beta_1 \) integrin chains. In the case of IH3-1 cells mAbs to \( \alpha_v \)-integrins and the \( \beta_3 \)-subunit could partially block the binding (Oleszewski et al., 1999).

We additionally tested the ability of shed L1 to serve as a substrate for haptotactic cell migration using Transwell chambers. L1 from all three sources was able to support cell migration of B16F10 cells which was blocked by the mAbs against the \( \alpha_5 \) and \( \beta_1 \) subunit but not by control antibodies (Fig. 3A and B). The \( \alpha_v \beta_3 \) expressing IH3-1 cells did not migrate at all under these conditions. Collectively, these results suggested that the shed L1 retained its ability to serve as an integrin substrate.

**L1 is released without a cytoplasmic tail**

We investigated why the released L1 antigen was smaller in size than the cell surface expressed molecule. The difference was still seen when the immunoprecipitates were treated with Endoglycosidase F to remove N-linked glycans ruling out the possibility of differential glycosylation (not shown). Release of transmembrane proteins often occurs due to enzymatic cleavage close to the membrane. It was therefore tested whether the released L1 contained a cytoplasmic tail. Fig. 4A shows that an antibody against the C terminus of L1 only precipitated the membrane form but not the medium-released L1 indicating that the cytoplasmic tail was missing.

Biosynthetic labelling was used to identify the cytoplasmic cleavage fragment retained in the membrane. B16F10 cells were labelled for 2 hours with \([^{35}S]\)methionine/cysteine for 2 hours and then chased for 24 hours in normal tissue culture medium to allow cleavage to occur. Cells were lysed and immunoprecipitated with mAb 74-5H7 specific for the...
cytoplasmic tail of L1 (Lemmon et al., 1989). As shown in Fig. 4B an approx. 16 kDa fragment was identified. As the cytoplasmic tail and transmembrane portion of L1 comprises a calculated relative mass of 14.6 kDa, this observation suggested that the released L1 had lost both cytoplasmic and most likely the transmembrane portion which were retained in the cell membrane.

**L1 release is blocked by a metalloproteinase inhibitor**

We observed that the monocytic cell line ESb-MP also released L1 from the surface which could not be blocked with PMSF or a cocktail of conventional proteinase inhibitors (Complete™ Boehringer Mannheim, Germany). Shedding did not, however, occur at 4°C further suggesting the involvement of a shedding protease. In addition to L1 these cells express L-selectin allowing a direct comparison of L1 and L-selectin shedding in the same cell type. L-selectin shedding usually requires PMA treatment of the cells. We therefore examined release of L1 and L-selectin in the presence or absence of PMA. As shown in Fig. 5A, the basal L-selectin shedding level was clearly upregulated by PMA (approximately twofold). In contrast, the release of L1 was, if at all, only poorly effected in the presence of PMA (Fig. 5B).

Hydroxamate-based metalloproteinase inhibitors have been used for the analysis of shedding phenomena of diverse molecules including L-selectin. We analyzed the effect of TAPI (Immunex compound 3), a well studied compound for blocking metalloproteinases. For this experiment ESb-MP cells were labeled and incubated for 1 hour in the presence of PMA and different doses of inhibitor. Supernatants were subsequently collected and precipitated first with anti-L-selectin and then with anti-L1. As demonstrated in Fig. 5A, the release of L-selectin was partly blocked in the presence of TAPI in agreement with the literature (Arribas et al., 1996, Bennet et al., 1996). Strikingly, TAPI inhibited the release of L1 more efficiently (Fig. 5A). The IC50-value for TAPI blocking of L1 shedding was approx. 2.5 µM whereas the respective value for L-selectin was between 25-50 µM. This is higher than the reported value of 1 µM for L-selectin shedding from human neutrophils (Bennet et al., 1996).

The effect of other metalloproteinase inhibitors on L-selectin and L1 release was also examined. At a concentration of 5 mM, 1,10 phenanthroline inhibited both L1 and L-selectin release by approx. 30%. EDTA at 5 mM did not show any effect on the release (data not shown).

Release of L1 was noticed also in other cell lines of mouse or human origin. Fig. 6 shows that released L1 was present in the medium of mouse J558 cells transfected with human L1. Clearly evident was the shedding of L1 in human and mouse melanoma cell lines Mel 63 or B16F10, respectively, as well as neuroblastoma N2A (not shown). The L1 fragments recovered from the medium in each case showed a similar size and the release process was inhibited by TAPI. There was only marginal shedding in mouse spleen cells and the human endothelial cell line ECV 304. The results suggested that the mechanism of metalloproteinase-mediated L1 release was conserved among different cell types and could operate across species barriers.

The rate of L1 release in B16F10 cells was estimated by densitometric analysis of band intensities. Approximately 5% of the cell surface expressed L1 could be recovered from the medium after 1 hour of incubation at 37°C (mean of 3 experiments).

**L1 release is distinct from L-selectin shedding**

The data presented in Fig. 5 had suggested that L1 and L-selectin cleavage from the membrane could be blocked by the hydroxamate-based metalloproteinase inhibitor TAPI. (A) Analysis of L-selectin (B) analysis of L1 shedding. ESb-MP cells were cell-surface iodinated and incubated for 1 hour at 37°C in RPMI 1640 in the presence (lanes 3 to 6) or absence (lane 2) of 100 ng/ml PMA. TAPI was present at the following final concentrations: lane 4: 50 µM; lane 5: 25 µM; lane 6: 5 µM. Note that DMSO used as solvent for TAPI did not have any effect. Lane 1: cell surface form for L-selectin or L1, respectively.
TAPI although with different IC50 values. This suggested that different mechanisms were responsible for the shedding. A recent study has shown that the PMA induced L-selectin shedding is impaired in cells derived from TACE −/− mice (Peschon et al., 1998). In addition, recombinant TACE can cleave a peptide representing the cleavage site of L-selectin suggesting that TACE is mediating L-selectin shedding (Peschon et al., 1998). To investigated whether L1 was cleaved by the same mechanism as L-selectin we carried out transfection studies. As shown in Fig. 7, L1 release was evident in transfected CHO cells using cell surface iodination (Fig. 7A) or biosynthetic labelling with [35 S]Met/Cys (Fig. 7B). The L1 release could be blocked by TAPI as expected (data not shown). In contrast, human L-selectin transfected into CHO cells did not show significant shedding in the short term assay (Fig. 7A) and little L-selectin was detectable in the medium of biosynthetically labelled cells (Fig. 7B). In the presence of PMA the CHO cells did not significantly upregulate the release of L1 or L-selectin (Fig. 7B).

To rule out the possibility that the human L-selectin expression plasmid was defective we also carried out transfection studies in COS-7 cells. As shown in Fig. 7B both L1 and L-selectin release were clearly detectable in COS-7 cells. The release of both molecules occurred spontaneously and was only weakly upregulated in the presence of PMA.

Previous studies have shown that human L-selectin can indeed be shed from CHO cells (Arribas et al., 1996). To examine why L-selectin was not cleaved in the CHO subline used here, we performed northern blot analysis with a mouse TACE specific probe. As shown in Fig. 8 the probe hybridized to a 4 kb mRNA species which was expressed at high levels in several of the cell lines tested. In contrast, expression of TACE in CHO cells was only very low. Separate experiments showed that the probe hybridized strongly to mRNA derived from hamster spleen ruling out the possibility of weak cross-hybridization (not shown). These results suggested that the failure of CHO cells to shed L-selectin was most likely due to the lack of TACE and that L1 release was independent of TACE expression.

**DISCUSSION**

The release of L1 adhesion molecule from the cell surface has been noticed under different circumstances. Early studies have shown that cultured PC12 rat pheochromocytoma cells and
sympathetic neurons can release increased amounts of L1 into the medium in response to nerve growth factor (Richter-Landsberg et al., 1984). Similarly, soluble forms of L1 are released from cultured mouse cerebellar cells (Sadoul et al., 1988). Also melanoma M21 cells spontaneously release L1 into the medium at a rate of approx. 10^4 molecules/cell/hour (Montgomery et al., 1996) and stimulation of neutrophils with PMA causes rapid loss of L1 from the cell surface (Hubbe et al., 1993). The molecular mechanism of L1 release and the protease(s) involved in this process have been unknown. In the present report we provide evidence that (i) L1 can be released from the cell surface of several cell types, (ii) the released L1 is not degraded and retains its ability to support integrin-mediated cell adhesion and migration; (iii) the process is blocked by the metalloproteinase inhibitor TAPI; (iv) the mechanism of L1 release is distinct from L-selectin shedding and does not involve TACE.

Our starting observation was that L1 on B16F10 melanoma cells or other cell lines of human or mouse origin was released into the tissue culture medium. The released product was approx. 15-20 kDa smaller than the cell surface L1. This finding and the observation that the remaining membrane associated fragment had a size of approx. 16 kDa suggested that the cleavage site is located in the ectodomain of L1 most likely juxtaposed to the transmembrane region. This conclusion is supported by our recent finding that a truncation of the complete cytoplasmic tail of L1 does not significantly affect the release into the medium (S. Beer, unpublished). The released form of L1 described in this report may be identical to the previously described soluble L1-180 which is released from membranes of mouse brain following incubation in PBS for 2 hours at 37°C (Sadoul et al., 1988). It seems therefore likely that primary brain cells possess a similar shedding machinery as described here for established cell lines.

A striking finding was that the release of L1 was sensitive to the metalloproteinase inhibitor TAPI. This compound is a representative of a family of hydroxamic acid-based zinc-metalloprotease inhibitors that can block conventional metalloproteinases as well as membrane protein secretases which has been termed ‘ectodomain shedding’ (Hooper et al., 1997; Turner and Hooper 1999). TACE is involved in the shedding of a diverse group of membrane-anchored proteins including pro-TNF-α, TGF-α, L-selectin and the p75 TNF receptor (Peschon et al., 1998). ADAM-10 can cleave pro-TNF-α and the Alzheimer’s β-amyloid precursor protein (Rosendahl et al., 1997; Lammich et al., 1999). Many other molecules are released from the membrane, however, the shedding enzymes involved are less well studied. It is possible that a closely related family of metalloproteinases controls these shedding events but the number of proteases involved is unknown. Studies with shedding deficient CHO mutant cells have suggested the existence of some common components for the machinery of ectodomain shedding (Arribas et al., 1996, 1997). The mutant CHO cells (M2 cells) were established by mutagenesis and selection for lack of TNF-α shedding but proved to be also deficient in the shedding of several other molecules. A recent study from this group has shown that the mutant cells were not defective in TACE but rather in a component that controls TACE activity (Merlos-Suarez et al., 1998). The component mutated in M2 cells appears to control only a subset of shedding proteases since Notch processing, which depends on the activity of ADAM-10/Kuzbanian, was normal in these cells (Merlos-Suarez et al., 1998). These observations are relevant for the data presented here showing that our CHO cells could release L1 but not L-selectin. In our case a low expression of TACE was likely to be responsible for the defect. This is supported by recent evidence, that retransflection of L-selectin-CHO cells with mouse TACE can restore the PMA inducible release of L-selectin (S. Beer unpublished results). Our observation of intact L1 shedding in CHO cells is in agreement with the results of Merlos-Suarez et al. that other ADAMs are active in CHO cells. The cell surface release of L1 described in this paper has common as well as distinct features from previous studied ectodomain shedding events. In agreement is that the L1 cleavage occurs close to the membrane and can be blocked by the metalloproteinase inhibitor TAPI. However, in contrast to other systems the L1 release is only poorly upregulated by PMA treatment. Further studies will be necessary to identify the metalloproteinase involved in L1 release as well as the exact cleavage site.

Could released L1 be of biological significance? In situ, released L1 has been detected in association with the extracellular matrix of murine sciatic nerve (Martini and Schachner, 1986) and within intratumor laminin strands of human tumors growing in nude mice (Montgomery et al., 1996). It seems possible that ECM proteins can capture and retain released L1. In support of this, selective binding between L1 and laminin has been demonstrated in vitro which, at least in part, is mediated by the HNK-1 carbohydrate epitope (Hall et al., 1993, 1997). Our recent results demonstrate that L1 via the 1st Ig-like domain can interact with the brain specific proteoglycan neurocan (Oleszewski et al., 1999). Still, the physiological function of matrix-embedded L1 remains uncertain. Conceivably, matrix-embedded L1 gradients could locally promote targeted cell migration as a substrate for integrin adhesion. Such a role is consistent with the results presented in this report showing that shed or recombinant L1 can support haptotactic migration of B16F10 cells and the results from previous work showing that L1 can promote αvβ3 mediated migration of human tumor cells or activated T lymphocytes (Montgomery et al., 1996; Duczmal et al., 1997). Released L1 may also promote intratumor angiogenesis by interacting with αv integrins on sprouting vessels (Felding-Habermann et al., 1997). Finally, recent experiments by Yip et al have shown that the RGD motif in L1 can promote neurite outgrowth via interaction with αvβ3 integrin (Yip et al., 1998). L1 released by brain cells could be therefore an important substrate for the navigation of neural cells.

We thank Dr Stefan Rose-John for helpful suggestions at the beginning of this study and Dr Sammar Marei for valuable discussions. We are also thankful to Klaus Hexel for help with FACs sorting, Dr Rüdiger Pipkorn for peptide synthesis, Dr Vance Lemmon for L1-J558 cells and mAb 74-5H7, Dr Roy Black and Immunex for providing reagents and to Dr Hideo Yagita for monoclonal antibodies.

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