Role of Src Kinases in the ADAM-mediated Release of L1 Adhesion Molecule from Human Tumor Cells*  

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Paul Gutwein, Matthias Oleszewski, Sabine Mechtersheimer, Nancy Agmon-Levin‡, Kerstin Krauss, and Peter Altevogt§

From the Tumor Immunology Programme, 0710, German Cancer Research Center, D-69120 Heidelberg, Germany

The ectodomain of certain transmembrane molecules can be released by proteolysis, and the solubilized antigens often exert important biological functions. We demonstrated before that the L1 adhesion molecule is shed from the cell surface. Here we show that L1 release in AR breast carcinoma cells is mediated by a member of the disintegrin metalloproteinase (ADAM) family of proteases. Up-regulation of L1 shedding by phorbol ester or pervanadate involved distinct mechanisms. Pervanadate induced shedding and rounding-up of cells from the substrate, which was blocked by the Src kinase inhibitor PP2. Tyr phosphorylation of the L1 cytoplasmic tail and the Src kinase Fyn was observed following pervanadate treatment. Up-regulation of L1 release and activation of Fyn occurred also when cells were detached by EDTA suggesting that the regulation of L1 shedding by this pathway was linked to cell morphology and adhesion. The phorbol 12-myristate 13-acetate-induced shedding was inhibited by the protein kinase C inhibitor bisindolylmaleimide I and by PD98059, a specific inhibitor of the mitogen-activated protein kinase pathway. Soluble L1 binds to the proteoglycan neurocan and in bound form could support integrin-mediated cell adhesion and migration. We propose that the release of cell-associated adhesion molecules such as L1 may be relevant to promote cell migration.

Many transmembrane proteins can undergo cleavage and release of their ectodomain into the medium (for review see Refs. 1–3). These proteins are diverse in structure and function and comprise molecules such as TNF-α (4–8), FasL (9), interleukin-6 receptor (10, 11), T-selectin (12–14), pro-TGF-α, and the β-amyloid precursor protein (15–17). Although there is evidence for a physiological function of many released molecules, a general significance for ectodomain shedding is still disputed (2). Recently, members of the ADAM metalloproteinase family, which are membrane proteins composed of a disintegrin and metalloproteinase domain, were shown to be important in ectodomain release (for review see Refs. 3 and 18). TACE/ADAM17 mediates the membrane release of TNF-α, l-selectin, TGF-α (8), and TRANCE (tumor necrosis factor-related activation-induced cytokine), a TNF family member involved in osteoclastogenesis and dendritic cell survival (19). ADAM10/Kuz has been described as an α-secretase for the cleavage of β-amyloid precursor protein (20), and ADAM9 is involved in the phorbol ester-induced ectodomain shedding of membrane-anchored heparin-binding EGF-like growth factor (HB-EGF) (21, 22). Certain released molecules can be cleaved by more than one enzyme, and some enzymes can cleave more than one substrate. For example, TNF-α cleavage can also be mediated by ADAM10 (23), and α-secretase activity for β-amyloid precursor protein has been attributed to TACE/ADAM17 (24) and ADAM9 (25). It is not known how the protease(s) select their substrate, because consensus cleavage sites have not been identified. In addition to the proteolytic function, some members of the ADAM family like ADAM15 and ADAM2 can support integrin binding via their disintegrin domain (26, 27). It is increasingly recognized that ADAMs represent a novel group of membrane proteases that are important for cellular interactions under physiological and pathophysiological conditions (28).

We have recently shown that a soluble form of the L1 cell adhesion molecule can be released from mouse and human tumor cells by membrane proximal cleavage (29). L1 is a 200–220-kDa type I membrane glycoprotein of the immunoglobulin (Ig) superfamily expressed in neural, hematopoietic and certain epithelial cells (30–33). L1 supports homophilic and integrin-mediated cell binding and can interact with high affinity with neurocan, a proteoglycan that occurs in the brain and activated T lymphocytes (34–36). L1 shed from B16F10 melanoma cells remained intact and could serve as substrate for integrin-mediated cell adhesion and migration (29). In addition to the cell surface localization, L1 occurs in the extracellular matrix of brain and tumor cells (37, 38). A recent study has shown that L1 offered in solution or as substrate can deliver survival signals to growing neurons (39). Thus, the release of L1 from the cell surface could play an important biological role and could be a prerequisite for matrix integration. However, the enzyme(s) involved in the release process and the regulation are still unknown.

We showed before that L1 release was inhibited by the hydroxamate-based metalloproteinase inhibitor TAPI (Immunex compound 3) (29). This compound cannot distinguish between conventional matrix metalloproteinases and ADAMs. Moreover, a common feature of ectodomain shedding by ADAMs is the strong up-regulation by substances such as PMA or pervanadate. In contrast, L1 shedding was only poorly up-regulated by PMA when cells were in suspension (29). In search of

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§ To whom correspondence should be addressed: Tumor Immunology Programme, G0100, German Cancer Research Center, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany. Tel.: 06221-423714; Fax: 06221-423702; E-mail: P.Altevogt@dkfz-heidelberg.de.

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the enzymatic activity, we have now analyzed the regulation of L1 release and investigated a possible role of cell attachment in PMA and pervanadate up-regulation. We find that up-regulation of L1 shedding is observed in attached cells but not when in suspension. Detachment by EDTA and the rounding up of cells induced by pervanadate strongly enhances L1 release and were accompanied by Fyn activation. The pervanadate-induced effects on cell morphology and L1 release were blocked by the Src kinase inhibitor PP2. Additionally, our data provide evidence that L1 cleavage is mediated by an ADAM and that released L1 can be immobilized by binding to neurocan and becomes an integrin substrate. These findings establish a mechanism by which L1 can be converted from a membrane bound into a matrix-associated form.

MATERIALS AND METHODS

Cells—The human neuroblastoma cell line Kelly was obtained from Prof. Manfred Schwab, DKFZ Heidelberg. The breast tumor cell line AR was obtained from Dr. Brigitte Guckel, University of Tubingen. MED-B1 cells were described before (40). CHO cells stably transfected with human L1-CHO (hL1-CHO) or mouse L1-CHO (mL1-CHO) have been described (29). A mouse L1 mutant protein with a truncation of the cytoplasmic tail was constructed using polymerase chain reaction. Primers were designed to introduce a STOP codon after amino acid S1149 in the L1 tail was constructed using polymerase chain reaction. Primers were with human (hL1-CHO) or mouse L1 (mL1-CHO) have been described.

Chemicals and Antibodies—The following mAbs were used and have been described in previous publications (40, 41): mAbs 324 against the membrane form and that the cytoplasmic tail was missing vector (41). For all polymerase chain reactions the Pfu turbo polymerase (Stratagene) 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 (41). Shed L1 from the tissue culture supernatant of human AR breast carcinoma cells was purified using a mAb UJ 127.11 affinity column as described previously for mouse L1 (40).

Analysis of L1-Neurocan Binding—For the analysis of L1-neurocan interaction, 1 μg of recombinant neurocan (gift from Dr. U. Rauch, University of Lund, Sweden) was adsorbed to Immobilon membranes using a dot blot apparatus as described before (37). Membranes were incubated with released L1 from AR cells, and the binding was analyzed by subsequent incubation of the membrane with mAb UJ 127.11 followed by peroxidase-conjugated goat anti-mouse IgG and ECL detection (Amersham Pharmacia Biotech). Neurocan (1 μg/ml) was also coated to LABTEK slides for 16 h at 4 °C. Wells were blocked with 3% BSA in PBS for 20 min at room temperature, washed with Hank’s balanced salt solution containing 10 mM Hepes, 2.5 mM Ca2+ and Mg2+, and incubated for 45 min with affinity purified shed L1 (1–1 μg/ml) or purified L1-Fc (1–1 μg/ml). Wells were then washed and used for the cell binding assay. For binding, MED-B1 cells (5 × 104/ml) were suspended in Hank’s balanced salt solution containing 10 mM Hepes and 0.5 mM Mn2+, and 0.2-ml aliquots were added to the coated slides. The binding assay was performed for 30 min at room temperature. 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.

Transmembrane Domain—For the haptotactic cell migration assay, the purified shed L1, human L1-Fc fusion protein (both at ~2 μg/ml), or BSA for control was coated for 90 min to the backside of Transwell chambers (Costar, 6.5-mm diameter, 5-μm pore size). 2.5 × 104 AR breast or OAW42 ovarian carcinoma cells in RPMI medium containing 0.5% BSA were seeded into the upper chamber and were allowed to transmigrate to the lower compartment for 16 h at 37 °C. To quantitate transmigrated cells, the inner chamber was removed and cleaned carefully with a cotton swab to remove the nonmigrated cells. Migrated cells adherent to the backside of the membrane were stained with crystal violet solution. The membranes were extensively washed in water, and the remaining stain was eluted with 10% acetic acid. The eluted dye was measured at 595 nm in an enzyme-linked immunosorbent assay reader.

Biochemical Analysis—Samples were separated by SDS-polyacrylamide gel electrophoresis under nonreducing conditions and transferred to an Immobilon membrane using semi-dry blotting. After blocking with 5% skim milk in Tris-buffered saline, the blots were developed using the respective primary antibody (mAb UJ127.11 for human L1 or mAb UJ127.11 for human L1 or a polyclonal antibody for mouse L1) followed by peroxidase-conjugated secondary antibody and ECL detection. Tyr phosphorylation in AR cells was analyzed by incubating cells for the indicated length of time at 37 °C in the presence of PMA or pervanadate. Cells were then lysed in lysis buffer containing 10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 2% Nonident P-40, 1% deoxycholate in the presence of Na3VO4 for blocking of phosphatases. Lysates were immunoprecipitated with mAb UJ 127.11 coupled to Sepharose or anti-Fyn coupled to agarose, washed, and eluted from the Sepharose by boiling in nonreducing SDS sample buffer. The samples were then separated by SDS-polyacrylamide gel electrophoresis, blotted to Immobilon membrane, and probed with a phosphostryrosine-specific mAb and ECL detection. Intensities of protein bands were quantitated using the Lumimager® (Roche-Diagnostics).

RESULTS

Role of ADAMs in L1 Release—Previous reports have shown that melanoma cells and other tumor cell lines can release L1 from the cell surface both in vitro and in vivo (29, 38). To further investigate the mechanism, we chose human Kelly melanoma cell lines that constitutively express ADAMs (29) and human breast carcinoma cells. Both cell lines expressed L1 at the cell surface and released it into the medium. Using mAbs to the ectodomain or cytoplasmic tail of L1, we found that L1 in the medium was ~16 kDa smaller than the membrane form and that the cytoplasmic tail was missing (data not shown). These results confirm our previous observation that L1 is released by membrane proximal cleavage (29).

The hydroxamate-based metalloproteinase inhibitor TAPI (Immunex compound 3) was shown to inhibit L1 release (29). This inhibitor can block conventional matrix metallo-
teinases as well as ADAMs. To discern which metalloproteinase was involved in L1 release, we analyzed the effect of TIMPs and several other protease inhibitors on the shedding of AR cells in suspension. As shown in Table I, TIMP-1 and TIMP-2 were unable to block the L1 release from AR cells. It should be noted that the concentration used can block conventional matrix metalloproteinases (44) and the ectodomain shedding of Her2 (43). L1 release was also blocked by 1,10-phenanthroline, the metalloproteinase inhibitors BB-3103 and Ro 31–9790 (data not shown). In the presence of 10 mM EDTA but not EDTA. A small but consistently observed effect was seen in the presence of 10 mM dithiothreitol. Serine proteinase inhibitors did not show any effect on the L1 release. The failure of TIMPs to block shedding is consistent with the notion that ADAMs rather than conventional MMPs are involved in L1 release.

Up-regulation of L1 Shedding by Phorbolester and Pervanadate—In several antigenic systems ectodomain shedding can be up-regulated by PMA treatment of the cells. We reported before that L1 shedding in ESB-MP or B16F10 cells was only poorly up-regulated by PMA when cells were in suspension (29). We investigated a possible role of cell attachment in PMA regulation. As shown in Fig. 1, A and C, at 100 ng/ml PMA the basal level of L1 release in AR cells was increased ~7-fold in attached cells (A). In contrast, there was only a minor relative increase when cells were in suspension (C). Similar results were obtained with hL1-CHO cells (data not shown). We also tested Kelly cells for enhanced L1 shedding. As shown in Fig. 1, B and D, Kelly cells did not up-regulate L1 release in response to PMA when attached, and only a weak increase was seen in suspension.

Pervanadate is another agent that can up-regulate ectodomain shedding of various molecules. As shown in Fig. 2A, L1 shedding was indeed up-regulated by pervanadate and further controls indicated that H2O2 or vanadate alone were not effective. In attached AR cells, pervanadate up-regulated shedding ~6-fold (Fig. 2B), and a 4-fold increase was seen in Kelly cells (Fig. 2C). Similar findings were made in hL1-CHO cells (not shown). Thus, in contrast to the results with PMA, pervanadate up-regulated L1 release also in Kelly cells. In addition, pervanadate up-regulation was only observed when cells were attached and little effect was seen in suspension (Fig. 2, D and E). Further experiments indicated that both PMA- and pervanadate-induced shedding of L1 were fully blocked by the metalloproteinase inhibitors BB-3103 and Ro 31–9790 (data not shown).

Pervanadate Treatment Causes Changes in Cell Shape—We noticed that pervanadate treatment, much in contrast to PMA, induced a rounding up of cells that were spread and attached in the beginning. This was observed for all cell types analyzed and is demonstrated for AR cells in Fig. 3 (upper panel). We asked whether detachment per se increased the rate of shedding independent of PMA or pervanadate addition. To test this, we compared the basal amount of shed L1 in attached or suspension cells using identical numbers of AR cells. Surprisingly as shown in Fig. 4A, detachment of cells by EDTA/PBS already up-regulated L1 release by ~8-fold, and this increase was comparable in magnitude to the PMA-induced shedding seen in attached cells. Similar up-regulation of shedding was observed when Kelly cells were suspended (data not shown). Thus, the failure to observe up-regulated shedding of L1 in suspension was because of the fact that the release process was already activated in the first place and further increase was not possible.

**PMA and Pervanadate Induce L1 Release by Distinct Pathways**—To dissect the different modes of induction for L1 release, we searched for differential effects of kinase inhibitors. As shown in Fig. 3 (lower panel), the pervanadate-induced rounding up of AR cells was completely inhibited in the presence of the Src kinase inhibitor PP2. The negative control PP3, which blocks the activity of the epidermal growth factor receptor kinase, was without effect (not shown). Other inhibitors, like the protein kinase C inhibitors staurosporine and bisindolylmaleimide I or the MAP kinase kinase inhibitor PD98059, did not block the rounding up of cells (not shown). These data suggested that pervanadate presumably activated Src kinases normally retained in an inactive form by dephosphorylation and that activated Src kinases were responsible for the morphological changes.

We next investigated the effect of the inhibitors on the up-regulation of L1 release in attached AR cells. As shown in Table II, staurosporine and bisindolylmaleimide I blocked PMA-induced L1 shedding; however, they had no effect on the pervanadate-induced L1 release. On the other hand, the Src kinase inhibitor PP2 affected only the pervanadate-induced L1 shedding.

In contrast to adherent cells, PP2 was unable to inhibit the L1 release when AR cells were in suspension, even when higher concentrations of inhibitor were used (data not shown). We considered the possibility that EDTA/PBS-mediated cell detachment lead to a rapid activation of Src kinases and the shedding process and that at later time points PP2 was ineffective. We therefore examined the state of phosphorylation of Src kinases in pervanadate-treated or EDTA/PBS-detached AR cells. As shown in Fig. 4B, the Src kinase Fyn was Tyr-phosphorylated in pervanadate-treated but not in PMA-treated AR cells (compare lanes 3 and 5). Tyr phosphorylation of Fyn was blocked in the presence of PP2 (lane 4). EDTA/PBS detachment of AR cells also activated Fyn as detected by the state of Tyr phosphorylation (lane 2). Thus, activation of Fyn together with the induction of morphological changes was a common feature of pervanadate or EDTA/PBS detachment-induced L1 shedding.

**PMA-induced Shedding of L1 Is Dependent on the MAP Kinase Pathway**—Recent studies on the shedding of HB-EGF, TGF-α, and i-selectin have provided evidence for an important role of the MAP kinase(s) in the regulation of ectodomain release (21, 45). To investigate a potential role of this signaling cascade in L1 release, we analyzed the effect of PD98059, a specific inhibitor for the activation of MAP-kinase(s). As shown in Fig. 5, preincubation of AR cells or hCHO-L1 cells with PD98059 at a dose of 45 μM reduced PMA-induced L1 release by ~50%, whereas pervanadate-induced L1 shedding remained unaltered.

**Role of L1 Phosphorylation in the Up-regulation of L1 Release**—The cytoplasmic tail of L1 can be phosphorylated on Tyr or Ser/Thr residues (46). To study whether L1 phosphorylation...
was associated with the regulation of L1 shedding, we investi-
gated changes of Tyr phosphorylation following PMA or per-
vanadate treatment in attached AR cells. As shown in Fig. 6
A, pervanadate but not PMA treatment caused an increased level
of Tyr phosphorylation in L1. As both substances augmented
L1 release, these data implicated that Tyr phosphorylation of
L1 was not an absolute requirement.

To further study whether the cytoplasmic tail of L1 was
essential for the up-regulation of L1 release, we used CHO cells
stably transfected with mouse L1 (ml1-CHO) and a mutant
form in which most of the cytoplasmic tail except three amino
acids was deleted (ml1 Δcyt-CHO). The L1 cell surface expres-

FIG. 1. PMA-induced L1 shedding is dependent on cell attachment. Attached AR (A) or Kelly cells (B) were incubated in the presence or
absence of the indicated amount of PMA in tissue culture plates in Dulbecco’s modified Eagle’s medium without fetal calf serum for 1 h (attached).
AR cells (C) and Kelly cells (D) were also detached by EDTA/PBS and analyzed for shedding in suspension (suspension). Media were analyzed for
shed L1 using Western blot analysis with mAbs to the ectodomain of human L1 followed by ECL detection. Band intensities were quantitated by
densitometric scanning setting control values of medium alone as 100%.

FIG. 2. Induction of L1 release by pervanadate. The assay was performed as described in the legend to Fig. 2 except that pervanadate was
used instead of PMA. (A) L1 shedding is specific for pervanadate (Per) and is not seen with H2O2 or vanadate alone (V). Media were analyzed for
shed L1 using Western blot analysis with mAbs to the ectodomain of human L1 followed by ECL detection. B, attached AR cells; C, attached Kelly
cells; D, suspension AR cells; E, suspension Kelly cells. Band intensities were quantitated by densitometric scanning setting control values of
medium alone as 100%.

FIG. 3. Effect of pervanadate on cell
shape. Cells were treated with pervana-
date for the indicated length of time in the
absence (upper panels) or presence (lower
panels) of Src kinase inhibitor PP2 (50
μM).
shedding of L1 in AR cells. Cells were preincubated for 1 h with the indicated concentration of inhibitor (45 mM) before the addition of PMA or pervanadate. PD. PD088059. Media were analyzed for shed L1 using Western blot analysis with mAbs to the ectodomain of human L1 followed by ECL detection. Band intensities were quantitated by densitometric scanning setting control values of medium alone as 100%.

**Table II**

| Inhibitor     | Specificity          | Concentration | Inhibition of PMA-induced L1 shedding | Inhibition of PMA-induced L1 shedding |
|---------------|----------------------|---------------|--------------------------------------|-------------------------------------|
| Staurosporine | Protein kinase C     | 100 nM        | %                                    | %                                   |
| Bisindolylmaleimide | Protein kinase C | 10 μM        | 0                                    | 100                                 |
| PP2           | Src family of tyrosine kinases | 100 μM | 86.2                                 | 0                                   |

**Fig. 4. Role of MAP kinase signaling in L1 release.** Effect of the MAP kinase kinase inhibitor PD98059 on PMA- or pervanadate-induced shedding of L1 in AR cells. Cells were preincubated for 1 h with the indicated concentration of inhibitor (45 μmol) before the addition of PMA or pervanadate PD. PD088059. Media were analyzed for shed L1 using Western blot analysis with mAbs to the ectodomain of human L1 followed by ECL detection. Band intensities were quantitated by densitometric scanning setting control values of medium alone as 100%.

**Fig. 5. Cell detachment induces L1 shedding and activates Fyn.** A, shedding of L1 was analyzed on the identical number of AR cells (5 × 10⁵) either when attached or when in suspension after release by PBS/EDTA. A control of PMA-treated AR cells was included. Media were analyzed for shed L1 using Western blot analysis with mAbs to the ectodomain of human L1 followed by ECL detection. Band intensities were quantitated by densitometric scanning setting control values of medium alone as 100%. Lane 1, attached AR cells; lane 2, attached AR cells treated with PMA; lane 3, AR cells in suspension after EDTA/PBS. B, AR cells were treated for 1 h as indicated below, and lysates were prepared in the presence of Na₂VO₄ to preserve phosphorylation. Anti-Fyn immunoprecipitates from lysates were resolved by SDS-polyacrylamide gel electrophoresis and blotted, and Tyr phosphorylation (PY) was tested using a specific mAb followed by ECL detection. Lane 1, attached AR cells; lane 2, PBS/EDTA-treated AR cells in suspension; lane 3, attached AR cells treated with pervanadate; lane 4, attached AR cells treated with pervanadate plus 50 μM PP2; lane 5, attached AR cells treated with PMA. Total loading of Fyn was determined by blotting with anti-Fyn antibodies followed by ECL detection.

**DISCUSSION**

The present paper sheds new light on the membrane proximal cleavage of L1 adhesion molecule by showing that (i) L1...
release is mediated by a member of the ADAM metalloproteinase family; (ii) it is up-regulated by PMA, pervanadate, and EDTA/PBS-mediated detachment of cells; (iii) in AR cells activation of Fyn together with the induction of morphological changes are common features of pervanadate and EDTA/PBS-induced L1 shedding; and (iv) that released L1 can interact with neurocan and serve as substrate for integrin-mediated adhesion and migration.

ADAMs appear to play a prominent role in the membrane proximal release of many shed molecules; however, it is difficult to discriminate between conventional matrix metalloproteinases and ADAMs. Inhibition analysis with recombinant TACE/ADAM17 have revealed that a variety of hydroxamate-based compounds as well as TIMP-3 but not TIMP-1, -2, or -4 could block cleavage of a TNF-α peptide bearing the TACE cleavage site (49). TIMP-3 was also able to block the PMA-induced release of L1-selectin from human leukocytes (50). We have shown before that L1 release appeared to be independent of TACE as it was not impaired in TACE-deficient CHO cells (29) as well as in fibroblasts from TACE−/− mice.2 We present now suggestive evidence that L1 release is mediated by another member of the ADAM family. The failure of TIMP-1 and -2 to affect release and the strong blocking observed with hydroxamate inhibitors like Ro 31–9790 or BB-3103 are in support of this notion. Unfortunately, TIMP-3 was not available for our analysis. We also demonstrate that shedding can be activated by PMA in AR cells or L1-CHO transfectants but only when attached to their matrix. Interestingly, a recent study on PMA-induced shedding of membrane-anchored HB-EGF has also shown that shedding occurred only when cells were attached (22). However, in contrast to our findings, no shedding was seen when cells were in suspension, but the PMA inducible release was restored when cells had attached again (22). The reasons for these disparate findings are not known. As in many other systems, the release of L1 was also up-regulated in cells treated with pervanadate. Pervanadate is a strong inhibitor of phosphatases, which can activate kinases normally kept in an inactive form by phosphatases. Again, up-regulation of L1

2 S. Mechtersheimer and S. Beer, unpublished results.

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**Fig. 6. Phosphorylation of L1 after PMA or pervanadate treatment.** A, attached AR cells were treated with PMA or pervanadate for the indicated length of time and lysed in the presence of Na3VO4 followed by immunoprecipitation with Sepharose-conjugated mAb to human L1. After blotting, Tyr phosphorylation of L1 was tested using a specific mAb followed by ECL detection. B, analysis of PMA or pervanadate (PER) induced shedding in ml1-CHO and ml1Δcty-CHO cells. Attached cells were stimulated for 1 h, and media were analyzed for shed L1 using Western blot analysis with an antibody to the ectodomain of mouse L1 followed by ECL detection. IP, immunoprecipitate.

**Fig. 7. Functional characterization of shed L1.** A, shed L1 can bind to neurocan. Recombinant neurocan (1 mg/ml) was adsorbed to Immobilon membrane using a dot blot apparatus and subsequently incubated with conditioned medium containing shed L1 from AR cells. The conditioned medium was serially diluted in RPMI 1640 (N.d., nondiluted). L1 bound to neurocan was detected using the L1 mAb and ECL detection. Purified human L1-Fc (1 mg/ml) served as positive control, and plain medium was used as negative control. B, L1 bound to neurocan supports cell binding. Neurocan was adsorbed to glass slides that were then blocked with BSA before the addition of affinity purified shed L1 from AR cells or purified L1-Fc. MED-B1 cells were allowed to bind in the presence of Mn2+ ions to activate integrins. Blocking mAbs to the integrin chains αv, β3, or β1 were used at a final concentration of 10 μg/ml. C, shed L1 supports cell migration. Shed-L1, L1-Fc, or BSA for control was coated to the backside of Transwell chambers. AR or OAW42 cells were seeded into the top chamber and allowed to transmigrate for 16 h at 37 °C. Each determination was done in quadruplicate, and transmigrated cells were stained from the backside of the filter. The dye was eluted from the filter and measured at 595 nm. The amount of dye is proportional to the number of transmigrated cells. Results are given as mean values ± S.E.
shedding was only seen when cells were attached, and only marginal enhancement was observed in suspension cells. The pervanadate- or PMA-induced up-regulation of L1 shedding followed distinct pathways as revealed by inhibition analysis. The PMA-induced shedding was blocked only by protein kinase C inhibitors, whereas the pervanadate-induced shedding of L1 was inhibited by PP2, a potent and selective inhibitor of the Src family of protein Tyr kinases. Pervanadate treatment of AR cells caused Tyr phosphorylation of L1, which was not seen in PMA-treated cells. Moreover, attached Kelly cells did not up-regulate L1 release in response to PMA but did so in response to pervanadate. These findings already indicated that the PMA- and pervanadate-mediated signaling pathways leading to enhanced L1 shedding were distinct.

An important observation was that up-regulation of L1 shedding occurred in cells that were detached from the substrate by EDTA/PBS. Similar observations have been made before for the metallocproteinase-dependent shedding of the cell surface proteoglycan syndecan (48, 51). The induction of L1 shedding by pervanadate and the EDTA-mediated detachment were similar as they were accompanied with drastic changes of the cytoskeleton and activation of the Src kinase Fyn in AR cells. Importantly, the PP2 inhibitor could block the morphological changes as well as the L1 shedding only in attached cells, whereas it was ineffective in suspension cells. This suggested that activation of Fyn was an early event in the signaling cascade leading to up-regulated L1 release. An involvement of Src kinases in the regulation of shedding processes has not been reported so far. Our observation that Fyn activation and morphological changes can induce up-regulation of L1 release established a link between the activity of ADAMs and the status of cell adhesion and cytoskeletal organization. Indeed, an important relationship between Src kinases, integrins, and the cytoskeleton is increasingly recognized (for review see Ref. 52. Src family tyrosine kinases bind to components of the focal-adhesion complex, where their activity is regulated by integrin-ligand binding (53). Moreover, Src kinases were found to be necessary for integrin but not platelet-derived growth factor receptor signal transduction (54). Felsenfeld et al. (55) reported a regulation of integrin-cytoskeleton interaction by the Src kinase, which was selective for the vitronectin receptor but not for the fibronectin receptor α5β1. Also c-Src overexpression enhances cell matrix adhesion and migration (56). How Src kinase activation and cytoskeletal changes can activate ADAM-mediated release of substrate molecules is presently unknown.

Several recent studies have addressed the signaling pathway leading to the shedding of transmembrane molecules. Gechtman et al. (22) studied the release of HB-EGF transfected in CHO cells and found that PMA-induced shedding was blocked by the MAP kinase kinase inhibitor PD98059. Fan and Derynck (45) reported that TGF-α shedding induced by PMA, serum addition, or via growth factor receptors involved the MAP kinase pathway. In addition, the fMet-Leu-Phe-induced shedding of 1-selectin from neutrophils was blocked by inhibitors of p38 MAP kinase signaling (45). Moreover, the basal level of TGF-α release in CHO cells involved the p38 MAP kinase signaling because it could be blocked with the specific inhibitor SB202190. It was concluded that the activation of extracellular signal-regulated kinase and p38 MAP kinase signaling pathways might represent a general physiological mechanism to induce shedding (45). In contrast, Izumi et al. (21) reported that protein kinase C-δ is involved in the PMA-induced ectodomain shedding of HB-EGF in Vero-H green monkey kidney cells. It was proposed that protein kinase C-δ and MDC9 (ADAM9) could directly interact and thereby regulate shedding (21). The discrepancy between these findings could be because of alternative signaling pathways. Our results on L1 shedding support this notion. The PMA-induced shedding of L1 was partially blocked by the MAP kinase kinase inhibitor PD98059 and is therefore in agreement with the first two studies. However, the induction of L1 shedding by pervanadate involved Fyn activation and was independent of MAP kinases.

In this context it is interesting to note that a novel regulatory pathway of Src family kinase activity by protein kinase C-δ has been reported in a mast cell line (57). We can at present not exclude the possibility that Fyn and protein kinase C-δ cooperate in the induction of L1 shedding by this second pathway.

It is generally believed that the cleavage of membrane proteins by ADAMs requires the presence of the membrane-anchored enzyme and its substrate in cis in the same membrane (18). A still open question is how the protease selects the substrate and how both are brought together. The shedding of L1 has certain features that are not shared by other release mechanisms. One important difference is the amount of released molecules. In many examples, shedding can be followed at the cell surface after treatment with substances like PMA or more physiological stimuli. For example, PMA-induced cleavage of HB-EGF, which appears to involve ADAM9 (21), can be followed by cell surface fluorescence, and nearly 80% of material is lost within 1 h of incubation (21). Similar observations have been made for the induced 1-selectin (49) or syndecan cleavage (50), which appear also to be quite rapid. In contrast, PMA-induced L1 release cannot be followed at the cell surface using for example FACS staining, implicating that the number of newly synthesized molecules reaching the cell surface is similar to the number of released molecules. On the other hand, it is clear from our previous work using cell surface iodinated cells that cleavage indeed occurs at the cell surface as iodinated molecules can be recovered from the medium (29). This points to a rate-limiting element in the enzymatic release process of L1, which is distinct from the examples given above. Our present observation that the rate of shedding in suspension was only marginally augmented by PMA or pervanadate is in support of this notion. One possibility is that ADAM and substrate have to meet in a membrane compartment and that access to this compartment is limited. Our results suggest that the cytoskeleton and cell adhesion play an important role in regulation the rate of cleavage.

Finally, we addressed the question whether a biological role for released L1 can be found. We present evidence that shed L1 of human tumor cells could bind to neurocan and in its bound form could still serve as integrin substrate. Thus, masking of the sixth domain containing RGD sites because of steric hindrance can be excluded. This is interesting in light of previous reports showing that neurocan binding can interfere with homophilic L1-L1 binding (34, 35). Given the high affinity of L1-neurocan binding it is conceivable that L1 can be trapped and presented by neurocan. Previously, released L1 has been detected in association with the extracellular matrix of the murine sciatic nerve (38) and within intratumor laminin strands of human tumors growing in nude mice (37). It seems possible that extracellular matrix proteins like laminin and proteoglycans like neurocan can capture and retain released L1. These findings establish a mechanism by which L1 can be converted from a membrane bound into a matrix-associated form. It remains to be addressed in which biological settings extracellular matrix-retained L1 is of significance.

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Role of Src Kinases in the ADAM-mediated Release of L1 Adhesion Molecule from Human Tumor Cells
Paul Gutwein, Matthias Oleszewski, Sabine Mechtersheimer, Nancy Agmon-Levin, Kerstin Krauss and Peter Altevogt

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