The transmembrane and multidomain neural adhesion molecule L1 plays important functional roles in the developing and adult nervous system. L1 is proteolytically processed at two distinct sites within the extracellular domain, leading to the generation of different fragments. In this report, we present evidence that the proprotein convertase PC5A is the protease that cleaves L1 in the third fibronectin type III domain, whereas the proprotein convertases furin, PC1, PC2, PACE4, and PC7 are not effective in cleaving L1. Analysis of mutations revealed Arg^845 to be the site of cleavage generating the N-terminal 140-kDa fragment. This fragment was present in the hippocampus, which expresses PC5A, but was not detectable in the cerebellum, which does not express PC5A. The 140-kDa L1 fragment was found to be tightly associated with the full-length 200-kDa L1 molecule. The complex dissociated from the membrane upon cleavage by a protease acting at a more membrane-proximal site of full-length L1. This proteolytic cleavage was inhibited by the metalloprotease inhibitor GM 6001 and enhanced by a calmodulin inhibitor. L1-dependent neurite outgrowth of cerebellar neurons was inhibited by GM 6001, suggesting that proteolytic processing of L1 by a metalloprotease is involved in neurite outgrowth.

Proteolytic processing of cell-surface proteins is of prime importance for regulating the functional properties of these proteins (for reviews, see Refs. 1–5). Cleavage of recognition molecules at the cell surface has been implicated in neuronal migration, neurite outgrowth, and synaptic plasticity (6–13). Among the neural adhesion molecules, L1 has been shown to undergo proteolytic cleavage, which has been suggested to be involved in several functions of this molecule.

L1 is a member of the immunoglobulin superfamily consisting of immunoglobulin-like domains and fibronectin type III repeats (for reviews, see Refs. 14 and 15). In the central nervous system, L1 is expressed only by post-mitotic neurons and mainly on non-myelinated axons, whereas in the peripheral nervous system, it is expressed by neurons as well as by non-myelinating Schwann cells. L1 is also expressed by non-neural cells, including normal and transformed cells of hematopoietic and epithelial origin. L1 is involved in neuronal migration, neurite outgrowth, and myelination (for review, see Ref. 14) as well as axon guidance, fasciculation, and regeneration (16, 17). Furthermore, it enhances cell survival (18) and synaptic plasticity (19). The importance of L1 in nervous system development is underscored by the abnormal phenotypes of L1 mutations in humans and mice (for review, see Ref. 20). L1 engages in homophilic and heterophilic cell interactions (for reviews, see Refs. 14 and 15). Homophilic binding partners are the RGD-binding integrins and TAG-1/axonin-1 (20), F3/F11/contactin, NCAM, CD9, CD24, and phosphacan (Ref. 21 and references therein). These interactions are likely to depend on the presentation of the L1 molecule either as a membrane-bound form or as a proteolytic fragment, which has been described in various forms (Ref. 22 and references therein). The 140- and 80-kDa fragments resulting from cleavage within the third fibronectin type III (FNIII)1 domain (23) have been generated in vitro by trypsin (24) or plasmin (21). The third FNIII domain containing two RGD-independent integrin-binding sites (21) is involved in homophilic binding (25), multimerization (21), and L1-dependent neurite outgrowth (26). Cleavage within this domain by plasmin reduces multimerization and RGD-independent integrin binding (21). The 180- and 50-kDa fragments result from membrane-proximal cleavage of the membrane-spanning 200- and 80-kDa L1 forms, respectively, by a metalloprotease, most likely of the ADAM (a disintegrin and metalloprotease) family (27, 28). This cleavage step has been proposed to be required for cell migration (28). Because specific proteolytic processing of L1 is important for regulation of neuronal migration and neurite outgrowth, we have searched for the proteases responsible for cleaving L1 at the two sites and investigated some of the structural and functional consequences of this proteolytic cleavage.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—GM 6001 and leupeptin were purchased from Calbiochem (Bad Soden, Germany), and aprotinin and 1,10-phenanthroline were from Sigma (Taufkirchen, Germany). The calmodulin inhibitor CGS 9343B was a gift from Novartis Consumer Health (Nyon, Switzerland). Complete™ EDTA-free protease inhibitor mixture was obtained from Roche Molecular Biochemicals (Mannheim, Germany). Polyclonal anti-L1 antibodies were obtained from rabbits immunized with a protein A-purified L1-Fc fusion protein consisting of the extracellular domain of mouse L1 and the Fc portion of human IgG1 (18). Rat monoclonal antibody 555 (29) reacts with an epitope at the border between the FNIII homologous repeats 2 and 3. Anti-glyceraldehyde-3-phosphate dehydrogenase antibody was purchased from Chemicon.
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(Hohein, Germany). All secondary antibodies were obtained from Di-anova (Hamburg, Germany).

**DNA Mutagenesis**—Mutagenesis of L1 was performed using the QuickChange site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands). Mouse L1 cDNA was cloned into pGEM2 (Promega, Mannheim), and the resulting vector was used as a template in PCR-based mutagenesis. The PCR primers used for mutagenesis had the following sequences: 5′-tggc gag gcg cag cag cag cag cag acg atc cac aac age-3′ and 5′-gct ttt gtt gat agt gct gca gct gtt ctt gct gct gcc ctc cca-3′. Bases in boldface indicate exchanges in the mutated L1 cDNA.

**Cell Culture**—Mouse neuroblastoma cells (Neuro2a) were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 2 mM glucose, 2.2 mg/ml NaHCO3, 100 μg/ml transferrin, 10 μg/ml insulin, 4 mM tyrosine, 30 mM Na2SO4, 0.027 trypsin inhibitor units/ml aprotinin, 5 μU/ml penicillin, and 5 μg/ml streptomycin. Cultures of hippocampal neurons were also prepared from 6–8-day-old C57BL/6J mice as described (30). In brief, cerebella were digested with a trypsin/DNase solution and mechanically dissociated. The cells were resuspended and cultured in serum-free basal medium culture medium containing 1 mg/ml mouse L1 immunoaffinity-purified from adult mouse brain (35) in a 1:2 mixture of 5% horse serum, 6 mM glucose, 200 μg/ml insulin, 30 mM Na2SO4, 0.027 trypsin inhibitor units/ml aprotinin, 5 IU/ml penicillin, and 5 μg/ml streptomycin. Cerebellar granule neurons were prepared as described by Fischer et al. (30) in modified Eagle’s medium containing 10% horse serum, 10% fetal calf serum, 1 mM sodium pyruvate, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). Cerebellar granule neurons were cultured in Dulbecco’s medium containing 10% fetal calf serum, 10% horse serum, 10% fetal calf serum, 100 units/ml aprotinin, 5 IU/ml penicillin, and 5 μg/ml streptomycin.

**Purification of Overexpressed Proteins**—Protein pellets were resuspended in sample buffer. Cells were homogenized in homogenization buffer (0.32 M sucrose, 50 mM Tris-HCl, 150 mM NaCl, 50 mM Tris-HCl, 1 mM CaCl2, 1 mM MgCl2, 100 μg/ml leupeptin, and 0.1% Triton X-100). The gradient was centrifuged at 14,000 g for 1 h at 4 °C. After centrifugation, proteins from the supernatants were concentrated by methanol/chloroform precipitation. Cells were also homogenized in phosphate-buffered saline (pH 7.4) and further centrifuged at 100,000 g for 10 min at 4 °C. For SDS-PAGE, the supernatants were diluted with sample buffer.

**Infection of HEK293 Cells Using a Vaccinia Virus System**—For expression of wild-type and mutated L1 via vaccinia virus, the corresponding cDNAs were cloned into the p�M601 expression vector (36). HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. For expression of the different protease inhibitors, cells were infected with pM601 constructs carrying the cDNA inserts coding for mouse PC1, PC2, and PC5A; for rat PC7; and for human PACE4 and furin (38). After infection of cells with 1 plaque-forming units/cell for 2 h in 5 ml of phosphate-buffered saline and 0.01% bovine serum albumin, cells were maintained for 48 h in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Cells were then washed twice with serum-free medium and further incubated in 6 ml of the absence of serum. Cells were harvested by centrifugation at 500 × g for 10 min at 4 °C and resuspended in radioimmunoprecipitation assay buffer containing 1% Nonidet P-40 and Complete™ protease inhibitor mixture. Cell lysates were then subjected to sonication for 1 min at an amplitude of 100% (UP50, Hielscher, Stuttgart, Germany) and centrifuged at 14,000 × g for 30 min at 4 °C. Culture supernatants were clarified by centrifugation at 100,000 × g for 1 h at 4 °C. Clear cell lysates and culture supernatants were concentrated by acetone precipitation. Protein pellets were resuspended in radioimmunoprecipitation assay buffer containing Complete™ protease inhibitor mixture.

**Western Blot Analysis**—For analysis of the proteolytic L1 products, Neuro2a cells and mouse brains from adult wild-type mice were used. The Neuro2a cells and total mouse brain were homogenized in 1 ml NaHCO3 0.2 mM CaCl2 0.2 mM MgCl2 and 1 mM spermidine (pH 7.9) and centrifuged at 600 × g for 15 min at 4 °C. After recentrifugation of the supernatant at 25,000 × g for 45 min at 4 °C, the proteolytic supernatants were centrifuged at 17,000 × g for 15 min at 4 °C. Supernatants were clarified by centrifugation at 100,000 × g for 1 h at 4 °C. Culture supernatants and culture supernatants were concentrated by acetone precipitation. Protein pellets were resuspended in sample buffer.

**Subfractionation and Sucrose Gradient Analysis**—Brains or different brain regions (hippocampus and cerebellum) from adult C57BL/6J mice were homogenized in 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 10 mM MgCl2, and 1 mM EDTA containing 1% Nonidet P-40 resuspended in RPMI 1640 medium (PAA Laboratories, Colbe, Germany) and subdivided into 1-ml aliquots. These aliquots were incubated at 37°C for different times with or without the metalloprotease inhibitors GM 6001 (10 μM) and 1,10-phenanthroline (50 μM), the serine and cysteine protease inhibitor leupeptin (10 μM), and Complete™ EDTA-free protease inhibitor mixture (which inhibits a broad range of serine and cysteine proteases). As a control, aliquots were incubated at 4 °C. The samples were then centrifuged at 100,000 × g for 1 h at 4 °C. Supernatants were concentrated by acetone precipitation. Protein pellets were resuspended in sample buffer.

**In Vitro Assay of Proteolytic Processing**—For analysis of the proteolytic L1 products, Neuro2a cells and mouse brains from adult wild-type mice were used. The Neuro2a cells and total mouse brain were homogenized in 1 mM NaHCO3 0.2 mM CaCl2 0.2 mM MgCl2 and 1 mM spermidine (pH 7.9) and centrifuged at 600 × g for 15 min at 4 °C. After recentrifugation of the supernatant at 25,000 × g for 45 min at 4 °C, the proteolytic supernatants were centrifuged at 17,000 × g for 15 min at 4 °C. Supernatants were clarified by centrifugation at 100,000 × g for 1 h at 4 °C. Culture supernatants and culture supernatants were concentrated by acetone precipitation. Protein pellets were resuspended in sample buffer.

**Subfractionation and Sucrose Gradient Analysis**—Brains or different brain regions (hippocampus and cerebellum) from adult C57BL/6J mice were homogenized in 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 10 mM MgCl2, and 1 mM EDTA containing 1% Nonidet P-40 resuspended in RPMI 1640 medium (PAA Laboratories, Colbe, Germany) and subdivided into 1-ml aliquots. These aliquots were incubated at 37°C for different times with or without the metalloprotease inhibitors GM 6001 (10 μM) and 1,10-phenanthroline (50 μM), the serine and cysteine protease inhibitor leupeptin (10 μM), and Complete™ EDTA-free protease inhibitor mixture (which inhibits a broad range of serine and cysteine proteases). As a control, aliquots were incubated at 4 °C. The samples were then centrifuged at 100,000 × g for 1 h at 4 °C. Supernatants were concentrated by acetone precipitation. Protein pellets were resuspended in sample buffer.
FIG. 1. A, schematic representation of the different L1 forms in mouse brain. The extracellular domain of the full-length transmembrane 200-kDa form of L1 consists of six immunoglobulin-like domains and five FNIII domains. Cleavage within the third FNIII domain generates the 140-kDa fragment and a transmembrane 80-kDa fragment. Membrane-proximal cleavage of the 200-kDa form and the 80-kDa fragment leads to the generation of 180- and 50-kDa fragments, respectively, and, in addition, to a 30-kDa transmembrane stump. B, the putative proprotein convertase recognition motif in the third FNIII domain of L1. The sequences of third FNIII domain of human, rat, and mouse L1 (Swiss Protein Database accession numbers P32004, Q05695, and P11627) are shown. The putative proprotein convertase recognition motif in the L1 sequence, which is conserved in all sequences, is depicted in boldface. In the mouse wild-type L1 sequence, this motif precedes the cleavage site identified by sequencing the N terminus of the 80-kDa L1 form (23), which is highlighted in gray.

**RESULTS**

**L1 Is Proteolytically Cleaved by PC5A in the Third FNIII Domain**—In mouse brain, the full-length 200-kDa L1 molecule is cleaved at a site within the third FNIII domain, generating 140- and 80-kDa fragments (Fig. 1A). A membrane-proximal cleavage generates 180- and 30-kDa fragments from the full-length 200-kDa L1 molecule and 50- and 30-kDa fragments from the 80-kDa fragment (Fig. 1A). Because the third FNIII domain of L1 contains a putative proprotein convertase recognition and cleavage motif (Fig. 1B), we investigated whether the 140-kDa fragment could be generated by proprotein convertases (37).

Mouse neuroblastoma Neuro2a cells express the full-length transmembrane 200-kDa form of L1 and a 180-kDa proteolytic fragment, but not the 140-kDa L1 fragment (data not shown). Therefore, this cell line was used for transient transfection with the proprotein convertases furin, PC1, PC2, PACE4, PC5A, and PC7. As reported previously, control experiments have shown that each transfected proprotein convertase is expressed in similar amounts as a functional enzyme (39, 40). Cells were maintained overnight in serum-free culture medium, and cells and cell culture supernatants were then collected separately. Western blot analysis using polyclonal antibodies against the extracellular part of mouse L1 showed the full-length 200-kDa form in the cell pellet upon mock transfection (Fig. 2A, lane 1), whereas the 180-kDa fragment was present in both the cell pellet (Fig. 2A, lane 1) and culture supernatant (Fig. 2B, lane 1). The 140-kDa fragment was not detectable in the cell pellet or culture supernatant. Similar results were obtained upon transfection with PC1 (Fig. 2A and B, lanes 2), PC2 (lanes 3), and PACE4 and PC7 (data not shown). In contrast, after transfection with PC5A, high levels of the 140-kDa form and low levels of the 200- and 180-kDa forms were found in the cell pellet (Fig. 2A, lane 4). Only the 140-kDa fragment was detectable in the culture supernatant (Fig. 2B, lane 4). Transfection with furin resulted in the appearance of smaller amounts of the 140-kDa form in comparison with transfection with PC5A, whereas the 200- and 180-kDa forms were predominant in the pellet (Fig. 2A, lane 5). The supernatant of furin-transfected cells contained mainly the 180-kDa fragment, whereas the 140-kDa fragment was detectable in minor amounts (Fig. 2B, lane 5). These results indicate that PC5A cleaves L1 very efficiently to generate the 140-kDa form, whereas furin is not as efficient as PC5A in generating this fragment. The proprotein convertases PC1, PC2, PACE4, and PC7 are not capable of cleaving L1.

**Mutation of the Proprotein Convertase Recognition Motif Abrogates Generation of the 140-kDa L1 Fragment by PC5A—N-terminal sequencing of the 140-kDa fragment (23) indicated that this fragment is generated by proteolytic cleavage at Arg<sup>425</sup> (Fig. 1B). To verify this site as one that is cleaved by PC5A, the L1 sequence<sup>420</sup>RKHSSKR<sup>445</sup>, resembling the proprotein convertase recognition motif, (R/K)X<sub>2,4,6</sub>(K/R), was mutated to<sup>420</sup>RKHSSSS<sup>445</sup> (Fig. 1B). The L1-deficient cell line HEK293 was used for expression of mouse wild-type and mu-
tated L1 using a vaccinia virus system. Western blot analysis using polyclonal anti-L1 antibodies showed the 200- and 140-kDa forms in the cell pellet after expression of wild-type L1 (Fig. 3A, lane 1). Significant amounts of the 180- and 140-kDa forms were found in the supernatants (Fig. 3A, lanes 3), indicating the presence of L1-processing enzymes in HEK293 cells. Coexpression of wild-type L1 and PC5A resulted in a strong increase in the amount of the 140-kDa fragment in the cell pellet (Fig. 3A, lane 2). Only the 140-kDa form was found in the supernatant (Fig. 3A, lane 4), underscoring the efficient cleavage of L1 by PC5A. Coexpression of wild-type L1 with furin, PC1, PC2, PACE4, or PC7 showed no increase in the amount of the 140-kDa fragment (data not shown), confirming that PC5A is the most efficient L1-cleaving proprotein convertase.

This difference in cleavage efficiency is not due to different expression levels of each proprotein convertase, as shown previously in other studies (38, 41, 42). In contrast to the expression of wild-type L1, no 140-kDa fragment was found in the cell pellet (Fig. 3B, compare lanes 2 and 1) or in the culture supernatant (compare lanes 5 and 4) after expression of the mutated L1 molecule. Even after coexpression of mutated L1 and PC5A (Fig. 3B, lanes 3 and 6) or the other proprotein convertases (data not shown), the 140-kDa form was not detectable. These results clearly demonstrate that the proprotein convertase PC5A cleaves L1 at Arg45.

The 140-kDa L1 Fragment Is Generated in the Hippocampus, but Not in the Cerebellum—Previous studies showed that, in cultured cerebellar neurons, the 140-kDa fragment is not detectable (24). Because PC5A is highly expressed in the hippocampus, but not or only weakly in the cerebellum (44, 45), we investigated whether the 140-kDa form is generated in the hippocampus. Homogenates of adult mouse hippocampus and cerebellum or of cultured early postnatal hippocampal and cerebellar neurons were subjected to Western blot analysis using monoclonal anti-L1 antibody, which recognizes an epitope at the border between the second and third FNIII domains. The full-length membrane-spanning 200-kDa L1 form was detectable in the two brain regions (Fig. 4, lanes 1 and 2) and cultured neurons (lanes 3 and 4). The 140-kDa fragment was detectable only in the hippocampus and cultured hippocampal neurons (Fig. 4, lanes 1 and 3), but not in the cerebellum and cultured cerebellar neurons (lanes 2 and 4).

Dimers Consisting of the Transmembrane 200-kDa L1 Form and the Tightly Associated 140-kDa Fragment Are Released from the Membrane by a Metalloprotease—The generation of the 140-kDa fragment was analyzed in more detail in an in vitro assay using a crude membrane fraction from adult mouse brain. After incubation of the membrane fraction at 4 or 37 °C for 2 or 4 h, the membranes were pelleted. Pellets and supernatants were subjected to Western blot analysis using polyclonal anti-L1 antibodies. Membrane pellets contained high amounts of the 200-, 140-, and 50-kDa forms (Fig. 5A, lanes 2, 4, and 6). After incubation at 4 °C, only low amounts of the 180- and 140-kDa fragments were found in the supernatants (Fig. 5A, lane 1), whereas incubation at 37 °C yielded significantly higher amounts of these fragments in the supernatant (lanes 3 and 5). After incubation in the presence of the metalloprotease inhibitor GM 6001, neither the 180- nor 140-kDa fragment was detectable in the supernatant (Fig. 5B, compare lanes 1 and 3). Instead, the 180- and 50-kDa fragments were found in the supernatants (Fig. 5A, lanes 3), where the generation of the 180- and 140-kDa fragments is more likely to be due to the release of the 180- and 140-kDa fragments. Another metalloprotease inhibitor (1,10-phenanthroline) reduced the shedding of both fragments (Fig. 5B, compare lanes 1 and 5). Together, these results indicate that a metalloprotease is involved in the cleavage of the transmembrane 200- and 80-kDa forms, resulting in the generation and release of the 180- and 50-kDa fragments. Furthermore, the membrane-proximal cleavage of the transmembrane forms promotes the release of the 140-kDa fragment. These observations imply that the 140-kDa fragment is tightly associated with the full-length transmembrane L1 molecule and that cleavage of...
the 200- and 80-kDa forms by the metalloprotease results in the release of the soluble 180-, 140-, and 50-kDa fragments. To further investigate whether the 200-kDa form complexes with the 140-kDa fragment, sucrose gradient velocity sedimentation analysis was performed. A detergent extract from the brain homogenate was separated in a continuous sucrose gradient, and fractions were analyzed by Western blotting using monoclonal anti-L1 antibody. The 200- and 140-kDa forms were present in different fractions throughout the gradient. Based on the sedimentation of the molecular mass markers, the 200- and 140-kDa forms in fractions 4–8 correspond to the 200- and 140-kDa monomers, whereas the 200- and 140-kDa forms in fractions 12–14 sedimented at a molecular mass of ~350 kDa, which is the expected molecular mass of a 200/140-kDa heterodimer (Fig. 6A). Significant amounts of the 200-kDa form were present in fractions 16–17 (Fig. 6A), which correspond to a molecular mass of ~500 kDa and may represent a high molecular mass complex.

Western blot analysis after sucrose gradient analysis of detergent-solubilized membranes from mouse brain showed a major portion of the 200- and 140-kDa forms in fractions 12–14, corresponding to a molecular mass of ~350 kDa (Fig. 6B), the expected molecular of a 200/140-kDa heterodimer. Only a minor portion of the 200- and 140-kDa forms was present in fractions 4–8 (Fig. 6B), where the 200- and 140-kDa monomers are expected to migrate. The observation that a large portion of the 200- and 140-kDa forms was detectable in fractions 16–20 indicates that the two forms are associated in a high molecular mass complex. Western blot analysis after sucrose gradient analysis of a soluble protein fraction prepared from the mouse brain homogenate revealed that the soluble 140- and 180-kDa fragments were present only in fractions 4–9 (Fig. 6C), indicating that the soluble fragments migrate at molecular masses corresponding to the 140- and 180-kDa monomers. These results suggest that the membrane-associated 200/140-kDa heterodimers are released from the membrane upon cleavage of the 200-kDa form by a metalloprotease and that the resulting 180/140-kDa heterodimeric complex dissociates after release from the cell surface.

Cleavage of L1 by a Metalloprotease Is Increased by a Calmodulin Inhibitor—As mentioned above, neuroblastoma Neuro2a cells did not generate the 140-kDa fragment, but
released the 180-kDa fragment into the culture supernatant (Fig. 2, A and B). This finding prompted us to investigate this particular membrane-proximal proteolytic process leading to the formation of the soluble 180-kDa fragment. When Neuro2a cells were cultured in the presence of the metallocprotease inhibitor GM 6001, release of 180-kDa fragment into the culture supernatant was strikingly inhibited (Fig. 7, compare lanes 1 and 2), whereas leupeptin, an inhibitor of trypsin-like and cysteine proteases, had no effect (compare lanes 1 and 3). This finding confirmed that the 180-kDa fragment is generated by a metalloprotease activity.

Because it has been reported that processing by a metalloprotease of several cell-surface receptors, such as the transforming growth factor-α receptor (46), amyloid precursor protein (46), tyrosine receptor kinase TrkA (46), and L-selectin (47), is increased by a calmodulin inhibitor, we investigated whether cleavage of L1 and release of the 180-kDa fragment are affected by the calmodulin inhibitor CGS9343B. The production and release of the 180-kDa fragment were increased in the presence of this inhibitor (Fig. 7, compare lanes 1 and 4). As an internal control and for normalization, the cell lysates were analyzed by Western blot analysis using anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Fig. 7, lanes 1–4).

The Metalloprotease Inhibitor GM 6001 Inhibits L1-dependent Neurite Outgrowth—Cerebellar neurons from L1-deficient mice do not show neurite outgrowth on substrate-coated L1 (Ref. 48 and references therein), indicating that L1-induced neurite outgrowth is mainly mediated by homophilic interaction and signaling via neuronal cell surface-expressed L1. This finding allowed us to investigate the effect of GM 6001 on L1-induced neurite outgrowth and to determine whether processing of L1 by a metalloprotease is required for L1-induced neurite outgrowth. Microexplants of early postnatal mouse cerebellum were maintained in the presence or absence of GM 6001 either on poly-L-lysine or on substrate-coated L1. Explants maintained on poly-L-lysine showed no significant difference in neurite length in the presence or absence of GM 6001 (Fig. 8, A, B, and G). Explants maintained on substrate-coated L1 showed a decrease in neurite length of ~40% in the presence of GM 6001 compared with explants maintained in its absence (Fig. 8, C–G), suggesting that a metalloprotease activity is required for L1-mediated neurite outgrowth. Explants were also grown on poly-L-lysine or substrate-coated L1 in the presence or absence of the serine and cysteine protease inhibitor leupeptin and the serine protease inhibitor aprotinin (Fig. 8G). These protease inhibitors did not influence the L1-mediated neurite outgrowth or the neurite outgrowth on the control substrate.

These results confirm that shedding of L1 by a metalloprotease is involved in L1-mediated neurite outgrowth.

DISCUSSION

Full-length L1 is converted to N-terminal 140-kDa and C-terminal membrane-spanning 80-kDa complementary proteolytic fragments (24) by proteolytic cleavage in the third FNIII domain (23). This domain is functionally important because it contributes to homophilic binding (25) and multimerization of L1, neurite outgrowth, and RGD-independent integrin binding to the β1-integrin subunit (21). Two motifs (VQVKHLR27 and GSRKHSSKR34; see Fig. 2A) within the third FNIII domain are involved in RGD-independent integrin binding to L1. Substitution of the dibasic RK and KR in the
837GSQRKHSKR845 motif results in reduced multimerization of L1 molecules and diminished integrin binding (21). The functional activity of this particular RGD-independent integrin-binding motif appears to be regulated by proteolytic cleavage within this sequence motif: cleavage by plasmin within this motif disrupts multimerization and RGD-independent integrin binding. Plasmin, the end product of the plasminogen/plasminogen activator cascade, cleaves L1 within the 837GSQRKHSKR845 motif at Lys841 and Lys844, leading to the generation and release of a 140-kDa fragment (21). These cleavage sites are both different from the cleavage site obtained by N-terminal sequencing of the 80-kDa form (23), indicating cleavage sites are both different from the cleavage site obtained in vivo.

A similar result was obtained for the processing of integrin pro-α-subunits: PC5A is more active than furin, whereas PC1, PC2, PACE4, PC7, and PC5B are inactive (38). In addition, although furin and other proprotein convertases do not efficiently process members of the transforming growth factor-β superfamily, PC5A appears to be responsible for the in vitro processing of these proteins (49). The low processing efficiency of furin could be due to the fact that furin preferentially cleaves substrates with the recognition motif RX(R/K)R (50), and the sequence HXXKR, which is present in L1, is a poor substrate (51).

The particular spatial and temporal expression patterns of PC5A in the brain (45, 52, 53) overlap with those of L1 (54), whereas furin is ubiquitously expressed in all brain areas and cell types (55). In contrast to furin, PC5A expression is restricted to neurons (53), as has been shown for L1 (for review, see Ref. 20). Proteolysis of L1 by PC5A in the hippocampus might be involved in synaptic plasticity underlying hippocampus-dependent spatial learning. Indeed, generation of the 140-kDa fragment is observed in the hippocampus, which expresses PC5A, whereas in the cerebellum, which does not show detectable PC5A expression, this fragment is not generated. Interestingly, both PC5A (56) and L1 are up-regulated after a lesion in the peripheral nervous system; and thus, the processing of L1 by PC5A could be relevant in regeneration, which is highly L1-dependent (see, for instance, Ref. 17). However, our results do not exclude that other proteases, such as plasmin, cleave L1 in the third FNIII domain, resulting in the generation of different 140-kDa fragments.

A second proteolytic site has been shown to exist in L1, leading to the formation of a 180-kDa fragment. This site within an unknown cleavage sequence is localized close to the plasma membrane and is susceptible to cleavage by a metalloprotease. Recently, it has been suggested that the metalloprotease ADAM10 cleaves L1 at this site and generates a 180-kDa fragment (57). In the present study, we have shown that the metalloprotease inhibitor GM 6001 inhibits formation of this 180-kDa fragment and interferes with neurite outgrowth. Interestingly, calmodulin inhibitors enhance proteolytic cleavage at this site, as has been shown for other functionally important cell-surface receptors regulating cytokine, neurotrophin, and cell recognition (46, 47). Our observation thus provides further evidence that intracellular signaling via calcium influences release of recognition molecule fragments from the cell surface. These fragments may then diffuse into the extracellular matrix to interact with partner molecules and thereby modulate the cellular environment. The release of the 180-kDa fragment entails the release of the 140-kDa fragment, which remains tightly associated with the membrane by interacting with the full-length L1 molecule. A prerequisite for this concerted release of the complex of the 180- and 140-kDa fragments appears to be the dimerization of full-length L1 at the cell surface, as shown in this study.

cis-Dimerization has been reported for other recognition molecules belonging to the families of cadherins (58); integrins (for review, see Ref. 59); selectins (60); and immunoglobulins, such as ICAM-1 (intercellular adhesion molecule-1) (61), P0 (62), PEACAM-1 (63), JAM-1 (64), nectin (65), trichitin (66), CD4 (67), and CEACAM-1 and CEACAM-2 (68). Dimerization thus appears to be important for signal transduction, as has been shown for E- and C-cadherins as well as for the neural N-cadherin (Ref. 58 and references therein). Furthermore, dimerization of selectins has been shown to enhance adhesive tethers (60). Interestingly, dimerization of CEACAM-1 and CEACAM-2 is regulated by calmodulin and calcium ions (68), highlighting the importance of inside-out signaling mechanisms. ICAM-1 exists predominantly as a dimer at the cell surface and binds in this dimeric state with greatly enhanced affinity to the integrin LFA-1 compared with its monomeric form (43).

The shedding of the L1 dimers from the cell surface could have several consequences. Because the 180/140-kDa dimer dissociates into its monomers after release from the cell surface, the soluble diffusible 140- and 180-kDa fragments could constitute important ingredients in the extracellular matrix, possibly playing different functional roles. It might thus be conceivable that L1-synthesizing cells build concentration gradients of either soluble or matrix-embedded adhesion molecules that modulate cell migration and axon guidance by “conditioning” the cellular environment for L1 homophilic and heterophilic interactions with the surface of adjacent cells. Another possibility is that the proteolytic processing uncovers binding sites of the residual transmembrane fragments for different ligands. We thus support the idea that proteolytically processed L1 may at least at two functions: modification of the extracellular milieu and of transmembrane signaling via the residual L1 receptor stumps.

Acknowledgments—We are grateful to Galina Dityateva for hippocampal cell cultures and Tanja Schneegans for support concerning the cerebellar microexplant cultures.

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