The L1 cell adhesion molecule and its soluble form are tumor-associated proteins and potential markers for tumor staging as well as targets for therapeutic intervention. Soluble L1 is produced by metalloprotease-mediated ectodomain shedding of L1. We investigated effects of hepatocyte growth factor (HGF), a growth factor shown to increase invasiveness of renal carcinoma cells, on ectodomain shedding of L1 from these cells. All of the tested L1-positive renal carcinoma cell lines released a 180-kDa form of L1 into the medium. In the presence of serum, addition of HGF led to a dose-dependent increase in L1 shedding with a maximum reached at 5 ng/ml. In contrast, L1 shedding was inhibited by giall cell line-derived neurotrophic factor (GDNF). The tyrosine kinase inhibitor Genistein reduced basal and HGF-stimulated L1 shedding, indicating that protein phosphorylation is involved. To investigate the role of the L1 intracellular domain, two mutants of the L1 cytoplasmic part were constructed. L1trun lacking the complete intracellular domain showed enhanced basal shedding. In a L1YH mutant, containing the mutation tyrosine 1229 to histidine that deletes the ankyrin binding motif of L1, basal shedding was reduced. Disruption of actin assembly by cytochalasin D also reduced shedding of L1. These results indicate that the cytoplasmic domain regulates basal shedding of L1, and association with the cytoskeleton through the L1 ankyrin binding site is involved. HGF stimulated L1 shedding in both mutants, indicating that receptor-mediated phosphorylation in the L1 cytoplasmic domain is not required for HGF-stimulated shedding.

The L1 cell adhesion molecule (L1) was originally described as a protein of the nervous system, and mutated forms of L1 are linked to severe neurological pathologies referred to as MASA syndrome. A non-neuronal isoform of L1 lacking exons 2 and 27 is expressed at low levels in the normal adult human kidney (2) as well as during renal development (3). In contrast, high expression of L1 was found in 8 of 12 renal tumor cell lines and in some renal tumors (2). It was shown that the cell-bound form of L1 can serve as a target for radioimmunodiagnosis of metastatic neuroblastoma (4), and L1 may also be a marker and therapeutic target for certain renal cancers.

In cultured cell lines originating from a number of different tumors the L1 ectodomain was found to be cleaved proximal to the cell membrane by metalloproteases, followed by release of soluble L1 into the medium (5–7). Ectodomain shedding of L1 could be stimulated by phorbol esters and vanadate, indicating that phosphorylation events regulate L1 release (6). In cell culture soluble L1 substrate was found to stimulate cellular motility and migration (8). Release of soluble L1 is not restricted to cells in culture as soluble L1 was recently detected in serum samples of patients with ovarian and uterine tumors and is believed to lead to increased cell migration and metastatic spread in these malignancies (9).

Here we investigated whether hepatocyte growth factor (HGF) and glial cell line-derived neurotrophic factor (GDNF) influence shedding of L1 from renal carcinoma cells. Changes in the normal expression patterns of HGF and GDNF are associated with abnormal proliferation in the human renal system. While in the normal human kidney, HGF and c-Met are expressed at very low levels, increased HGF and c-Met expression is found in about 90% of renal cell carcinomas (10–12), and overexpression of HGF/c-Met enhances cellular motility of renal carcinoma cells in vitro and in vivo (13, 14). GDNF is overexpressed in renal dysplasia (15) and in collecting duct cysts (16). During kidney organogenesis, HGF and GDNF, as well as L1 are important for ureteric bud outgrowth and branching morphogenesis (17). In vivo, L1 knock-out mice show improper growth and branching patterns of ureteral branches (18), and blocking of HGF function inhibits proliferation and branching in kidney organ cultures (17).

Effects of growth factors on L1 could be mediated by direct interaction of receptors with the L1 extracellular domain. This is suggested by the findings that L1-mediated cell adhesion activates the fibroblast growth factor (FGF) receptor (19) and the epidermal growth factor (EGF) receptor (20) tyrosine phosphorylation, respectively, the latter an effect which appears to involve L1/EGF receptor trans interactions (20). On the other hand, the cytoplasmic domain of L1 contains several phosphorylation sites and it was shown before that phosphorylation events induced by growth factors regulate the interaction of the L1 family member neurofascin with the ankyrin/actin network (13, 14). GDNF is overexpressed in renal dysplasia and therapeutic target for certain renal cancers.
domain of L1 on basal and HGF-stimulated release of soluble L1, we found that the cytoplasmic domain of L1 and its interaction with the actin cytoskeleton regulate basal, but not HGF-stimulated L1 shedding.

MATERIALS AND METHODS

Cell Culture—The human embryonic kidney cell line HEK293 and the human renal carcinoma cell lines A-498 and Caki-2 were obtained from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. The human renal carcinoma cell line Foehn was obtained from the Institute of Pathology, University of Zurich, Switzerland. KTCTL-2 and KTCTL-53 were from the German Center for Tumor Research, Heidelberg, Germany. The cell lines RCC-W1, RCC-W2, RCC-GW, RCC-FG2, and RCC-MF were obtained from the Cell Lines Service and Cellbank in Eppelheim, Germany. The human embryonic kidney cell line 293T, originally referred to as 293tA1609neo (24, 25), was kindly provided by Prof. K. Ballmer, PSI, Villigen, Switzerland. This cell line is a derivative of HEK293 cells into which the gene for the temperature-sensitive SV40 T-antigen mutant tsA1609 has been inserted, which leads to a large production of replication-competent T antigen at 37 °C (25).

HEK293 and 293T cells were maintained in Dulbecco’s modified Eagle’s medium, Foehn, KTCTL-2, and KTCTL-53 cells in RPMI medium, A-498 cells in MEM containing 1 mM sodium pyruvate, and all other cell lines in McCoy medium at 37 °C and 7.5% CO2. All media were supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin, 0.5 μg/ml fungizone). All media and additives were obtained from BioConcept (Allschwil, Switzerland).

Reagents and Antibodies—Human recombinant HGF and GDNF and cytchalasin D were obtained from Sigma. Genstein was purchased from Calbiochem (Juro, Lucerne, Switzerland). The anti-L1 antibody chCE7 directed against the ectodomain of L1 was constructed, expressed, and purified as previously described (26, 27).

The anti-L1 antibody UJ127.11 directed against the ectodomain of L1 was purchased from Neo Markers (P. H. Stehelin, Basel, Switzerland). The anti-C-C motif antibody 74H7 directed against the cytoplasmic domain of L1 was from CRP Inc. (Eurogentech, Seraing, Belgium). The anti-c-Met antibody C-12 and the anti-c-Ret antibody C-19 were obtained from Santa Cruz Biotechnology (LabForce, Nunningen, Switzerland). The anti-mouse IgG-horseradish peroxidase conjugate W042B was from Promega (Catalys, Wallisellen, Switzerland). The anti-rabbit IgG-horseradish peroxidase and anti-goat IgG-horseradish peroxidase antibody conjugates were from Santa Cruz Biotechnology (LabForce). Protein G-Sepharose 4 FF was obtained from Amersham Biosciences. All other chemicals were purchased from Fluka (Buchs, Switzerland).

Cell L1 and Immunoprecipitation—Cells were washed twice with PBS and detached with 1 mM EDTA in PBS. After centrifugation for 5 min at 500 × g, cells were lysed in 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1% Nonidet P-40 (lysis buffer) containing CompleteTM Protease Inhibitor Mixture (Roche Diagnostics) and incubated on ice for 10 min. Cell lysates were centrifuged for 10 min at 16,000 × g at 4 °C, and the protein concentration of the supernatant was measured using the DC Protein Assay from Bio-Rad.

For immunoprecipitation of L1 and c-Met, 500 μg of protein in 300 μl of lysis buffer were preclaried with 20 μl of protein G-Sepharose suspension (10% in lysis buffer, PBS) for 30 min at 4 °C. After preclariation, lysates were incubated with 20 μl of PGS and the corresponding antibody (2 μg of chCE7 for L1, 1 μg of C-12 for c-Met) for 2 h at 4 °C. Protein G-Sepharose beads were washed twice with lysis buffer and once with Tris-HCl, pH 7.5 and analyzed by Western blotting.

For analysis of soluble L1 in the medium, cell supernatant was preclaried with 50 μl of PGS for 30 min at 4 °C. Immunoprecipitation of soluble L1 was performed overnight with 6 μg of chCE7 and 50 μl of PGS at 4 °C. Samples were centrifuged for 5 min at 17,000 × g and 4 °C. Protein G-Sepharose beads were washed as described and analyzed by immunoblotting using mAb UJ127.11.

Separation of Triton X-100-soluble and -insoluble Membrane Fraction—Isolation of Triton X-100-insoluble membrane fractions was performed as described in Ref. 28 with modifications. Foehn cells on 15-cm plates were detached with 1 mM EDTA in PBS, and cells were centrifuged for 5 min at 4 °C. Cells were resuspended in 200 μl of TNE buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA) including CompleteTM Protease Inhibitor Mixture using a Dounce homogenizer. Homogenates were centrifuged at 200 × g and 4 °C for 5 min. The supernatants were centrifuged at 100,000 × g for 1 h at 4 °C.

The pellets were resuspended in 200 μl of TNE buffer, pH 11, containing 1% Triton X-100 and incubated on ice for 30 min followed by centrifugation at 100,000 × g for 1 h at 4 °C. The Triton X-100-insoluble material was dissolved in 50 μl of TNE buffer containing 1% SDS, and 450 μl of TNE buffer was added. For analysis of L1 and c-Met distribution, both supernatant and dissolved pellet were subjected to immunoprecipitation and Western blotting.

For analysis of L1 distribution after HGF stimulation, Foehn cell cultures with a confluency of 80% were serum-starved overnight. Cells were stimulated for 30 min with starve- medium containing 10 ng/ml HGF or 10% FCS as control, and analysis of Triton X-100-soluble and -insoluble membrane fractions was performed as described above.

Western Blot Analysis—Protein samples or immunoprecipitates were membrane fractionated in sample buffer and boiled for 5 min. Samples were separated by SDS-polyacrylamide gel electrophoresis under reducing conditions on 7.5% gels, except for detection with mAb chCE7 where non-reducing conditions are required. The proteins were transferred to polyvinylidene difluoride membranes (Millipore) by semi-dry electrophotoblotting, and the membranes were blocked for 1 h at room temperature with 1% bovine serum albumin (BSA) in Tris-buffered saline (TBST, 20 mM Tris-HCl, 500 mM NaCl, 0.05% Tween-20, pH 7.5). The membranes were incubated with the respective primary antibody in 1% BSA in TBST for 1 h at room temperature or overnight at 4 °C, washed three times with TBST for 15 min, and probed with the corresponding horseradish peroxidase-conjugated secondary antibodies (1:20,000 in 1% BSA in TBST) for 1 h at room temperature or overnight at 4 °C. After TBST for 15 min, immunodetection was performed using the enhanced chemiluminescence kit (Pierce). Primary antibodies were used in a 1:1000 (chCE7), 1:100 (UJ127.11), 1:200 (74H7, c-Met, c-Ret) dilution.

L1 Cell Surface Expression—Cell surface expression of L1 in transfected 293T cells was determined by measuring binding sites for mAb chCE7. mAb chCE7 was labeled with 125I as described previously (2). 24 h after transfection of 293T cells, medium was changed, and cells were incubated for 3 h. 0.5 × 10^6 cells/ml were incubated in a total volume of 0.5 ml of 0.5% BSA in PBS, 100,000 cpm of 125I-chCE7 and increasing concentrations of unlabeled chCE7 (75–1200 ng) overnight at 4 °C. Cells were washed twice with the washing buffer and once with PBS, and cell-associated radioactivity was counted in a γ-counter. Saturation curves were analyzed by the Scatchard method.

Proliferation Assay—RCC-GW cells were seeded in triplicate at 50,000 cells per well on 6-well plates in 2 ml of serum-free medium. Either HGF (20 ng/ml) or GDNF (10 ng/ml) were added, and cells were stimulated for the indicated time points. Cells were removed with 0.05% EDTA in PBS and detached with 1 mM EDTA in PBS, and cells-associated radioactivity was counted in a γ-counter. Scattering Assay—100,000 RCC-GW cells per well were plated on 6-well plates either in full medium alone supplemented with 20 ng/ml HGF and 10 ng/ml GDNF, respectively. Cells were stimulated for 24 h, and scattering was documented using a microscope with digital equipment.

Analysis of L1 Shedding—For analysis of L1 release from carcinoma cell lines, the indicated cells were seeded on 10-cm plates and incubated for 5–17 h. Medium was changed and eventually supplemented with either HGF at the indicated concentrations, 50 ng/ml GDNF or 100 μg Genistein, respectively. Medium was collected at the indicated time points and passed to analysis.

Cloning of L1 Mutants—The DNA for human full-length L1 (a gift from Dr. V. Lemmon, University of Miami, FL) was cloned into the EcoH/Xhol restriction site of pcDNA3.1+ (Invitrogen, Basel, Switzerland). Recombinant baculovirus DNA was purified from the baculovirus infected host insect cells. The point mutation Tyr1229 to His was created by overlap extension. The SacI/XhoI DNA fragment of the L1wt was replaced by mutated SacI/XhoI fragments. All mutations were confirmed by DNA sequencing (Microsynth, Balgach, Switzerland). The sequences of oligonucleotides used for mutagenesis in this study are available on request.

Transient DNA Transfection and Analysis of L1 Shedding—Transient DNA transfection was performed as described below (27). 293T cells were seeded on 10-cm plates and incubated for 7 h. Transfection was performed using the calcium phosphate method. In brief, 5 μg of cdNA was dissolved in 400 μl of H₂O and 61 μl of 2 M CaCl₂ were added. The DNA mixture was dropped into 500 μl of 2× BBS (HEPES-buffered...
saline: 50 mM HEPES pH 7.1, 280 mM NaCl, 1.5 mM Na2HPO4, and the calcium phosphate DNA precipitate was allowed to stand at room temperature for 20 min. 10 ml of fresh complete medium was added to the cells before the transfection mixture was added, and cells were incubated overnight.

Cells were washed twice with PBS, split into four 6-cm plates and incubated for 24 h. 2 ml of fresh medium was added and either supplemented with 10 ng/ml HGF or 2 µM cytochalasin D, respectively. Cells were incubated for 3 h and medium was collected and filtered through a sterile filter. Immunoprecipitation and analysis of soluble L1 was performed as described before.

**RESULTS**

**Expression of L1, c-Met, and c-Ret in Human Renal Carcinoma Cells**—To find suitable renal carcinoma cell lines to study the effects of HGF on production of soluble L1, a number of human renal carcinoma cell lines and human embryonic kidney cells were screened for expression of L1, using Western blot analysis with mAb chCE7. Of the 11 tested cell lines 7 were found to express L1, some of them to a high extent (Fig. 1A). In some cell lines, the 140-kDa cleavage product of L1 was found to be associated with full-length L1, as described before by Kalus et al. (7). Renal carcinoma cell lines and embryonic kidney cells (HEK293 and 293T) were then screened by Western blotting for the presence of c-Met and c-Ret to find L1-positive cells which also respond to HGF and GDNF. The extent of c-Met- and c-Ret expression varied widely in different renal carcinoma cell lines (Fig. 1, B and C). In some cells (Fig. 1B, lanes 3, 4, 7, and 8) the 170 kDa proform of c-Met (c-Met pro) could be detected in addition to the 145-kDa β-subunit of mature c-Met (c-Met β), indicating strong overexpression of c-Met in these cell lines.

As L1 is known to associate with cytoskeletal proteins through its intracellular domain, we investigated the distribution of L1 between the Triton X-100 soluble and insoluble membrane fractions in Foehn renal carcinoma cells. Immunoprecipitation showed that most of L1 partitions in the Triton X-100-soluble membrane fraction (S) and a smaller amount is present in the insoluble fraction (P), which includes cytoskeletal proteins and lipid rafts (Fig. 1D, lanes 1 and 2). No significant changes in this distribution pattern of L1 could be observed after stimulation of serum-starved Foehn cells with 10 ng/ml HGF for 30 min (Fig. 1D, lanes 3 and 4). Similar to L1, expression of the β-subunit of c-Met in Foehn cells was found to be stronger in the Triton X-100 soluble membrane fraction (S) than in the insoluble fraction (P) whereas the proform of c-Met was shown to be expressed to a similar extent in the soluble and insoluble membrane fraction (Fig. 1E).

**Effects of HGF and GDNF on Proliferation and Scattering of RCC-GW Cells**—In order to test cellular responses to HGF and GDNF, the effect of these factors on proliferation and scattering of the L1- and c-Met/c-Ret receptor-positive cell line RCC-GW was investigated. It is known that serum can mask proliferative effect of HGF (30), therefore cell growth was measured in three independent experiments over a time period of 96 h in serum free medium alone or in the presence of 20 ng/ml HGF and 10 ng/ml GDNF, respectively. Fig. 2A shows in a typical experiment that both factors led to a significant increase in cell number, however the effect of GDNF appeared at

![Figure 1](http://www.jbc.org/)

**Fig. 1.** L1, c-Met, and c-Ret expression in renal carcinoma cell lines. **A,** 100 µg of cell lysate from different renal carcinoma cell lines and HEK293 cells were separated by SDS-PAGE under non-reducing conditions and subjected to Western blot analysis using anti-L1 mAb chCE7. 7 of 11 investigated cell lines expressed the 200-kDa band representing full-length L1. **B,** immunoprecipitates of c-Met from different renal carcinoma cell lines and HEK293 and 293T cells were analyzed by immunoblotting with anti-c-Met mAb C-12. 7 of 9 investigated cell lines expressed the 145-kDa β-subunit of mature c-Met (c-Met β). The 170 kDa proform of c-Met (c-Met pro) was found in high expressing cell lines (lanes 3, 4, 7, 8). **C,** 100 µg of cell lysate from different renal carcinoma cell lines and HEK293 and 293T cells were subjected to Western blotting followed by detection with anti-c-Ret mAb C-19. The 150 kDa c-Ret protein was found in 7 of 9 investigated cell lines. **D,** L1 distribution in detergent soluble and insoluble membrane fractions. Foehn cells were grown to a confluency of 80%, serum-starved overnight and either stimulated with 10% FCS (lanes 1 and 2) or 10 ng/ml HGF (lanes 3 and 4) for 30 min. Isolated membranes were extracted with 1% Triton X-100, and soluble (S, lanes 1 and 3) and insoluble (P, lanes 2 and 4) membrane fractions were analyzed for L1 expression by immunoprecipitation of L1 with mAb chCE7 followed by Western blotting using mAb UJ127.11. No changes in the distribution pattern of L1 after HGF stimulation could be observed. **E,** c-Met distribution in detergent-solu-
later times than the effect of HGF. HGF is also known as “scatter factor,” and addition of 20 ng/ml HGF to complete medium led to a strong scattering effect and elongation of RCC-GW cells within 24 h (Fig. 2, B, panel 2), whereas 10 ng/ml GDNF showed no scattering over a time period of 96 h (Fig. 2, B, panel 3). These results show that RCC-GW cells do not only express the HGF and GDNF receptors, but also respond to these growth factors.

**Influence of Growth Factors on L1 Shedding from Renal Carcinoma Cells**—L1 is known to be released from the cell surface of various tumor cell lines due to membrane proximal cleavage by metalloproteases (5, 7, 31). To study shedding of L1 from the cell surface of renal tumor cells, immunoprecipitation of soluble L1 from cell culture medium with mAb chCE7 followed by immunoblotting with mAb UJ127.11 was performed. RCC-GW cells expressing full-length L1 (200 kDa) were used as a control (Fig. 3, A and B, lane 1). All of the tested cell lines released a 180-kDa form of L1 into the medium (sL1) and the amount of soluble L1 correlated with the extent of L1 expression (Fig. 3A). Shedding of L1 was also observed in the L1-expressing neuroblastoma cell line SK-N-AS. A time course of L1 release from RCC-GW cells showed that when the cells were supplied with fresh culture medium, soluble L1 was faintly detectable in the medium after 1 h and shedding increased over 48 h (Fig. 3B). No concomitant decrease in L1 expression on the cell surface was seen over the whole time period (data not shown).

To evaluate effects of HGF and GDNF on L1 shedding, RCC-GW cells were stimulated by 100 ng/ml HGF or 50 ng/ml GDNF for 24 h. Addition of HGF to complete medium including 10% FCS strongly stimulated the release of soluble L1 into the medium (Fig. 3C), accompanied by a decrease of cell surface expression of L1 (data not shown). Densitometric analysis of the intensities of the 180-kDa Western blot bands using Aida© software (Raytest GmbH, Straubenhardt, Germany) revealed a 2-fold increase of L1 shedding after HGF stimulation compared with basal levels \( (n = 3, \text{av} = 1.96, \text{S.D.} = 0.23, p = 0.02) \). When GDNF (50 ng/ml) was added to complete medium inhibition of L1 shedding was observed (Fig. 3C). Increasing concentrations of HGF in a range from 1 to 50 ng/ml led to a dose-dependent increase of L1 shedding, with a maximum reached at 5 ng/ml (Fig. 3D), as confirmed by three independent experiments. Basal shedding of L1 was found to be similar in serum free and complete medium (Fig. 3E, lanes 1 and 2), while no stimulation of L1 shedding by HGF in serum-free medium was observed (Fig. 3E, lanes 3 and 4), indicating that components present in the serum are involved in HGF-stimulated shedding of L1 but not in basal L1 release. These results demonstrate, that L1 is shed from the cell surface of renal carcinoma cells and that release of L1 is stimulated by physiological concentrations of HGF but not by GDNF.

In order to investigate if shedding of L1 is mediated by tyrosine phosphorylation, we analyzed basal and HGF-stimulated L1 shedding from Foehn cells in the presence of the broad range tyrosine kinase inhibitor Genistein. We found that both basal and HGF-stimulated shedding of L1 was suppressed after addition of 100 μg/ml Genistein for 3 h (Fig. 3F). The inhibition of L1 shedding was not due to a toxic effect on the cells, because cell viability measured by the trypan blue assay after 3 h of incubation with Genistein was found to be similar to that in control cells (85%). These results indicate that protein phosphorylation regulates the release of soluble L1 from the cell surface.

**Influence of Mutations in the L1 Cytoplasmic Domain on L1 Shedding**—We constructed L1 mutants and transiently expressed them in 293T cells (Fig. 4) to address the role of the intracellular domain in L1 shedding. In contrast to HEK293 cells, these cells express replication-competent T antigen, which leads to high expression of transfected L1 mutants and high amounts of soluble L1 in the medium. A first mutant was cloned missing all but 3 amino acids (KRS) of the cytoplasmic domain (L1trun). A second mutant with an exchange of tyrosine 1229 to histidine (L1YH) was generated. This mutation known from the MAS syndrome abolishes the tyrosine phosphorylation-mediated intracellular binding of L1 to ankyrin (32) and enhances lateral motility of L1 (23). All L1 mutant proteins were expressed in equal amounts in 293T cells as verified by immunoblotting of cell lysates with
HGF Stimulates Ectodomain Shedding of L1

Effects of HGF, GDNF, and Genistein on L1 ectodomain shedding from renal carcinoma cells. A, L1 shedding from the surface of different L1-expressing cell lines. The indicated cell lines were grown on 10-cm plates in the corresponding medium. Medium was collected after 24 h, and soluble L1 was immunoprecipitated from cell culture medium with mAb chCE7. Analysis of immunoprecipitates was performed by immunoblotting using mAb UJ127.11. Lane 1, RCC-GW cell lysate, control; lane 2, SK-N-AS, neuroblastoma cell line; lanes 3–5, renal carcinoma cell lines; lane 6, embryonic kidney cells. All investigated cell lines released the 180-kDa soluble L1 (sL1) fragment into the medium. B, time course of L1 shedding from RCC-GW cells. Medium was collected at the indicated time points and soluble L1 was immunoprecipitated from cell culture medium with mAb chCE7 followed by Western blot analysis using mAb UJ127.11. Lane 1, RCC-GW cell lysate, control. Soluble L1 (sL1) was detectable in the medium after 1 h and increased up to 48 h. C, L1 shedding from RCC-GW cells is stimulated by HGF. Cells were incubated for 24 h in complete culture medium (lane 1), supplemented with 100 ng/ml HGF (lane 2), or 50 ng/ml GDNF (lane 3). Analysis of soluble L1 was performed by immunoprecipitation from cell culture medium with mAb chCE7 followed by immunoblotting with mAb UJ127.11. The release of the 180-kDa cleavage product of L1 (sL1) was enhanced after HGF stimulation compared with control, whereas soluble L1 decreased in the medium after GDNF treatment. The 140-kDa cleavage fragment of L1 known to associate with full-length L1 was found together with the 180-kDa product in the medium. The positions of the different L1 fragments are indicated to the right. D, HGF stimulation of L1 shedding from RCC-GW cells is dose-dependent. Cells were incubated for 24 h in complete medium containing the indicated HGF concentrations. Amount of released L1 (sL1) was determined by immunoprecipitation with mAb chCE7 from mAb UJ127.11 24 h after transfection (Fig. 5A, Ln1 ecto). Analysis of 293T cell lysates with mAb 74–5H7 directed against the cytoplasmic part of L1 revealed strong reactivity with full-length L1 (L1wt) and L1YH (Fig. 5A, Ln1 cyt). As expected, no reactivity with L1 lacking the whole intracellular domain (L1trun) was detectable. No endogenous L1 expression was detectable in 293T cells transfected with the vector pcDNA3.1+ (Fig. 5A, lane 1).

To investigate the amount of basal shedding of the L1 mutants, 293T cells were transfected, incubated overnight and transferred to 6-cm plates. After 24 h, medium was changed and cell culture supernatant was analyzed for soluble L1 after 3 h. RCC-GW cell lysate with full-length L1 was used as control (Fig. 5B, lane 1). We found that 293T cells expressing the truncated form of L1 (L1trun) showed enhanced shedding compared with 293T cells transfected with full-length L1 (L1wt) (Fig. 5B). In contrast, the L1YH mutant released less soluble L1 into the medium than L1wt cells. As it is known that the mutation of Tyr-1229 to histidine in L1 leads to enhanced endocytosis when transfected into rat B35 neuroblastoma cells (31), we investigated if the observed reduced shedding of L1 in L1YH cells is due to altered trafficking. The amount of L1 cell surface expression was determined by Scatchard analysis with mAb 125I-chCE7 after 3 h of incubation in fresh medium. Two independent experiments showed a similar number of binding sites for mAb chCE7 in L1wt and L1YH cells (data not shown). These results strongly suggest that the cytoplasmic domain of L1 exerts a regulatory function on basal L1 shedding, partly mediated through its interaction with ankyrin. Ankyrin links L1 via spectrin to the actin cytoskeleton (21). To elucidate the role of the actin cytoskeleton in L1 shedding, we treated transfected 293T L1wt cells for 3 h with cytochalasin D at a concentration known to completely suppress F-actin polymerization in the periphery of cells (23). Viability of cells was determined by the trypan blue viability test and was found to be similar in treated and untreated cells (95%). We found that cells treated with cytochalasin D showed reduced basal shedding of L1 compared with untreated L1wt cells (Fig. 5C, lanes 1 and 2). To determine if cytochalasin D treatment influences the cell surface expression of L1, Scatchard analysis with mAb 125I-chCE7 was performed. Two independent experiments showed a similar number of binding sites for mAb chCE7 in untreated L1wt cells and in L1wt cells treated with 2 μM cytochalasin D for 3 h (data not shown). Our results indicate that the association of L1 with a dynamic pool of actin regulates basal L1 shedding. Addition of HGF to cytochalasin D-treated L1wt cells led to a large increase in L1 shedding (Fig. 5C, lane 3). In order to investigate whether the stimulating effect of HGF on L1 shedding is affected by mutations in the cytoplasmic domain of L1, we stimulated mutant 293T cells 24 h post-transfection for 3 h with 10 ng/ml HGF. The extent of stimulation of L1 release by HGF was found to be smaller in mutant 293T cells than in cell culture medium followed by immunoblotting with mAb UJ127.11. Lane 1, RCC-GW cell lysate, control; lane 2, marker. HGF stimulated L1 cleavage in a dose-dependent manner with the maximum reached at 5 ng/ml HGF. E, HGF stimulation of L1 shedding requires complete medium. RCC-GW cells were incubated either in serum-free medium (lanes 1 and 3) or in medium supplemented with 10% FCS (lanes 2 and 4), and 100 ng/ml HGF was added to one of each sample (lanes 3 and 4). Soluble L1 was immunoprecipitated from cell culture medium after 24 h with mAb chCE7 and analyzed by immunoblotting using mAb UJ127.11. No stimulating effect of HGF on L1 shedding could be observed in serum-free medium. F, L1 shedding is inhibited by the tyrosine kinase inhibitor Genistein. Foehn cells were incubated for 3 h in complete medium (lanes 1 and 2) or supplemented with 10 ng/ml HGF (lanes 3 and 4). Addition of 100 μM Genistein (lanes 2 and 4) led to reduced L1 shedding in both basal and HGF-stimulated cells. Molecular masses of standards are indicated to the left.
renal carcinoma cells. Nevertheless we found that all L1 mutants including L1trun showed enhanced shedding of L1 after HGF stimulation (Fig. 5D). We therefore conclude that stimulation of L1 shedding by HGF is due to a mechanism that does not require the cytoplasmic domain of L1 and is independent of actin assembly.

DISCUSSION

Ectodomain shedding of cell adhesion proteins such as carcinoembryonic antigen (CEA) (33, 34), NCAM (35, 36), or CD44 (37, 38) is linked to strong overexpression of these proteins in certain types of epithelial cancers, and the soluble forms of these proteins may serve as clinical tumor markers. We found previously that the cell adhesion molecule L1 is overexpressed in some renal tumors (2). Here we show that L1 overexpression is found in many renal carcinoma cell lines and L1 is shed from the surface of these cells. A time course of basal L1 shedding showed that detectable levels of soluble L1 in the medium were found after 1 h and soluble L1 increased up to 48 h, whereas no concomitant change in the cell surface expression of L1 was found. The release of L1 from the cell surface does not lead to L1 down-regulation and is therefore more likely to serve as a means for production of soluble L1. In the nervous system, soluble L1 released from cultured neurons promotes neurite outgrowth and influences neuronal differentiation (7, 28). The release of soluble L1 from tumor cells may have effects on motility and spreading, as soluble L1 was shown to be deposited in the stroma of melanoma tumors and to support integrin-mediated cell adhesion and migration (5, 31). Recently, soluble L1 was detected in the serum of ovarian and uterine cancer patients and the levels of soluble L1 strongly correlated with the malignancy of the tumors (9).

The release of L1 from the cell surface may be a regulated process triggered by extracellular signals. In PC12 cells, nerve growth factor (NGF) was found to increase shedding of the NILE glycoprotein (39), which was later identified as the rat homologue of L1 (31). We found that in renal carcinoma cells, basal shedding of L1 is stimulated by HGF, a growth factor important for proper kidney development (17), which is strongly expressed in 90% of renal cell carcinomas (10, 11, 12) and increases invasiveness in renal carcinoma cells in vitro and in vivo (13, 14). HGF stimulated basal shedding of L1 at physiological concentrations with a maximum reached at 5 ng/ml HGF. This effect was dependent on the presence of serum in the cell culture medium, indicating an involvement of components of the serum in HGF stimulated shedding of L1. In contrast, L1 shedding was suppressed by GDNF, indicating the specificity of the effect of HGF. The renal carcinoma cell line used for these experiments (RCC-GW) responded to the two growth factors in terms of proliferation and scattering and showed no constitutive phosphorylation of c-Met (data not shown), indicating that the overexpressed receptors are not in an activated state.

L1 shedding may be regulated by protein phosphorylation because it is stimulated by agents such as phorbol esters or pervanadate, which activate cellular protein kinases (6). We found that the protein tyrosine kinase inhibitor Genistein inhibited shedding of L1, which also indicates that protein phosphorylation is involved. The L1 cytoplasmic domain contains several regulatory motifs, including serine and tyrosine phosphorylation sites, which could be targets for the action of HGF. One of the best characterized is tyrosine 1229 within the ankyrin binding site of L1. In neuroblastoma cells, Tyr-1229 in the L1 family member neurofascin was shown to be phosphorylated by NGF and bFGF, which led to inhibition of ankyrin binding, enhanced lateral mobility of L1 and scattering of neuroblastoma cells (22, 40).

We investigated two L1 mutants, either lacking the intracellular domain except for the membrane proximal KRS sequence (L1trun) or with the mutation tyrosine 1229 to histidine (L1YH) known to abolish ankyrin binding (32). In 293T cells expressing the truncated form of L1 lacking the intracellular domain (L1trun), basal shedding was found to be strongly increased compared with the full-length form of L1 (L1wt). This result shows that the cytoplasmic part of L1 exerts a negative regulatory role on L1 shedding, which is in contrast to the results of Gutwein et al. (6), who found that a truncated form of L1 showed no significant difference to L1wt in basal, FMA-, or pervanadate-induced L1 shedding. The L1YH mutant showed reduced basal shedding compared with L1wt cells, which was not caused by altered cell surface expression of L1, indicating that connection of L1 to the cytoskeleton through ankyrin binding is involved in the regulation of basal L1 shedding. We found that cytochalasin D treatment also led to reduced basal shedding of L1 compared with control, but did not affect L1 cell surface expression, which further supports the idea that interactions with the actin network regulate basal L1 shedding. It is known for the case of the CD44 cell surface protein that the intracellular part of CD44 is required for its association with...
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and cleavage by MT1-MMP and provides the connection of this complex to the actin cytoskeleton (41). In a similar manner, changes in L1 cytoskeletal attachments may regulate the interaction of L1 with the involved proteases.

The effect of HGF on L1 shedding was found to be independent of deletion of the complete cytoplasmic domain of L1, or of the mutation of tyrosine 1229 to histidine, indicating a mechanism independent of L1 cytoplasmic regulatory sites. The stimulating effect of HGF on L1 shedding was also unaffected by treatment with cytochalasin D. It is possible that HGF directly stimulates metalloprotease activity, as for instance in prostate cancer cells HGF has been shown to increase cleavage of E-cadherin through activation of MMP-7 (42). For L1 it is known that membrane-proximal cleavage occurs through a metalloprotease (5, 6, 7) and it is suggested that ADAM10 may be one of the involved proteases (8). Although ADAM10 was recently found to be unregulated by insulin-like growth factor 1 and epidermal growth factor in pancreatic carcinoma cells (43), no stimulation of ADAM10 by HGF has been described.

The data presented in this study show that basal shedding of L1 is regulated by its interaction with the actin cytoskeleton while HGF-stimulated L1 shedding is independent of the L1 cytoplasmic domain. It would be of interest to analyze cleavage fragments of L1 after basal and HGF-stimulated shedding to give insight if different proteases are involved in basal and HGF-stimulated L1 release.

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