Misregulation of Stromelysin-1 Expression in Mouse Mammary Tumor Cells Accompanies Acquisition of Stromelysin-1-dependent Invasive Properties*

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Stromelysin-1 is a member of the metalloproteinase family of extracellular matrix-degrading enzymes that regulates tissue remodeling. We previously established a transgenic mouse model in which rat stromelysin-1 targeted to the mammary gland augmented expression of endogenous stromelysin-1, disrupted functional differentiation, and induced mammary tumors. A cell line generated from an adenocarcinoma in one of these animals and a previously described mammary tumor cell line generated in culture readily invaded both a reconstituted basement membrane and type I collagen gels, whereas a nonmalignant, functionally normal epithelial cell line did not. Invasion of Matrigel by tumor cells was largely abolished by metalloproteinase inhibitors, but not by inhibitors of other proteinase families. Inhibition experiments with antisense oligodeoxynucleotides revealed that Matrigel invasion of both cell lines was critically dependent on stromelysin-1 expression. Invasion of collagen, on the other hand, was reduced by only 40–50%. Stromelysin-1 was expressed in both malignant and nonmalignant cells grown on plastic substrata. Its expression was completely inhibited in nonmalignant cells, but up-regulated in tumor cells, in response to Matrigel. Thus misregulation of stromelysin-1 expression appears to be an important aspect of mammary tumor cell progression to an invasive phenotype.

The matrix metalloproteinases (MMPs) are a family of extracellular matrix (ECM)-degrading enzymes that have been implicated in a variety of normal developmental and pathological processes, including tumorigenesis (1, 2). The MMP family comprises at least 15 members with different, albeit overlapping, substrate specificities. During activation of latent MMPs, their propeptides are cleaved and they are converted to a lower molecular weight form by other enzymes, including serine proteinases, and by autocatalytic cleavage.

Among the MMPs, stromelysin-1 (SL1) possesses the broadest substrate specificity (1). Despite increasing knowledge about its enzymatic properties and the regulation of its expression, little is known about its function. We have generated transgenic animals that express an autoactivating mutant of rat SL1 targeted to the epithelial compartment of the mammary gland (3). Phenotypically, SL1 transgenic mice display increased branching morphogenesis and lactogenic differentiation at prepubertal stages and premature involution during late pregnancy (3, 4). Branching morphogenesis requires the invasion of epithelial cells into the adipose tissue, a process reminiscent of invasion of stromal compartments by tumor cells. Strikingly, a large number of SL1 transgenic animals also develop mammary tumors of various histotytes, including invasive adenocarcinomas (5). Because tumor development is a late response of SL1 transgenic mice to overexpression of the transgene, it remains unclear whether SL1 plays a direct role in tumor growth and/or invasion or whether the observed tumors are a consequence of other molecular alterations in the microenvironment of the mammary gland before the onset of tumor growth. Studies performed with synthetic inhibitors of MMP activity and tissue inhibitors of metalloproteinases (TIMPs) have shown that suppression of MMP activity also suppresses tumor growth and metastasis (6, 7). In many cases, the level of SL1 expression in tumors of the mammary gland and other tissues is positively correlated with the degree of malignancy (8, 9). However, the only direct evidence for the nature of the MMPs involved was provided by the demonstration that function-blocking antibodies against gelatinase A and antisense inhibition of matrilysin expression decreased the invasiveness of tumor cells in a reconstituted basement membrane assay (10, 11). These studies encouraged us to investigate whether SL1 plays a direct role in invasion of ECM. We used two carcinoma cell lines, TCL1 and SCg6 that formed rapidly growing, invasive tumors in vivo and migrated through Matrigel and collagen gels in culture.
Antisense oligodeoxynucleotides (ODNs) against SL1 inhibited Matrigel invasion by TCL1 and SCg6 cells by more than 80% and collagen invasion by about 50%. Comparison of the regulation of SL1 expression by ECM in TCL1 and SCg6 cells with the nonmalignant, functional cell line SCp2 revealed striking differences that could play a role in the acquisition of an invasive tumor phenotype.

Experimental Procedures

Tumor Formation

0.25 x 10^6 SCp2, TCL1, or SCg6 cells suspended in Dulbecco’s minimum essential medium (DMEM)/F-12 (Life Technologies, Inc.) were injected into the number 4 mammary gland of athymic nude mice. Two weeks later, animals were sacrificed and tissue samples of the injected gland, adjacent skin and lung were fixed with 4% paraformaldehyde, blocked in paraffin, sectioned at 10 µM, and stained with hematoxylin and eosin.

Cell Culture

TCL1 cells were established from a metastatic mammary adenocarcinoma (from animal number M2-20-2-7-1-2) (5). A small piece of tumor tissue (0.5 mm x 0.5 mm x 0.5 mm) was excised and placed under the skin of a nude mouse. After 47 days, the tumor, which had grown to 11 mm x 13 mm x 8 mm, was excised, cut into small pieces, and cultured in DMEM/F-12 containing 10% fetal bovine serum (Life Technologies, Inc.), 5 µg/ml insulin (Sigma), and 50 µg/ml gentamicin (Life Technologies, Inc.). Mammary epithelial cells were enriched in these cultures by differential trypsinization as described (12). Cells were maintained in the same medium and passaged 10 times before the medium was replaced with DMEM/F-12 containing 5% fetal bovine serum, 5 µg/ml insulin, and 50 µg/ml gentamicin (growth medium). For all experiments TCL1 cells were used between passages 25 and 30. The origins of the mouse mammary carcinoma cell line SCg6 and the functionally normal mouse mammary cell line SCp2 have been described previously (13).

For routine passaging, all cell lines were cultured in growth medium. To study the regulation of MMP expression, cells were trypsinized with 0.05% trypsin and 0.02% EDTA in phosphate-buffered saline, pH 7.4 (PBS), centrifuged at 80 x g, and the cell pellet was resuspended in a chemically defined medium containing DMEM/F-12, 5 µg/ml insulin, 3 µg/ml prolactin (Sigma), 1 µg/ml hydrocortisone (Sigma), and 50 µg/ml gentamicin. Cells (5 x 10^5) were plated in 1 ml of chemically defined medium into six-well tissue culture dishes (Falcon) and maintained for 1–2 days. For preparation of reconstituted basement membrane gels, 200 ml of Matrigel (Collaborative Biomedical Products) were evenly spread into each well at 4 °C and subsequently allowed to gel in a humidified incubator for 30 min at 37 °C before addition of cells. Laminin-entactin, type IV collagen, fibronectin (all from Collaborative, 50 µg/ml), heparin (100 µg/ml, Sigma), aprotinin (0.3 µM, Sigma), pepstatin (1 µM, Sigma), leupeptin (1 µM, Sigma), or phosphorothioate ODNs (Bio-Synthesis) were added to the cell culture medium immediately after cell plating. SL1 antisense (5’-GAAGACCCCTTCTTTCTCAT-3’) and sense (5’-ATGAAAATGAAAGGTCCTTC-3’) ODNs were used at a concentration of 3.2 µM, SL3 antisense (5’-ACAGGCCGGCCGTCCAT-3’) ODNs were used at 8 mM, and collagenase-3 antisense (5’-CAGGATATGCTGATGCAT-3’) ODNs were used at 32 µM. The hydroxamic
acid metalloproteinase inhibitor 3-(N-hydroxycarbamoyl)-(2R)-isobutylpropionyl-L-tryptophan methylamide (GM6001) and an inactive structural homologue (N-tert-butyloxycarbonyl)-L-leucine-L-tryptophan methylamide (GM1210) (gifts from Dr. R. Galardy and Glycomed Corp., Alameda, CA; Ref. 14) were dissolved at a concentration of 40 mM in Me₂SO and added to the culture medium after cell plating at a final concentration of 10 µM, unless otherwise indicated. GM6001 is a general inhibitor of all metalloproteinases with $K_i$ values < 100 nM (14, 15). It has a $K_i$ of 27 nM against SL1 (15). None of the components influenced attachment, proliferation, or trypan blue-quantifiable survival rate of the cells (data not shown).

**Invasion Assays**

Invasion assays were modified from published procedures (16) and performed in a modified Boyden chamber with polyethylene terephthalate filter-inserts for 24-well plates containing 8-µm pores (Collaborative). Filters were coated on ice with 10 µl of Matrigel at 6 to 8 mg/ml protein or 15 µl of type I collagen (Vitrogen, Celtrix Laboratories) at 2.6 mg/ml. 1 x 10⁵ cells were plated in 200 µl of chemically defined medium into the upper chamber. The lower chamber was filled with 300 µl of medium. Proteinase inhibitors and ODNs were added immediately after cell plating to both chambers at concentrations described above. After 18 h in culture, cells were fixed with 5% glutaraldehyde in PBS and stained with 0.5% toluidine blue (Sigma) in 2% Na₂CO₃. Cells on the upper side of the filter as well as the ECM were removed with paper towels, and the cells found on the lower side of the filter were counted by microscopic inspection. Six visual fields, 1 mm² each, were counted per experiment. The results were averaged, and the mean of three experiments is shown for each treatment.

**Immunofluorescence Labeling**

For immunodetection of E-cadherin, 1 x 10⁵ cells were grown for 2 days in chemically defined medium on glass coverslips coated with 5 µg/ml poly-L-lysine (Sigma). Cells were washed once with PBS and fixed with 5% formalin in PBS (Sigma) for 10 min. They were then washed twice with PBS containing 50 mM NH₄Cl, and once with PBS, for 10 min each. For immunodetection of keratin and vimentin in cells grown on glass coverslips, medium was removed and cells were fixed and permeabilized by incubation with 50% methanol, 50% acetone at -20 °C for 15 min. After rinsing three times with PBS, nonspecific binding sites were blocked by incubation with PBS containing 0.1% fatty acid-free bovine serum albumin (Sigma), 0.2% Triton X-100, and 0.025% Tween 20 (blocking buffer 1) for 2 h at ambient temperature. Cells were then treated with mouse monoclonal antibody against E-cadherin (1:100 dilution, Transduction Laboratories), mouse monoclonal antibody against vimentin (1:200, Sigma), or rabbit polyclonal antisera against bovine keratins (1:10, Dako) in blocking buffer 1. After incubation for 2 h at ambient temperature, cells were briefly washed five times with PBS and incubated with Texas Red-conjugated goat anti-mouse (1:100, Caltag) or donkey anti-rabbit (1:100, Amersham) antibodies in blocking buffer 1 for 1 h at ambient temperature, washed five times with PBS, and mounted with Vectashield (Vector Laboratories).
Zymograms, Immunoblots, and Analysis of General Protein Secretion

Chemically defined culture medium conditioned by cells for 2 days was processed for casein substrate gels as described (17). In brief, conditioned medium was mixed with Laemmli sample buffer (18) without reducing agents, incubated for 15 min at 37 °C, and separated on 8.8% sodium dodecyl sulfate (SDS)-polyacrylamide slab gels (18) containing 1 mg/ml a-casein (Sigma). After electrophoresis, gels were incubated twice for 30 min with 2.5% Triton X-100 and subsequently for 3 days at 37 °C in 100 mM Tris-HCl, pH 7.4, 15 mM CaCl₂. Gels were stained with Coomassie R-250 and destained with 10% acetic acid, 30% methanol. Clear zones emerged against a blue background, indicating proteinolytic activity. For densitometric analysis of caseinase activity in zymograms, video images of three zymograms for each condition were analyzed with an Eagle Eye II image analysis system (Stratagene) by comparing the average pixel values of cleared zones. Statistical comparison was performed with Student’s t test. For characterization of caseinases, 1,10-phenanthroline (1 mM) or 4-aminophenylmethylmercuric acetate (1 mM) was incubated with the samples for 30 min at 37 °C before addition of sample buffer (17, 18); 1,10-phenanthroline at 1 mM was also included in all incubation steps after gel electrophoresis.

For detection of SL1 by immunoblotting, 4 ml of conditioned medium was concentrated by acetone precipitation (19) and resuspended in 100 µl of Laemmli sample buffer. An equivalent of conditioned medium from 1 x 10⁶ cells was separated in each lane of 8.8% SDS-polyacrylamide slab gels (18). Transfer onto Immobilon-P membranes (Amersham) was performed as described (20). Membranes were blocked overnight at 4 °C with 250 mg/liter Tween 20 in Tris-buffered saline, pH 7.5 (TBST), containing 15% skim milk powder and 5% bovine serum albumin (blocking buffer 2) and then treated with sheep antiserum against SL1 (1:10,000, a generous gift from Dr. H. Nagase, University of Kansas) for 2 h at ambient temperature in blocking buffer 2. Membranes were washed five times (10 min each wash) with TBST and incubated for 1 h at ambient temperature with peroxidase-conjugated anti-sheep antibodies (1:1, 500, Dako) in blocking buffer 2. After five washes (10 min each) with TBST and two brief rinses with Tris-buffered saline, bands were visualized with an ECL detection system (Amersham) according to the manufacturer’s instructions. For analysis of protein secretion in the presence of SL1 antisense ODNs, cells were maintained for 24 h in the chemically defined medium in the presence or absence of SL1 antisense or sense ODNs. Then the medium was replaced with methionine-free DMEM (Life Technologies, Inc.) with or without ODNs and with 100 mCi/ml [35S]methionine. After another 8-h incubation, 200 µl of conditioned medium from an equivalent of 0.25 x 10⁶ cells was concentrated by acetone precipitation, resuspended in 15 µl of Laemmli sample buffer, and separated on 6% SDS-polyacrylamide slab gels. Gels were vacuum-dried and exposed to Kodak autoradiography films for 4 h.

Northern Blot Hybridization

Total RNA was extracted with TRIzol (Life Technologies, Inc.) according to the manufacturer’s instructions. RNA was resuspended in FORMAzol (Molecular Research Center) and resolved on 1% (w/v) agarose gels containing 2.2 M formaldehyde. RNA was transferred by capillary blots onto MAGNA MT nylon membranes (MSI). Blots were prehybridized with 50% deionized formamide, 1 x Denhart’s solution, 4 x SSPE (1 x SSPE = 180 mM NaCl, 10 mM NaH₂PO₄, 1
mM EDTA, pH 7.5), 20 mM potassium phosphate, pH 6.5, 1% SDS, and 400 µg/ml salmon sperm DNA at 45 °C. Hybridization was carried out under the same conditions but in the presence of 10% (w/v) dextran sulfate (Nycomed AS) and 3 x 10^6 dpm/ml [32P]dCTP (Amersham)-labeled cDNA probes generated by random priming (Rediprime kit, Amersham) according to the manufacturer’s instructions. Blots were washed in 2 x SSPE containing 0.1% SDS, 0.5 x SSPE containing 0.1% SDS and 0.1 x SSPE containing 0.1% SDS, each step for 1 h at 55 °C. The cDNA fragments used for random priming for SL3 (21), matrilysin (22), MT-MMP (21), gelatinases A (21) and B (21), TIMP1 (21), TIMP3 (23), and 28 S RNA (21) have been described. cDNA for mouse SL1 was cloned with SL1-specific primers as specified in the following section. All blots shown in Fig. 5 were hybridized twice for detection of MMP or TIMP expression and once for detection of 28S RNA for internal standardization. In between hybridizations, radioactive material bound to the filters was removed by boiling blots for 1 min in water.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Southern Hybridization

Total RNA was extracted with TRIzol (Life Technologies, Inc.) according to the manufacturer’s instructions. RNA was resuspended in diethyl pyrocarbonate-pretreated water and reverse transcribed for 60 min at 37 °C in a volume of 20 µl with 10 units/µl Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) in 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.5 mM dTTP, 12.5 ng/µl oligo(dT)12-18 (Life Technologies, Inc.). PCR amplification was performed in a total volume of 50 µl with 2 ng/µl reverse transcription RNA, 0.025 unit/µl Taq DNA polymerase (Life Technologies, Inc.), 1 μM 5’(sense)-primer, 1 μM 3’(antisense)-primer (all ODNs from Bio-Synthesis), 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2 mM MgCl2, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP. Each PCR cycle was performed at 94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min with cycle numbers indicated in the figure legends. For each PCR amplification, only one primer pair was included in the reaction mixture. cDNAs were amplified with the following primer pairs: GCTGCCATTTCTAATAAAGAGA as 5'-primer and GCACCTCTTTTCACAAGAG as 3'-primer for amplification of mouse SL1, GCAGCCATTTTCTTTAAAGACC as 5’-primer and CCACCTCAGTGCCGAAGTT as 3’-primer for the rat SL1 transgene, TTCTCCACAAGCCAGCTAAG as 5’-primer and TGACTCAATCGAAGAGACca as 3’-primer for mouse SL2, GACATTCTGGAAAGTTATCC as 5’-primer and ACTCTCACAATGCGATTACTC as 3’-primer for mouse collagenase-3, and TGCAATGACGAGTAGTGC as 5’-primer and GTACCACTCGCAAGAAGC as 3’-primer for mouse TIMP2. PCR amplification products were resolved on 2.0% agarose gels. To verify the identity of the amplified sequences, Southern hybridizations were performed with ODNs complementary to the mRNA sequence of the gene examined: GAAACCCAAATGCTTCAAAGTCAGCATCCA for mouse and rat SL1, GGTCTCAGGTCTTGGAGAGGTATCC as 5’-primer and ACTCTCAAATGCGATTACTC as 3’- primer for collagenase-3, and TTGCAATGACGAGTAGTGG for TIMP2. Gels were incubated with 0.5 M NaOH, 1.5 M NaCl for 30 min, 1.0 M Tris-HCl, pH 8.0, 1.5 M NaCl for 30 min and subsequently vacuum-blotted onto Hybond N+ membranes (Amersham) in the presence of 10 x SSC (1 x SSC = 0.15 M NaCl, and 0.015 M sodium citrate). PCR products were UV-cross-linked onto the membranes and prehybridized for 12 h at 55 °C with 5 x SSC, 0.1% (w/v)
lauroylsarcosine, 0.02% SDS, and 1% (w/v) blocking reagent for nucleic acid hybridization (Boehringer Mannheim). The same solution was used for hybridization for 2–4 h at 55 °C with 2 fM digoxigenin-labeled oligonucleotides. Oligonucleotides were labeled and detected with a digoxigenin oligonucleotide 3’-end labeling and a luminescent detection kit (both from Boehringer Mannheim), respectively, according to the manufacturer’s instructions.

Results

**Mouse Mammary Cell Lines TCL1 and SCg6, but Not SCp2, Are Tumorigenic**

The mouse tumor cell lines TCL1 and SCg6 used here to study invasion and MMP expression in culture, formed invasive carcinomas when injected into mammary glands of athymic nude mice. The tumors generated by both TCL1 and SCg6 cells 2 weeks after injection were undifferentiated spindle cell tumors that invaded mammary fat tissue and entrapped mammary ducts (Fig. 1, a and b). Tumor foci were also found in the skin adjacent to the injected mammary gland and in the lung (Fig. 1, c and d). Consistent with this in vivo phenotype, neither SCg6 nor TCL1 cells underwent lactogenic differentiation when plated on a reconstituted basement membrane (Ref. 13 for SCg6 cells, and data not shown for TCL1 cells). In contrast, the functionally normal mammary epithelial cell line SCp2 formed organized alveolar structures and secreted milk proteins when maintained in an appropriate microenvironment (13, 24). SCp2 cells did not form tumors when injected into athymic mice (data not shown). Consistent with their epithelial origin, SCp2 cells expressed E-cadherin and cytokeratins but not vimentin (Fig. 2) (13). TCL1 cells expressed vimentin and low levels of cytokeratins, but did not express E-cadherin (Fig. 2). Like TCL1 cells, SCg6 cells expressed vimentin but not E-cadherin. Cytokeratins in SCg6 cells could be detected only by two-dimensional electrophoresis of metabolically labeled cell extracts and not by conventional immunocytochemistry (Fig. 2). Thus, SCg6 and TCL1 carcinoma cells appear to have undergone epithelial-mesenchymal transdifferentiation (25).

**Matrigel Invasion Is Blocked by Metalloproteinase Inhibitors and Antisense ODNs to SL1**

To study the role of proteinases in tumor progression, we adopted a widely used assay that allows the assessment of tumor cell migration through Matrigel, a reconstituted basement membrane also known as Engelbreth-Holm-Swarm matrix (26). This assay mimics the early steps of tumor invasion in vivo, namely the degradation of, and migration through, basement membranes (27). When SCp2, TCL1, and SCg6 cells were plated onto Matrigel, only TCL1 and SCg6 cells exhibited invasive properties (Fig. 3a). To investigate whether proteinases were involved in Matrigel invasion of the tumorigenic cells, we performed invasion assays in the presence of the MMP inhibitor GM6001 and its inactive homologue GM1210, the serine proteinase inhibitor aprotinin, the serine and cysteine proteinase inhibitor leupeptin, and the aspartic proteinase inhibitor pepstatin (Fig. 3, b and c). GM6001 reduced invasion of both SCg6 and TCL1 cells by about 80%, while the other inhibitors and GM1210 had no effect (Fig. 3, b and c).

SL1 overexpression is frequently associated with tumor progression and invasion (8, 9). Because we hypothesize that SL1 may cause formation of invasive tumors in mice transgenic for SL1, we...
examined the effect of SL1-antisense ODNs on invasion of Matrigel by tumor cells. Antisense ODNs to SL1 largely inhibited invasion of Matrigel by TCL1 and SCg6 cells (Fig. 3d). SL1 sense ODNs and SL3 and collagenase-3 antisense ODNs had no effect on Matrigel invasion of either cell type (Fig. 3d). Immunoblots and casein zymograms showed that SL1 antisense, but not SL1 sense or SL3 antisense treatment, reduced the amount of the 57-kDa SL1 protein secreted into the culture medium (Fig. 4, a–c). Of the three non-SL1 caseinases of molecular masses of 53, 65, and 80 kDa, the latter two showed a reduction of caseinolytic activity in the presence of SL1 antisense ODNs but not with the other ODNs (Fig. 4, b and c). However, only the reduction in activity of the 80-kDa caseinase secreted by TCL1 cells and of the 65-kDa caseinase secreted by SCg6 cells were statistically significant, as determined by densitometric analysis of cleared zones in the zymograms (Fig. 4d). SL1 antisense ODNs did not affect general secretion of proteins, as evidenced by the pattern of radiolabeled proteins secreted into the culture medium (Fig. 4e). Thus, SL1 specifically interferes with production and/or secretion of caseinases. SCp2 cells did not secrete detectable levels of SL1 or other caseinases (Fig. 4a, and data not shown).

To characterize caseinases secreted by tumor cells, conditioned medium was incubated with the organomercurial 4-aminophenylmethylmercuric acetate, which activates the cysteine switch and converts latent MMPs into active species (28). This treatment led to the disappearance of 57- and 65-kDa bands and the appearance of 46- and 49-kDa bands (Fig. 4f), with the latter corresponding to the expected size of active SL1 (1). In the presence of the metalloproteinase inhibitor 1,10-phenanthroline, only the 53-kDa band remained (Fig. 4f). Thus, the 65-kDa caseinase can be classified as a latent MMP, and the 80-kDa caseinase as an active MMP. These results show that SL1 may be involved in the regulation of expression of MMPs. Unactivated conditioned medium from TCL1 cells did not show a 49-kDa band (Fig. 4b), suggesting that these cells do not secrete significant amounts of autoactivating rat SL1 transgene.

Matrigel Up-regulates Expression of SL1 in Tumorigenic Cells but Down-regulates It in Nontumorigenic Cells

SL1 mRNA was found in the functionally normal SCp2 cells, as well as in SCg6 and TCL1 carcinoma cells by Northern blot hybridization (Fig. 5a). Expression of SL1 by SCp2 cells was observed only when cells were cultured on tissue culture plastic and was suppressed when cells were plated on Matrigel. Down-regulation of SL1 expression in response to Matrigel was also observed with primary mammary epithelial cells (data not shown). In contrast, TCL1 and SCg6 cells contained high levels of SL1 mRNA only when they were in contact with Matrigel, but not with tissue culture plastic. By RT-PCR, however, SL1 mRNA was also detectable in SCg6 cells maintained on plastic but not in SCp2 cells maintained on Matrigel (data not shown). Since the probe used for Northern blots did not discriminate between mouse and rat SL1, we analyzed expression of endogenous mouse SL1 and rat SL1 transgene in TCL1 cells by RT-PCR with primers specific for mouse and rat SL1 (Fig. 5b). Whereas mouse SL1 was readily amplified after 25 PCR cycles, only a weak PCR product was observed with rat SL1-specific primers after 31 cycles. This was expected because the transgene was constructed with a whey acidic protein (WAP) promoter (3), which is down-regulated in culture (29, 30). Since, in addition, an active SL1 was not identified in TCL1 cells with zymograms or immunoblots (Fig. 4, a and b), as would be expected if the autoactivating rat SL1 transgene was produced, we conclude that
expression of the rat SL1 transgene in TCL1 cells is not necessary for SL1-dependent invasion in culture. Interestingly, primary fibroblasts from the mammary gland, analogous to TCL1 and SCg6 cells, which have aspects of a mesenchymal phenotype, synthesized more SL1 in the presence of Matrigel (data not shown).

In contrast to the dramatic and contrasting regulation of SL1 expression by Matrigel of SCp2 versus SCg6 and TCL1 cells, SL3, MT-MMP, matrilysin, TIMP1, and TIMP3 mRNAs, which were also expressed by all three cell types, were little, if at all, affected by the presence of Matrigel (Fig. 5a and data not shown). Gelatinases A and B were found only in SCg6 cells by both Northern blot hybridization (Fig. 5a) and RT-PCR (data not shown) and were up-regulated by Matrigel. Collagenase-3 was found in TCL1 cells but not in SCp2 or SCg6 cells by RT-PCR (Fig. 5b), where its expression appeared to be increased on Matrigel. SL2 was expressed by SCg6 and TCL1 cells but not by SCp2 cells and, in contrast to SL1, was downregulated by Matrigel (Fig. 5b). TIMP2 was expressed by all three cell types and not regulated by the presence of Matrigel (Fig. 5b).

To determine the specific ECM constituent that regulates SL1 and to rule out the influence of the various growth factors contained in Matrigel (31), we examined the role of purified ECM components in the regulation of SL1 expression. SCg6 cells were maintained on plastic substrata and treated with laminin-entactin, type IV collagen, fibronectin, or heparin, which were added to the culture medium. Expression of SL1 mRNA and its secreted protein was increased by laminin-entactin and heparin (Fig. 6). Type IV collagen and fibronectin had no significant effect on the synthesis of SL1 protein (Fig. 6, b and c). Similar results were obtained with TCL1 cells (data not shown).

In contrast to the up-regulation of SL1 in TCL1 and SCg6 cells, SL1 mRNA levels in SCp2 cells were slightly reduced in the presence of laminin-entactin and heparin (Fig. 6a). SL1 could not be detected with zymograms from conditioned medium of SCp2 cells maintained under any of the conditions mentioned above (data not shown). While the general pattern of expression was similar, the complete inhibition of SL1 mRNA expression in SCp2 cells seen on Matrigel (Fig. 5) could not be reproduced with isolated ECM constituents and might thus be the result of synergistic cooperation of several ECM components and a need for three-dimensional structure analogous to the conditions described for WAP expression and for the regulation of apoptosis in culture (4, 29, 30).

Because antisense inhibition of SL1 expression in tumor cells led to a significant decrease in Matrigel invasion, we predicted that an increase of SL1 synthesis and secretion by other means should also lead to an increase in invasion. We found that SL1 secretion could be augmented by omission of hydrocortisone from the culture medium (Fig. 7a and data not shown for TCL1 cells), as could be predicted from our previous observations that hydrocortisone suppresses SL1 expression in the mammary gland in vivo (21). SL1 secretion by SCg6 cells on Matrigel was also increased when cells were exposed to heparin, but was unaffected by laminin-entactin, type IV collagen, or fibronectin (Fig. 7a). Invasion of Matrigel was approximately doubled both in the presence of heparin and in the absence of hydrocortisone (Fig. 7b). In both cases, Matrigel invasion could be largely blocked by SL1 antisense ODNs, but not by SL1 sense or SL3
antisense ODNs (Fig. 7b). These findings are consistent with the importance of SL1 for invasion of reconstituted basement membranes.

**SL1 Antisense ODNs Inhibit Collagen Invasion Only Partially**

Because the molecular composition of basement membranes and interstitial stroma are fundamentally different, and SL1 is most effective in the degradation of basement membrane constituents but only weakly attacks stromal collagens (32, 33), we asked whether SL1 might also play a role in invasion of stromal ECMs. To mimic this event in culture, we seeded SCp2, TCL1, and SCg6 cells onto type I collagen gels. TCL1 and SCg6 cells invaded collagen with similar efficiency (Fig. 8), whereas SCp2 cells were again noninvasive (data not shown). Invasion of collagen for both cell types was inhibited by 50% by inhibitors of MMP activity, but not by aprotinin, leupeptin, or pepstatin (Fig. 8a). When invasion assays were performed in the presence of ODNs, invasion of collagen by TCL1 and SCg6 cells was reduced again by half by SL1 antisense ODNs (Fig. 8b). In addition, collagen invasion by TCL1 cells was reduced by half in the presence of collagenase-3 antisense ODNs, whereas invasion of SCg6 cells, which do not express collagenase-3 (Fig. 5), remained unaffected (Fig. 8b). Neither SL1 sense nor SL3 antisense ODNs affected collagen invasion by either cell type (Fig. 8b).

**Discussion**

Migration of cells through reconstituted basement membranes and type I collagen has been widely used as a model to study the invasive potential of tumor cells and the underlying molecular mechanisms. This rationale has led to the identification of a growing number of intracellular and extracellular factors that influence invasion either positively or negatively (1, 2). Although the emerging information is complex, it appears that the synthesis of MMPs by tumor cells and the surrounding stroma plays a pivotal role in tumor invasion. MMPs are involved in the destruction of the ECM and thus the removal of signals that hinder growth (34) and the physical barriers that impair invasion of tumor cells. Other, more indirect mechanisms of MMP action, such as exposure of cryptic signaling sites within ECM molecules and release or activation of growth factors (35), may also be operating.

Our results with TCL1 and SCg6 mouse mammary carcinoma cells provide evidence for an important role of SL1 in invasion of reconstituted basement membranes. Whereas inhibitors of serine, cysteine, and aspartic proteinases and antisense ODNs to SL3 and collagenase-3 did not affect Matrigel invasion, MMP inhibitors and SL1 antisense ODNs impaired invasion by 80% or more. There are at least three potential mechanisms by which SL1 could promote Matrigel invasion. First, it could act directly by proteolysis of ECM constituents, because it attacks almost all known ECM molecules (1). Second, SL1 could activate other MMPs, e.g. collagenase-3 (36), gelatinases A (37) and B (38), and matrilysin (39). Third, SL1 can process the tumor necrosis factor-α precursor (40), raising the possibility that SL1 has additional functions. These include the activation of growth factors and cytokines that in turn might alter the expression of genes relevant for invasion. In particular, the fact that one out of three non-SL1 caseinases decreased significantly upon exposure of the cells to SL1 antisense ODNs raises the interesting possibility that SL1 may positively regulate expression of other proteinases by
interfering with autocrine and/or paracrine signaling pathways. This notion is also supported by the finding that SL1-overexpressing transgenic animals exhibited elevated levels of endogenous SL1 and a number of other MMPs. Similarly, when mouse mammary epithelial cells were transfected with SL1 expression constructs, expression of a number of ECM-degrading enzymes was increased concomitant with the increase in SL1 expression. Although the detailed mechanism of SL1 action remains to be elucidated, its activity appears to be an overriding regulator of basement membrane penetration, because its inhibition results in dramatic inhibition of Matrigel invasion. We speculate that the combined effect of SL1 on degradation of ECM, proteolytic activation of other MMPs, and regulation of expression of other ECM-degrading proteinases may be responsible for its central role in invasion of basement membrane-like structures.

Recently, SL1 was detected in vivo in carcinoma cells of mammary tumors formed in WAP-ras, but not in WAP-myc, transgenic mice (41). The association of carcinoma cells with SL1 in WAP-ras transgenic mouse tumors that are undifferentiated and metastasizing, but not with tumors of WAP-myc transgenic mice that are differentiated and nonmetastasizing, supported previous findings (8, 9) that the level of SL1 expression may be correlated with malignant progression and, possibly, invasion. In addition, the same tumor regions that exhibited stromal invasion showed SL1-positive carcinoma cells, whereas in noninvasive areas, SL1 was found only in myoepithelial cells (41). In the same study, SL1 expression was found in invasive ductal carcinomas of the human breast. Thus, the dependence of TCL1 and SCg6 cells on SL1 for invasion in culture may now provide a direct proof for its involvement in gaining an invasive phenotype in vivo.

Expression of SL1 by TCL1 and SCg6 cells was strongly up-regulated by Matrigel, and by addition of laminin-entactin, the major basement membrane constituent. Heparin was also able to increase SL1 expression, suggesting that basement membrane heparan sulfate proteoglycans may participate in modulating SL1 expression in tumor cells. On the other hand, SCp2 cells stopped expressing SL1 when plated on Matrigel. The fact that SCp2 cells, and also primary mammary epithelial cells, lose expression of SL1 when in contact with reconstituted basement membrane components is consistent with the finding that, in disease-free, noninvoluting glands, where all epithelial cells are surrounded by an intact basement membrane, SL1 is not expressed by mammary epithelia (42). The complete loss of SL1 expression in SCp2 cells and the pronounced up-regulation of its expression in TCL1 and SCg6 cells in response to Matrigel suggest that misregulation of SL1 expression is a key event in malignant progression of mammary tumors. In fact, the pattern of SL1 expression in TCL1 and SCg6 cells resembles that of fibroblasts, which also increase SL1 expression in response to Matrigel and laminin-entactin. In this context, it appears that TCL1 and SCg6 cells, which express vimentin, very little keratin, and no E-cadherin, have acquired the property to up-regulate SL1 expression in response to ECM as part of the phenotypic changes associated with epithelial-mesenchymal transdifferentiation which in turn renders cells migratory and invasive (25). The differences between SCp2 and primary mammary epithelial cells, on the one hand, and TCL1 and SCg6 cells, on the other hand, in the expression of epithelial and mesenchymal marker proteins and the accompanying relationship between SL1 expression and invasion are reminiscent of the transition from squamous skin carcinomas to invasive spindle cell carcinomas, where SL1 expression is correlated with loss of epithelial characteristics (9).
MMPs other than SL1 that were positively affected by the presence of Matrigel were gelatinases A and B and collagenase-3. However, assuming similar modes of invasion for SCg6 and TCL1 cells, they are unlikely to be rate-limiting in mediating Matrigel invasion, because they were only found in either SCg6 or TCL1 cells. The other MMPs studied here were either not affected or down-regulated by Matrigel. The MMP inhibitors TIMP1, TIMP2, and TIMP3 were expressed by all cell types and subject to no or only moderate regulation by Matrigel.

The results on invasion of collagen gels by mammary tumor cells imply that MMP-independent mechanisms are important for stromal invasion. That SL1 antisense ODNs inhibited collagen invasion to the same extent as MMP inhibitors is remarkable, because native type I collagen is only a poor substrate for SL1 in vitro (32, 33). However, as in the case of Matrigel invasion discussed above, SL1 is likely to activate other MMPs that are more efficient in degrading interstitial collagens. Indeed, this mechanism might be operating in TCL1 cells, because collagen invasion was also reduced by half in the presence of collagenase-3 antisense ODNs and, furthermore, treatments that increased collagenase-3 expression promoted collagen invasion, whereas further up-regulation of SL1 synthesis did not (data not shown).

The stimulation of tumor growth and invasion in vivo by the injection of Matrigel or laminin has been attributed to increased angiogenesis (43). We propose that Matrigel itself and endogenous basement membrane components in situ may also stimulate tumor expansion by promoting expression of SL1 and thus subsequent basement membrane dissolution and stromal invasion. In this context, the misregulation of SL1 expression in tumor cells by basement membrane constituents might constitute an instructive switch for acquisition of invasive properties and, therefore, a powerful target for therapeutic intervention.

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Histology of tumors formed by TCL1 and SCg6 cells. Hematoxylin/eosin-stained paraffin sections of primary tumors (panels a and b), invaded skin (panel c) and lung metastasis (panel d) generated by TCL1 (panels a and c) and SCg6 (panels b and d) cells injected into the mammary gland of nude mice. The spindle cell tumors (T), entrapped adipose tissue (asterisks) and epithelial ducts (arrowheads) in the mammary gland are indicated.
Immunostaining of SCp2, TCL1, and SCg6 cells for E-cadherin, cytokeratin, and vimentin. Cells were maintained for 2 days on glass coverslips, fixed with 5% formalin, and processed for indirect immunofluorescence with antibodies against E-cadherin (E-cad), cytokeratins (keratin), and vimentin, followed by Texas Red-conjugated anti-mouse (for E-cadherin and vimentin) or anti-rabbit (for keratin) antibodies.
Invasion of Matrigel by SCp2, TCL1, and SCg6 cells and inhibition by proteinase inhibitors and antisense ODNs. SCp2, TCL1, and SCg6 cells were plated on Matrigel in modified Boyden chambers. After fixation with 5% glutaraldehyde and removal of cells that had not migrated, the number of cells that had migrated through Matrigel after 18 h of culture was quantified by counting the number of toluidine blue-stained cells per visual field. Panel a, comparison of invasion by SCp2, TCL1 and SCg6 cells. Panel b, TCL1 cells (black bars) and SCg6 cells (white bars) were maintained in the absence of proteinase inhibitors (control) or with GM6001 (10 µM), aprotinin (0.3 µM), leupeptin (1 µM), or pepstatin (1 µM). Panel c, SCg6 cells were maintained in the presence of GM6001 (white circles) or GM1210 (black circles) at increasing concentrations given in µM. Panel d, TCL1 cells (black bars) and SCg6 cells (white bars) were maintained in the absence of ODNs (control) or in the presence of SL1 antisense (3.2 µM, SL1 AS) or sense (3.2 µM, SL1 S) ODNs, SL3 antisense ODNs (8 µM, SL3 AS), or collagenase-3 antisense ODNs (32 µM, C-3 AS) ODNs. Results were normalized with control values set to 100 in b–d. Error bars indicate standard deviations from three experiments.
Effect of antisense ODNs to SL1 on expression of SL1 and other caseinases. Panel a, immunoblot with SL1-specific antibodies with conditioned medium of SCp2 (p2), TCL1 (T), and SCg6 (g6) cells maintained for 2 days in culture in the presence of 3.2 µM SL1 sense (S) or antisense (AS) ODNs. Arrowhead indicates position of the 57-kDa SL1 band. Panels b and c, casein zymograms run on conditioned medium from TCL1 (a) and SCg6 (b) cells maintained for 2 days in culture in the absence (2) or presence of SL1 antisense (3.2 µM, 1 AS), SL1 sense (3.2 µM, 1 S), or SL3 antisense (8 µM, 3 AS) ODNs. Caseinases migrating at 53, 57 (SL1), 65, and 80 kDa are indicated by arrowheads. Panel d, densitometric analysis of caseinolytic activity in cleared zones from zymograms of TCL1 and SCg6 cells maintained for 2 days in the absence of ODNs (control, black bars) or in the presence of antisense (white bars) or sense (dotted bars) ODNs to SL1 or antisense ODNs to SL3 (hatched bars). Error bars indicate standard deviations from three independent experiments. Asterisks indicate significant differences (p \leq 0.05) as compared to the control treatment. Panel e, autoradiogram of radiolabeled proteins from conditioned medium from SCg6 (lanes 1–3) and TCL1 (lanes 4–6) cells cultured in the absence of ODNs (lanes 1 and 4) or in the presence of SL1 antisense (lanes 2 and 5) or sense (lanes 3 and 6) ODNs separated on SDS-polyacrylamide gels. The positions of molecular size markers of 43, 68, 97.4, and 200 kDa are indicated. Panel f, the profile of secreted caseinases was analyzed by incubation of conditioned medium from SCg6 cells with 4-aminophenylmethylmercuric acetate (1 mM, A) or by incubation of the substrate gel in the presence of 1,10-phenanthroline (1 mM, P) in comparison to untreated zymograms (C). Caseinases migrating at 46, 49, 53, 57, 65, and 80 kDa are indicated. Negative images of zymograms are shown in b, c, and e.
Regulation of MMP expression in TCL1 and SCg6 cells by Matrigel. SCp2, SCg6, and TCL1 cells were maintained for 2 days on tissue culture plastic (P) or Matrigel (M), and the amount of mRNA produced was analyzed by Northern blot hybridization for SL1, SL3, matrilysin (ML), MT-MMP, gelatinases A (GA) and B (GB), and TIMP1 and TIMP3 (panel a) or by Southern analysis of RT-PCR products to discriminate between mouse SL1 (mSL1) and rat SL1 (rSL1) in TCL1 cells, and for SL2, collagenase-3 (C-3) and TIMP2 (panel b). Hybridization with a probe for 28 S ribosomal RNA for standardization of Northern blots is shown in panel a. The Northern blot for SL1 was reprobed for SL3 and subsequently hybridized for detection of 28 S RNA (28S - SL1,SL3). Similarly, MTMMP was detected on a blot first used for detection of matrilysin, TIMP1 on a blot first used for detection of gelatinase A, and TIMP3 on a blot first used for detection of gelatinase B. 19, 22, 25, 28, and 31 cycles of amplification were performed for each PCR reaction shown in panel b.
Regulation of MMP expression in SCp2 and SCg6 cells by basement membrane constituents. SCp2 and SCg6 cells were maintained for 2 days on plastic substrata in the absence (control) or presence of type IV collagen (col IV), laminin-entactin (LN-ENT), heparin, or fibronectin (FN). Panel a, RT-PCR and Southern analysis of SL1 and TIMP3 expression. 25, 30, and 35 cycles of amplification were performed for detection of SL1 in SCp2 cells and 21, 24, and 27 rounds of amplification for remaining samples. Panel b, negative image of casein zymogram of conditioned medium from SCg6 cells grown for 2 days on Matrigel or on plastic in the absence of ECM (control) or presence of laminin-entactin, type IV collagen, heparin, or fibronectin. The arrowhead indicates the position of latent SL1. Panel c, densitometric analysis of caseinolytic activity in cleared zones from zymograms of SCg6 cells maintained under the same conditions as in b. Error bars indicate standard deviations from three independent experiments. Asterisks indicate significant differences ($p < 0.05$) as compared to the control treatment.
**FIGURE 7**

Heparin and hydrocortisone effects on SL1 expression and Matrigel invasion. Panel a, negative image of casein zymogram of SCg6 cells maintained for 2 days on Matrigel and either left untreated (control) or treated with laminin-entactin (LN-ENT), heparin, type IV collagen (col IV), fibronectin (FN), or maintained without hydrocortisone (w/o HC). Arrowhead indicates migration of latent SL1. Panel b, TCL1 cells (black bars) and SCg6 cells (white bars) were plated on Matrigel, and the number of cells that had migrated was quantified as described for Fig. 3. Cells were maintained in standard medium (-), in the presence of heparin (+ heparin), or in the absence of hydrocortisone (w/o HC) either in the absence of ODNs (- ODN) or in the presence of SL1 antisense (SL1 AS) or sense (SL1 S) ODNs or SL3 antisense ODNs (SL3 AS). Results were normalized with control values for TCL1 and SCg6 cells in the absence of ODNs set to 100. Error bars indicate standard deviations from three experiments.
**FIGURE 8**

Effect of proteinase inhibitors and antisense ODNs on collagen invasion by TCL1 and SCg6 cells. TCL1 cells (black bars) and SCg6 cells (white bars) were plated on type I collagen gels, and the number of cells that had migrated was determined as described for Fig. 3. Cells were maintained in the absence of any treatment (controls in panels a and b) or in the presence of proteinase inhibitors GM6001, aprotinin, leupeptin or pepstatin, or in the presence of SL1 antisense (SL1 AS) or sense (SL1 S) ODNs, SL3 antisense ODNs (SL3 AS), or collagenase-3 antisense ODNs (C-3 AS). Error bars indicate standard deviations from three experiments.