Invasive Properties of Murine Squamous Carcinoma Cells: Secretion of Matrix-degrading Cathepsins Is Attributable to a Deficiency in the Mannose 6-Phosphate/Insulin-like Growth Factor II Receptor

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ABSTRACT

Penetration of basement membrane layers is a hallmark feature of metastatic tumor cells. The invasive propensity of murine SCC-VII squamous carcinoma cells is in part attributable to the extracellular action of the lysosomal cysteine proteinase cathepsin B. Although most noncancerous cells store this enzyme in the lysosomes, we found that SCC-VII cells release a large fraction (42%) of their newly synthesized procathepsin B into the culture medium. Procathepsins D and L, the precursors of other major lysosomal proteinases, are also secreted in significant amounts (24 and 75%, respectively). In contrast, normal mouse 3T3-L1 fibroblasts express only minor amounts of their newly synthesized procathepsins B (10%), D (<1%), and L (16%). Western blotting analysis revealed that SCC-VII cells are deficient in the 300 kDa mannose 6-phosphate/insulin-like growth factor-II receptor (M6P/IGF2R), a tumor suppressor with a central role in the intracellular transport of lysosomal enzymes. Consistent with the absence of M6P/IGF2R, SCC-VII cells lack dense lysosomes, with the bulk of intracellular acid hydrolases residing in late endosomes/prefusomes. On the other hand, the synthesis of the M6P recognition marker on lysosomal enzymes is not impaired in SCC-VII cells, because [33P]Pi is incorporated into the carbohydrate moieties of procathepsins B, D, and L. Furthermore, 69% of the phosphorylated N-linked oligosaccharides synthesized by SCC-VII cells carry phosphomonoester groups and as such constitute high-affinity ligands for M6P receptors. SCC-VII cells express the 46 kDa cation-dependent M6P receptor (MPR46), but intracellular retention of procathepsins B, D, and L is not affected by ammonium chloride and chloroquine, agents known to perturb the M6P receptor system. Our results indicate that failure to express the 300 kDa M6P/IGF2R may enhance the metastatic capacity of tumor cells by inducing the secretion of procathepsins B, D, and L.

INTRODUCTION

Malignant cancer cells display a distinct capacity to degrade extracellular matrix components, a feature indispensable for penetration of basement membranes and subsequent spreading to distant sites. Various proteinases have been implicated in tumor invasion and metastasis, including the lysosomal enzymes cathepsins B, D, and L (1–3). In most noncancerous cells, the subcellular location of these cathepsins is restricted to lysosomes. However, the proteinases are frequently redistributed in tumor cells to peripheral vesicles, promoting exocytosis of the enzymes (4). In addition, tumor cells secrete precursor forms of cathepsins B, D, and L into the pericellular fluid, because of leakage in the biosynthetic transport of the latent proenzymes to lysosomes. For cathepsin L, secretion was attributed to the intrinsic low affinity of the proteinase to its sorting receptors (5). Once released, self-activation of the individual proenzymes may occur (6). Intracellular trafficking of cathepsins to lysosomes depends on the presence of M6P in the carbohydrate moieties of the proteinases. These residues mediate binding to specific M6P receptors (7). Mutant cells with either impaired synthesis of the M6P recognition marker or a M6P receptor deficiency fail to retain their newly synthesized lysosomal hydrolases (8). Two distinct M6P-binding proteins occur in mammalian cells, the 300 kDa M6P/IGF2R and the 46 kDa cation-dependent MPR46. Besides representing the main targeting receptor for lysosomal enzymes, M6P/IGF2R binds IGF-II and TGF-β receptor (9). M6P/IGF2R participates in the degradation of IGF-II, a potent growth stimulant and mitogen that is often overproduced in tumors (9, 10). Furthermore, the receptor facilitates activation of latent TGF-β, a growth-suppressing cytokine (11). Because of this dual role in the control of cellular growth, M6P/IGF2R is considered a tumor suppressor (10). Indeed, the M6P/IGF2R gene is frequently mutated in liver, breast, and gastrointestinal cancers (12–14). Recently, direct evidence for a growth-suppressive role of M6P/IGF2R in tumors has been provided (15). However, the impact of M6P/IGF2R on tumor invasion and metastasis has not yet been investigated.

We have shown previously that the invasive properties of murine SCC-VII squamous carcinoma cells are in part attributable to the extracellular action of the major lysosomal cysteine proteinase cathepsin B (16). In this report, we demonstrate that SCC-VII cells are deficient in M6P/IGF2R, causing hypersecretion of cathepsin B and other lysosomal proteinases. Our results indicate that loss of M6P/IGF2R may promote the invasiveness of malignant tumor cells.

MATERIALS AND METHODS

Reagents. Tran35S]label metabolic labeling reagent (>1000 Ci/mmol) was obtained from ICN Pharmaceuticals (Costa Mesa, CA). 14HMannose (10–20 Ci/mmol), [33P]Pi, (>2500 Ci/mmol), UDP-[3H]galactose (5–20 Ci/mmol), unlabeled and 4C-methylated molecular mass standards, Amplify fluorographic reagent, Percoll, protein A-Sepharose 4B, Sephadex G-10, and Enhanced Chemiluminescence Western blotting reagents were obtained from Amersham Pharmacia Biotech (Rainham, United Kingdom). Endoglu-cosaminidase H and peptide N-acetylglucosamine were obtained from Boehringer Mannheim (Mannheim, Germany). Calf intestinal alkaline phosphatase, chloroquine, HRP (type VI-A), 4-nitrophenyl-β-N-acetylglucosaminide, 4-nitrophenyl phosphate, 3,3’,5,5’-tetramethylbenzidine, diethyl(2-hydroxypropyl)aminoethyl-Sephadex, leupeptin, E-64, sodium β-glycerophosphate, BSA (fraction V), and chicken egg ovalbumin (grade V) were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade.

Antibodies. The production and characterization of a rabbit antiserum against purified human liver cathepsin B has been reported previously (17). The antiserum has been shown to cross-react with all forms of the murine enzyme (16). Rabbit antiserum against rat M6P/IGF2R was generously supplied by Dr. Thomas Braulke (Georg-August University Göttingen, Germany).

Received 11/2/99; accepted 5/30/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported in part by Australian Research Council Grants 04/15/412/170, 04/15/412/284, and 04/15/412/343 (to L. M.).

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5 The abbreviations used are: M6P, mannose 6-phosphate; IGF-II, insulin-like growth factor II; IGF2R, IGF-II receptor; HRP, horseradish peroxidase; MPR46, 46-kDa cation-dependent M6P receptor; TGF, transforming growth factor; αPAR, urokinase-type plasminogen activator receptor.
Dr. Braulke also provided an anti-peptide antisera against the COOH terminus of murine MPR46, with the kind permission of Dr. Annette Hille-Rehfeld from the same department. Antisera against mouse cathepsins D and L were donated by Dr. John S. Mort (Shiriner’s Hospital for Crippled Children, Montreal, Quebec, Canada) and Dr. Ann H. Erickson (University of North Carolina, Durham, NC), respectively.

**Cell Culture.** Murine SCC-VII squamous carcinoma cells were propagated in Minimal Essential Medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37°C as described (16). Human HepG2 hepatoma cells and normal human GM5522 skin fibroblasts were obtained from the American Type Culture Collection (Rockville, MD) and the Human Genetic Mutant Cell Repository (Camden, NJ), respectively. Both cell lines were cultured as outlined above. Murine 3T3-L1 fibroblasts, supplied by Dr. David James (University of Queensland, Brisbane, Queensland, Australia), were maintained in DMEM supplemented with 10% bovine calf serum, 2 mM glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37°C. Murine NIH 3T3 fibroblasts were obtained from the American Type Culture Collection and propagated in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37°C. All tissue culture reagents were purchased from Life Technologies, Inc. (Gaithersburg, MD).

**Metabolic Labeling and Immunoprecipitation.** Confluent cell monolayers were metabolically labeled for 1 h with [35S]methionine (100 μCi/ml) or [35S]PPi (250 μCi/ml) and were subsequently chased for 4 h as described (17). When added, NH4Cl (10 mM) and chloroquine (30 μM) were present throughout the entire experimental procedure. Immunoprecipitation of antigens from labeled cell and medium extracts, followed by SDS-PAGE and fluorography, was performed as reported (17, 18). To determine the amount of label incorporated into individual polypeptides, the corresponding gel areas were excised from the membrane pellets were extracted with 20 mM sodium phosphate buffer (pH 7.4), 150 mM NaCl, 0.5% (w/v) Triton X-100, 1 μg/ml leupeptin, and 1 μg/ml E-64 for 30 min at 4°C prior to releasing. Soluble fractions and membrane extracts (100 μg of protein each) were separated by SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane (Hybond-C; Amer-sham Pharmacia Biotech) as reported (18). The membrane was probed with rabbit antibodies to the cytoplasmic domain of murine MPR46. Bound immunoglobulins were detected with HRP-conjugated goat anti-rabbit IgG immunoglobulins (Accurate, Westbury, NY) and Enhanced Chemiluminescence Western blotting reagents (16).

**Analysis of Phosphorylated Oligosaccharides.** SCC-VII cells (8 × 10⁶) were metabolically labeled for 8 h with [3H]mannose (1 mCi/ml) in 5 ml of glucose-poor culture medium (1 mM glucose) containing 10 mM NH4Cl. Medium proteins were collected by precipitation with 0.5 g/ml ammonium sulfate, redissolved in 2 ml of 20 mM sodium acetate buffer (pH 5.5), 5 mM sodium β-glycerophosphate, and dialyzed against the same buffer. The reten- tate was concentrated by ultrafiltration (10 kDa cutoff) and incubated with 5 milligrams of endogulosaminidase H as described (5). H-released oligosacchari- des thus released were isolated by ultrafiltration, desalted on a 1.5 × 7-cm column of Sephadex G-10 eluted with water, and finally fractionated on diethyl(2-hydroxypropyl)aminoethyl-Sephadex according to (5). To remove phosphomonoester groups, H-labeled oligosaccharides were treated with 1 unit of alkaline phosphatase in 0.1 mM Tris/HCl buffer (pH 8.0) for 1 h at 37°C. Phosphodiester linkages were cleaved by incubation in 2 mM acetic acid for 2 h at 80°C.

**Immunofluorescence Staining.** SCC-VII cells and NIH 3T3 fibroblasts grown on glass coverslips were fixed by incubation for 10 min in 4% paraformaldehyde in PBS. After blocking with PBS containing 2 mg/ml BSA for 1 h, the cells were incubated for 1 h with rabbit anti-mouse cathepsin D antiserum (diluted 1:50 in PBS containing 0.1% saponin). Nonimmune rabbit serum was used as a negative control. After a second blocking step in PBS containing 0.1% saponin and 5% fetal bovine serum (1 h), bound primary antibodies were detected by incubation for 1 h with fluorescein-conjugated, affinity-purified goat anti-rabbit immunoglobulin antibodies (Sigma) at a con- centration of 30 μg/ml in PBS containing 0.1% saponin. All steps were performed at room temperature. The immunostained cells were examined using a Zeiss Axiosvert 35 microscope with the appropriate filter combination.

**RESULTS**

**Biosynthesis of Cathepsin B in SCC-VII Cells and 3T3-L1 Fibroblasts.** To follow the biosynthesis of cathepsin B, SCC-VII cells and control mouse 3T3-L1 fibroblasts were metabolically labeled with [35S]methionine. Upon immunoprecipitation with an anti- serum to the purified enzyme, a protein of 42 kDa was detected in both cell types, which corresponds to cathepsin B, the latent precursor of the proteinase (not shown). When 3T3-L1 cells were chased, this precursor was found to be rapidly converted into mature single-chain cathepsin B (32 kDa), indicating delivery of the enzyme to lysosomes. In contrast to human cells (17, 18), the two-chain form of the proteinase was not produced, even after prolonged chase periods (not shown). Under normal conditions, only a small fraction (10%) of the newly synthesized proenzyme was secreted into the culture medium. Procathepsin B release was significantly stimulated by the presence of 10 mM NH4Cl (22%), albeit weaker than in human GM5522 fibroblasts (>60%; not shown). Similar results were obtained with 30 μM chloroquine (not shown). Both lysosomotropic bases are known to raise the pH in the lumen of endosomes and lysosomes, which perturbs M6P receptor-mediated trafficking of acid hydrodolases to these compartments. Furthermore, NH4Cl and chloro- quine inhibited procathepsin B processing in 3T3-L1 cells, which also requires an acidic environment (Fig. 1A).

In contrast to 3T3-L1 fibroblasts, SCC-VII cells released a substan- tial fraction (42%) of newly produced procathepsin B into the culture medium. The fraction (58%) retained inside the cells was processed to

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the mature enzyme (Fig. 1A). The heterogeneity of mature SCC-VII cathepsin B (32–36 kDa) was found to be attributable to differences in glycosylation, because removal of N-linked oligosaccharides by treatment with peptide N-glycosidase F yielded a discrete 31 kDa band (Fig. 1B). Interestingly, procathepsin B secretion by SCC-VII cells was not significantly enhanced by NH4 Cl (44%). Similar results were obtained with chloroquine (not shown). However, procathepsin B maturation was incomplete (86%) when SCC-VII cells were treated with either base, indicating that endosomal/lysosomal alkalinization had indeed occurred. Therefore, the residual retention of cathepsin B by SCC-VII cells is apparently mediated by a pH-insensitive mechanism that differs from the classic M6P receptor pathway.

Biosynthesis of Cathepsin L in SCC-VII Cells and 3T3-L1 Fibroblasts. The most prominent mammalian lysosomal cysteine proteinase besides cathepsin B is cathepsin L. In 3T3-L1 cells, only 16% of newly synthesized procathepsin L (37 kDa) was secreted into the culture medium, with the remainder being intracellularly retained. Unlike cathepsin B, the retained procathepsin L was processed in these cells via a single-chain intermediate (29 kDa) into the mature double-chain enzyme, as indicated by the appearance of its heavy chain (20 kDa). NH4Cl substantially stimulated procathepsin L secretion (62%). As for cathepsin B, residual intracellular procathepsin L did not undergo proteolytic maturation in the presence of the base (Fig. 2A).

In contrast, SCC-VII cells secreted 75% of their newly made procathepsin L into the culture medium. Any proenzyme retained by the cells was rapidly processed via the single-chain intermediate (29 kDa) into mature two-chain cathepsin L. The presence of NH4Cl did not significantly enhance procathepsin L release (78%). However, NH4Cl did inhibit the formation of the mature forms of the proteinase to 69%, which confirms that NH4Cl is effective in elevating the pH of lysosomes in SCC-VII cells (Fig. 2A).

Our results demonstrate that SCC-VII cells and 3T3-L1 fibroblasts...
sort procathepsin L less efficiently than procathepsin B to lysosomes. This discrepancy may be attributable to the intrinsic low affinity of procathepsin L for M6P receptors (5) or indicate the involvement of distinct M6P-independent sorting receptors, with preference for cathepsin B over cathepsin L.

**Biosynthesis of Cathepsin D in SCC-VII Cells and 3T3-L1 Fibroblasts.** Cathepsin D, the major aspartic proteinase in mammalian lysosomes, was found to be initially synthesized by SCC-VII cells and 3T3-L1 fibroblasts as a latent 46 kDa precursor. 3T3-L1 cells released <1% of newly made procathepsin D into the medium. All proenzymes retained by the cells were converted to mature single-chain cathepsin D (44 kDa). Treatment with NH₄Cl induced secretion of the latent proenzyme to some extent (21%), but most cathepsin D remained inside the cells even in the presence of the lysosomotropic agent. In contrast to cathepsins B and L, proteolytic maturation of intracellular procathepsin D in these cells was not significantly affected by NH₄Cl (Fig. 2B).

SCC-VII cells secreted substantial amounts (24%) of their newly synthesized procathepsin D. Any intracellularly retained proenzyme was processed into the mature single-chain form of the proteinase. Procathepsin D secretion was only slightly stimulated by NH₄Cl (33%). As in 3T3-L1 cells, NH₄Cl did not inhibit intracellular procathepsin D processing (Fig. 2B).

Our results indicate that SCC-VII cells and 3T3-L1 fibroblasts preferentially retain cathepsin D as compared with cathepsins B or L. This finding is consistent with an alternative, M6P-independent lysosomal targeting mechanism for cathepsin D, as it has been reported for human breast cancer cells (23).

**Phosphorylation of Lysosomal Enzymes in SCC-VII Cells.** Because SCC-VII cells hypersecrete, in addition to cathepsins B, D, and L, also the lysosomal marker β-N-acetylhexosaminidase (not shown), we investigated whether the cells are capable of forming M6P residues on lysosomal enzymes. Secreted procathepsin B (42 kDa) was immunoprecipitated and then treated with endogluco- and oligosaccharides (5). Treatment with endoglucosaminidase H resulted in the appearance of two novel polypeptides of 40 and 38 kDa reflecting the loss of one and two N-glycans, respectively. Complete deglycosylation of procathepsin B with peptide N-glycosidase F reduced the apparent molecular mass of the protein to 36 kDa, consistent with the removal of three N-linked oligosaccharide side chains. Similar results were obtained for procathepsin B synthesized in the presence of NH₄Cl. These data indicate that one or two of the three carbohydrate side chains of procathepsin B contain M6P residues (Fig. 3A).

To directly establish phosphorylation of lysosomal enzymes, SCC-VII cells and 3T3-L1 fibroblasts were metabolically labeled with [33P]orthophosphate in the presence of NH₄Cl (1) and SCC-VII cells (2) were metabolically labeled for 1 h with 100 µCi/ml [3H]mannose. [3H]-labeled glycoproteins secreted into the culture medium were immunoprecipitated and treated with endogluco- and oligosaccharides containing either one (9.8%) or two (4.2%) phosphodiesters, or either one (5.4%) or two (20.1%) phosphomonoesters. The identity and composition of each fraction were verified by milder acid treatment (which converts phosphodiester linkages into phosphomonoester linkages) and alkaline phosphatase.

**Fig. 3. Phosphorylation of lysosomal proteinases in SCC-VII cells.** A, SCC-VII cells were metabolically labeled with [33P]orthophosphate in the absence (−) or continuous presence (+) of NH₄Cl as described in the legend of Fig. 1. Procathepsin B was immunoprecipitated and then treated with endogluco- and oligosaccharides as outlined in Materials and Methods. B, 3T3-L1 fibroblasts (1) and SCC-VII cells (2) were metabolically labeled for 1 h with 100 µCi/ml [3H]mannose and subsequently chased for 4 h in the continuous presence of NH₄Cl as outlined in Materials and Methods. C, 3T3-L1 fibroblasts (1) and SCC-VII cells (2) were immunoprecipitated and treated with (+) or without (−) 0.2 unit of alkaline phosphatase (AlkPhos) for 16 h at 37°C prior to analysis by SDS-PAGE and fluorography as outlined in Materials and Methods.

When 33P-labeled procathepsin L was treated with peptide N-glycosidase F, all radioactivity associated with the protein was lost, demonstrating that the radiolabel had been indeed incorporated into the carbohydrate moiety of the proenzyme. Incubation with peptide N-glycosidase F led to complete deglycosylation of procathepsin L, because the treatment shifted the apparent molecular mass of the 33P-labeled cathepsin L precursor to 35 kDa. The 33P-label of procathepsin L also proved sensitive to alkaline phosphatase (Fig. 3C). The same results were obtained for procathepsin B (not shown).

To comprehensively assess the status of phosphorylated N-linked oligosaccharides, SCC-VII cells were metabolically labeled with [3H]mannose. 3H-Labeled glycoproteins secreted into the culture medium were isolated and treated with endogluco- and oligosaccharides containing either one (9.8%) or two (4.2%) phosphodiester linkages, both one phosphomonoester and one phosphodiester (6.3%), or either one (5.4%) or two (20.1%) phosphomonoesters. The identity and composition of each fraction were verified by mild acid treatment (which converts phosphodiester linkages into phosphomonoester linkages) and alkaline phosphatase.
line phosphatase treatment (which cleaves exclusively linkages involving phosphomonoester groups). These results demonstrate that 69% of the phosphorylated N-linked oligosaccharides synthesized by SCC-VII cells contain phosphomonoester groups that serve as high-affinity ligands for M6P receptors (7). This is in good agreement with data reported for human fibroblasts (22). Thus, it appears that phosphorylation of lysosomal enzymes is fully functional in SCC-VII cells.

Expression of M6P Receptors in SCC-VII Cells. Mammalian cells usually synthesize both known M6P receptors, M6P/IGF2R and MPR46. We have investigated the expression of both receptors in SCC-VII cells and 3T3-L1 fibroblasts. Upon immunoblotting with antibodies against MPR46, a diffuse 45-kDa protein was detected in membrane extracts of SCC-VII cells. A slightly smaller immunoreactive polypeptide (43 kDa) was present in 3T3-L1 membranes. No reaction was observed with the corresponding soluble protein fractions (Fig. 4A). It has been established that differences in glycosylation may account for cell type-specific molecular forms of MPR46 (24).

When extracts of 3T3-L1 fibroblasts labeled with [35S]methionine were immunoprecipitated with antibodies against M6P/IGF2R, a polypeptide of >250 kDa was detected. However, this protein was not present in extracts of SCC-VII cells. No cross-reactive polypeptides were detectable in the culture medium of either cell line, ruling out the possibility that shedding of cell surface-resident M6P/IGF2R was responsible for the absence of this protein in SCC-VII cell lysates (Fig. 4B).

Although both M6P receptors contribute to intracellular sorting of lysosomal enzymes, receptor-mediated uptake of exogenous M6P-containing ligands is exclusively mediated by M6P/IGF2R (7). We have shown previously that procathepsin B synthesized by human hepatoma HepG2 cells is efficiently internalized by autologous recipient cells and human fibroblasts in a M6P/IGF2R-dependent manner (18). As a functional test for the presence of M6P/IGF2R, NH4Cl-induced secretions of HepG2 cells labeled with [35S]methionine were offered to unlabeled SCC-VII cells and 3T3-L1 fibroblasts for receptor-mediated endocytosis. Although procathepsin B was internalized by 3T3-L1 cells in a M6P-inhibitable manner, no uptake was detectable in SCC-VII cells. Identical results were obtained for procathepsin D (data not shown). We conclude that SCC-VII cells lack any endogenous synthesis of M6P/IGF2R, which induces hypersecretion of newly synthesized lysosomal enzymes as well as impairs their recapture via receptor-mediated endocytosis.

The Formation of Mature Lysosomes Is Impaired in SCC-VII Cells. Recent studies have shown that M6P/IGF2R plays a key role in the biogenesis of lysosomes (8). To assess the status of lysosomes in SCC-VII cells, postnuclear organelles were separated by density-gradient centrifugation. When the distribution of cathepsin B in the gradient fractions was determined, a marked peak of enzyme activity was observed at a buoyant density of 1.06 g/ml (Fig. 5A). The same distribution was found for the lysosomal marker β-N-acetylgalactosaminidase and acid phosphatase, a lysosomal enzyme transported to these compartments in a M6P-independent manner (7, 21). In contrast, the bulk of the cathepsin B (and β-N-acetylgalactosaminidase) activity of control HepG2 cells was located in compartments with a density of >1.08 g/ml, as typical for mature lysosomes (Fig. 5B). However, the subcellular distribution of lysosomal enzymes in SCC-VII cells overlaps significantly with the locations of the Golgi marker galactosyltransferase and the endocytic tracer HRP (Fig. 5A), a feature reminiscent of late endosomal/prelysosomal compartments. In fact, mutant human fibroblasts with a defect in the formation of dense lysosomes exhibited the same sedimentation pattern as observed for SCC-VII cells (19).

In normal cells, lysosomes are usually located in the perinuclear region (8, 19). Such a pattern was observed when control NIH 3T3 fibroblasts were immunostained with anti-cathepsin D antibodies (Fig. 6A). In contrast, immunocytochemical detection of cathepsin D in SCC-VII cells revealed numerous vesicles distributed throughout the cytoplasm (Fig. 6B). A similar subcellular distribution of lysosomal markers was observed in M6P/IGF2R-deficient murine fibroblasts, where the labeled structures were found to resemble late endocytic compartments (8). Thus, SCC-VII “lysosomes” display at least some characteristics of late endosomes/prelysosomes, indicating impaired formation of mature lysosomes in these cells as a direct consequence of the absence of M6P/IGF2R.

DISCUSSION

Our results demonstrate that SCC-VII cells secrete lysosomal proteinases attributable to the absence of functional M6P/IGF2R. Similarly, lack of M6P/IGF2R synthesis has been observed for the highly deviated Morris 7777 hepatoma, a malignant rat tumor (24). Indeed, Morris 7777 hepatoma cells display enhanced secretion of procathepsin D as compared with normal rat hepatocytes (25). Interestingly,
transformation of murine BALB/3T3 fibroblasts with Moloney murine sarcoma virus triggers exocytosis of procathepsins B and L, concomitant with the loss of M6P/IGF2R activity (26). M6P/IGF2R deficiency has been also detected in leukemia cells, which secrete large quantities of lysosomal hydrolases (27). Hence, the release of matrix-degrading cathepsins as a consequence of M6P/IGF2R absence may represent a common feature of many tumor cells.

M6P/IGF2R plays a pivotal role in the formation of mature lysosomes (7, 8). SCC-VII cells fail to synthesize the receptor and thus lack a functional set of these organelles. Consequently, these cells store lysosomal enzymes in compartments with properties reminiscent of late endosomes/prelysosomes. Similar results were obtained for other M6P/IGF2R-negative tumor cell lines, such as murine J774 myeloid leukemia cells (28) and Morris 7777 hepatoma cells (29). Interestingly, cathepsin B was relocated to endosomal vesicles in human MCF-10A breast epithelial cells upon transfection with an oncogenic version of the c-Ha-ras gene (30). Furthermore, transformation of mouse NIH 3T3 fibroblasts with Kirsten murine sarcoma virus leads to redistribution of cathepsin L and other lysosomal enzymes to endosomes/prelysosomes (31). Because both latter cell types are not deficient in M6P/IGF2R, impaired formation of lysosomes may also occur in M6P/IGF2R-positive tumor cells.

Despite the lack of M6P/IGF2R, SCC-VII cells retain a large proportion of their newly synthesized lysosomal proteinases. Although the other known mammalian M6P receptor, MPR46 (8), is present in SCC-VII cells, the insensitivity of the intracellular transport of cathepsins B, D, and L to lysosomal alkalinization suggests that M6P-independent sorting of lysosomal enzymes may take place in these cells. Similarly, M6P-independent targeting mechanisms have been proposed for procathepsins D and L in normal and transformed mouse NIH 3T3 fibroblasts (32) and for procathepsin D in human breast cancer cells (23). Interestingly, procathepsin D transiently associates during its biosynthesis with prosaposin (33), a protein delivered to lysosomes via interaction with the low-density lipoprotein receptor-related protein (34). Thus, interaction of procathepsin D with endogenous prosaposin may account for its preferential retention in SCC-VII cells as compared with cathepsins B and L.

It has been reported recently that M6P/IGF2R interacts with uPAR through a domain distinct from its M6P- and IGF-II binding sites (35). In the absence of M6P/IGF2R, uPAR is not efficiently internalized, effectively increasing the number of surface binding sites for plasminogen activators, which may cause enhanced focal proteolysis. Because SCC-VII cells produce plasminogen activators (16), deficient uPAR internalization could further add to the proteolytic load in the pericellular environment of the cells. Hence, M6P/IGF2R deficiency may enhance the invasiveness of tumor cells by at least two means, elevated secretion of matrix-degrading cathepsins and increased amounts of cell-surface plasminogen activators. Interestingly, uPAR may also promote tumor cell invasion in a protease-independent manner through stimulation of integrin-mediated cell migration (36).

Fig. 5. Characterization of SCC-VII lysosomes. Postnuclear supernatants of SCC-VII (A) and HepG2 (B) cells were subjected to Percoll density-gradient centrifugation as described in “Materials and Methods.” Fractions (1 ml) were collected from the bottom of the tubes and analyzed for buoyant density (□), cathepsin B activity (○), and galactosyltransferase activity (●). Enzyme activities are expressed as the percentage of the total activity recovered in the fractions of each gradient. In A, endocytosed HRP emerged as a broad peak in fractions 9–12. In B, HRP distribution was essentially the same as for galactosyltransferase, with maximum activity in fraction 9.

Fig. 6. Subcellular localization of cathepsin D in SCC-VII cells and NIH 3T3 fibroblasts. Control NIH 3T3 fibroblasts (A) and SCC-VII cells (B) were fixed and immunostained with anti-cathepsin D antibodies as specified in “Materials and Methods.” The labeled compartments were visualized by fluorescence microscopy. Bars, 10 μm.

P. Ton and L. Mach, unpublished data.
The $\text{M6P/IGF2R}$ gene is often inactivated in human and animal tumors. Rodents appear particularly susceptible because $\text{M6P/IGF2R}$ displays monoleucine expression in mice and rats, with the paternal allele being repressed. In contrast, this genomic imprinting of $\text{M6P/IGF2R}$ is a polymorphic trait in humans, with both alleles being transcribed in most humans. Thus, inactivation of the $\text{M6P/IGF2R}$ locus generally requires two genetic events in humans but only one in mice (10). Interestingly, loss of one $\text{M6P/IGF2R}$ allele is a common feature of human hepatocellular and breast cancers (12, 13). $\text{M6P/IGF2R}$ contains several microsatellite sequences in its coding region, and deletions within the remaining $\text{M6P/IGF2R}$ allele arise frequently as a consequence of microsatellite instability (14). These mutations generally cause frame-shifts that result in premature termination of translation, giving rise to the synthesis of truncated, soluble receptors (12). However, $\text{M6P/IGF2R}$-related polypeptides were not detectable in the culture supernatants of SCC-VII cells, thus indicating that the molecular defect in the $\text{M6P/IGF2R}$ gene of these cells is probably not detectable directly related to microsatellite instability.

$\text{M6P/IGF2R}$ exerts at least two growth-suppressive functions: (a) the receptor accounts for the internalization and degradation of IGF-II, a powerful growth-stimulating factor; and (b) $\text{M6P/IGF2R}$ binds latent TGF-$\beta$ and stimulates activation of the growth-suppressive cytokine by recruitment of plasminogen and uPAR-bound plasminogen activators (37). In $\text{M6P/IGF2R}$-mutant gastrointestinal tumors, IGF-II degradation and TGF-$\beta$ activation are significantly reduced (38). Recently, transfection of wild-type $\text{M6P/IGF2R}$ cDNA into $\text{M6P/IGF2R}$-mutant human SW48 colorectal carcinoma cells was shown to suppress growth and induce apoptosis (15). Because our results indicate that $\text{M6P/IGF2R}$-negative tumor cells display an increased potential to degrade extracellular matrix components, inactivation of the $\text{M6P/IGF2R}$ gene may support growth as well as metastasis of malignant cancers.

ACKNOWLEDGMENTS

We express our gratitude to Drs. Ann H. Erickson, Annette Hille-Rehfeld, Thomas Braulke, David James, and John S. Mort for providing antibodies and cell lines. We also thank Melinda Abas and Prof. Josef Glössl for critical reading of the manuscript.

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Invasive Properties of Murine Squamous Carcinoma Cells: Secretion of Matrix-degrading Cathepsins Is Attributable to a Deficiency in the Mannose 6-Phosphate/Insulin-like Growth Factor II Receptor

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*Cancer Res* 2000;60:4070-4076.

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