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Biochemistry

A 170-kDa membrane-bound protease is associated with the expression of invasiveness by human malignant melanoma cells

(protease/plasma membrane/extracellular degradation/gelatinase/invasion)

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ABSTRACT **Malignant spreading of cancer cells requires cell surface proteases that cleave the crosslinked collagenous matrix of connective tissues. From correlating the morphologically defined invasiveness of tumor cells with the presence of specific membrane-associated proteases, we have identified a malignant human melanoma cell line, LOX, that invades crosslinked gelatin films *in vitro* and contains uniquely a neutral 170-kDa gelatinase in the cell membrane. A similar gelatinase was found in membranes recovered from culture media conditioned with LOX. The 170-kDa gelatinase is a wheat germ agglutinin-binding protein. The proteolytic activity is maximal at neutral pH, enhanced by EDTA and dithiothreitol, inhibited by the cysteine protease inhibitors *N*-ethylmaleimide, $HgCl_2$, and phenylmethylsulfonyl fluoride, and can bind to an organomercurial adsorbent, suggesting that it is a neutral sulfhydryl-sensitive protease. This 170-kDa gelatinase of LOX cells was not found in a control melanoma cell line, SK-MEL28, or in 32 other tumor cell lines that did not show extracellular gelatin degradation. Thus, we have identified a large membrane-bound protease that may be a specific marker molecule for melanoma cell invasiveness.**

The invasion of tumor cells through surrounding connective tissue is an important stage of metastasis requiring cell surface protease activity (1, 2). Tumor cell invasiveness has been linked with an increased production of extracellular matrix (ECM)-degrading enzymes, including plasminogen activators (for a review see ref. 3), lysosomal cysteine proteases (4, 5), collagenases (6), and glycosidases (7). Very little attention, however, has been paid to membrane proteases. Plasma membrane-associated proteases have been identified from biochemically defined "membrane" fractions, which were derived from mixed cell types in tissues (8–10). Studies from some tumors have shown that solid tumors contain heterogeneous cell types, and invading tumor cells represent less than 1/10,000 of the population (11). Thus, attempts to identify these proteases from *in situ* solid tumors have not been successful.

Isolation of membrane proteases from a homogeneous population of tumor cells in culture has shown some important results. Cell fractionation has localized cathepsin B-like activity to the plasma membrane of tumor cells (5). A chymotrypsin-like protease activity was also identified from tumor cell membranes (12, 13). In addition, the 100-kDa common acute lymphoblastic leukemia antigen (CALLA) has been identified as neutral endopeptidase 24.11, an integral zinc metalloendoproteinase (14), which may be identical to the neutral metalloendoprotease purified from the detergent-extracted membrane fraction of rabbit kidney tissue (8).

Previously, plasminogen activator was localized on the cell surface of Rous sarcoma virus (RSV)-transformed cells (15) and was shown to be involved in tumor metastasis (16). L. B. Chen and Buchanan (17), however, showed that plasminogen-independent fibrinolysis of cell surface proteins involved unidentified proteases produced by transformed cells. We have shown that RSV-transformed cells express invasiveness by locally degrading fibronectin-crosslinked gelatin films at rosette contact sites or invadopodia (2, 18, 19). The transformed cells express 120- and 150-kDa proteases, which degrade fibronectin, in association with the membrane (20). These proteases were present in extremely low quantity and the possibility that these proteases were associated with viruses in the RSV transformation model is difficult to rule out. To obtain a human tumor cell line that retains a stable invasive phenotype for identification of specific proteases, we have investigated the capacity of more than 32 human tumor cell lines to degrade immobilized ECM substrata upon contact of cells over a 24-hr period. Previously, we developed crosslinked gelatin films containing fluorescent and radiolabeled proteins covalently coupled to the surface of the substratum (2). This technique allows identification of tissue culture cells which express activated ECM-degrading proteases at sites of contact between cells and their substrata. In the present study, two human melanoma cell lines were selected for in-depth characterization of cell invasiveness and the presence of specific ECM-degrading proteases. LOX, a cell line originating from a xenograft of human amelanotic melanoma, induces a high incidence of lung metastasis after intravenous injection into athymic mice (21). SK-MEL28, a cell line originating from human melanotic melanoma, was used as the control for cell invasiveness because it has a more differentiated phenotype (22). Here, we show that the LOX cells invade crosslinked gelatin films *in vitro* and that the cells express uniquely a neutral sulfhydryl-sensitive 170-kDa gelatinase in the cell membrane.

MATERIALS AND METHODS

Cell Culture. The human amelanotic melanoma cell line LOX (21) was maintained with 1:1 mixture of Dulbecco's modified Eagle's medium and RPMI 1640 medium supplemented with 10% calf serum, 5% Nu-serum (Collaborative Research), 2 mM L-glutamine, penicillin at 1 unit/ml, and streptomycin at 10 μg/ml. The human melanotic melanoma cell line SK-MEL28 (22) was maintained in Eagle's minimal essential medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, nonessential amino acids at 0.1 mM, 2 mM L-glutamine, penicillin at 1 unit/ml, and streptomycin at 10 μg/ml. LOX and EJ cells were kindly provided by L. B.

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Abbreviations: ECM, extracellular matrix; DIC, differential interference contrast; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride; RSV, Rous sarcoma virus; WGA, wheat germ agglutinin.

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Chen (Dana–Farber Cancer Institute, Harvard Medical School, Boston). Other human tumor cell lines, including DLD1, SW620, HT1080, and SK-MEL28 cells, were purchased from the American Type Culture Collection.

Cell Assays for Localized Degradation of the Glutaraldehyde-Crosslinked Gelatin Film by Tumor Cells. Glutaraldehyde-crosslinked gelatin films coated on glass coverslips that were surface-coupled with fibronectin were prepared as previously described (2). Morphological identification of localized degradation of crosslinked gelatin films by tumor cells was done by differential interference contrast (DIC) microscopy using a Zeiss Photomicroscope III (Zeiss) as described (2).

Protease Assays and Gelatin Zymography. A detergent-soluble membrane fraction, a detergent-soluble nuclear fraction, and a water-soluble cytosol fraction were prepared as previously reported (15, 20). Serum-free conditioned media derived from cell culture were spun at low speed to remove floating cells and cell debris. Membranes from conditioned media were collected by centrifugation at 100,000 × *g* for 1 hr at 4°C and then extracted in 2% octyl glucoside in Tris-buffered saline (TBS; 50 mM Tris·HCl, pH 7.4/150 mM NaCl) as described (23) or extracted in 1% Triton X-114 in TBS and then partitioned into detergent and aqueous phases according to the previously described method (24). Total protein concentrations of various fractions were determined by BCA (bicinchoninic acid) assay (Pierce), using bovine serum albumin solutions as standards.

To analyze degradation of immobilized ECM proteins after SDS/PAGE, we used substrate zymography (20, 25) and fibrin overlay zymography (26). For substrate zymograms, protease substrates were gelatin (3 mg/ml, denatured type I collagen; Sigma), casein (2 mg/ml; Sigma), or human plasma fibronectin (0.5 mg/ml; New York Blood Center, New York). Fibrin overlay zymography was used to detect plasminogen activators and fibrinolytic proteases in the cellular fractions (26). Cellular proteins were first separated by electrophoresis on an SDS/6% polyacrylamide gel without reduction. The gel was washed with 2.5% Triton X-100 and then overlaid with an additional 1.25% agarose gel which contained fibrinogen (essentially plasmin free; Sigma) at 2.5 mg/ml, human thrombin (Sigma) at 0.4 unit/ml, and plasminogen (Sigma) at 27 μg/ml. After incubating at 37°C overnight, the fibrin-agarose gel was fixed and stained with 0.1% amido black in 70% methanol/10% acetic acid/20% water (vol/vol). Plasminogen activators and fibrinolytic proteases were demonstrated as negative bands in the fibrin gel.

Column Chromatography. The 170-kDa gelatinase was partially purified by molecular sieve chromatography on a Sephacryl S-200 column (Pharmacia LKB). The LOX membrane fraction (2.5-ml sample volume) was loaded onto a Sephacryl S-200 column (1.5 × 70 cm) equilibrated in 1% octyl glucoside in TBS at 4°C. The column was eluted with the same octyl glucoside buffer at a flow rate of 8 ml/hr and 2.4-ml fractions were collected. Fractions having absorbance at 280 nm were analyzed by gelatin zymography and those containing the 170-kDa protease were pooled for further purification.

The above fractions containing the 170-kDa protease were mixed with wheat germ agglutinin (WGA)-agarose (Vector Laboratories) by rotating overnight at 4°C. The WGA-agarose mixture was packed in a column, and the column was washed with 3 column volumes of 1% octyl glucoside/200 mM NaCl/5 mM EDTA in 10 mM Tris·HCl, pH 7.5. Adsorbed WGA-binding proteins were then eluted with 2 column volumes of 0.5 M *N*-acetyl-D-glucosamine (Sigma)/1% octyl glucoside/200 mM NaCl/5 mM EDTA in 10 mM Tris·HCl, pH 7.5 (27). Fractions containing proteins were combined and subjected to gelatin zymography.

Protease Inhibitors. Specific inhibitors for various proteases were included in gelatin zymography for the inhibition of the 170-kDa protease. The samples were preincubated with various inhibitors at 37°C for 30 min before they were applied to the gelatin gel for electrophoresis. After electrophoresis and washing, the gel slices were incubated in Hanks' balanced salt solution (HBSS; 0.4 mM Na_2HPO_4, pH 7.5/0.5 mM KH_2PO_4/5.25 mM KCl/1.25 mM $CaCl_2$/0.8 mM $MgSO_4$/136 mM NaCl) containing the same protease inhibitors as those used for pretreatment at 37°C overnight. These inhibitors are phenylmethylsulfonyl fluoride (PMSF), benzamidine, 1,10-phenanthroline, EDTA, *N*-ethylmaleimide (NEM), $HgCl_2$, and cysteine (Sigma), dithiothreitol (DTT; Serva), and leupeptin and pepstatin (Peninsula Laboratories).

RESULTS

In serum-containing medium, the crosslinked gelatin film remained unchanged when incubated without cells or with the control melanoma line SK-MEL28 (Fig. 1) and other human tumor cells (not shown). However, growth of LOX cells on the crosslinked gelatin film resulted in the appearance of surface indentations beneath the cells in a time-dependent manner (Fig. 1). More than 10% of LOX cells invaded the film at sites of cell contact 3 hr after cell plating, and more than 30% at 5 hr. The degraded spots increase in size as a function of time, such that large holes, more than 10 μm in diameter, formed after 1 day (Fig. 1). When LOX cells were cultured on a fluorescently labeled fibronectin-coated substratum, the cells formed discrete spots lacking fluorescent fibronectin beneath the cells prior to the formation of morphological indentations in the gelatin film (not shown). The appearance of these fibronectin-negative spots corresponds to an overall release of radiolabeled fibronectin into the medium as shown in chicken embryonic cells transformed by RSV (2). The observed ECM-degrading activity of LOX cells, however, was lacking when the cells were cultured in serum-free media. We tested the possibility that activation of cell surface plasminogen activator is directly involved in the localized degradation of the ECM by LOX cells. LOX cells degraded the fibronectin-gelatin film when the cells were cultured in media containing 5% plasminogen-depleted fetal calf serum prepared as described (28) and 20 mM ε-aminocaproic acid. Thus, the plasmin-dependent protease cascade is not essential for the capacity of LOX cells to degrade the ECM and invade the glutaraldehyde-fixed gelatin film. The LOX invasive phenotype appears to be stable, as we have not detected any change in this cell surface proteolytic activity during a period of 2 years.

To determine whether the observed ECM degradation by LOX cells is due to proteases associated with the plasma membrane, we prepared subcellular fractions from LOX cells and the control melanoma line SK-MEL28, and we assayed them for proteolytic activities by ECM substrate zymography. Fig. 2 shows that, on gelatin zymograms, a prominent

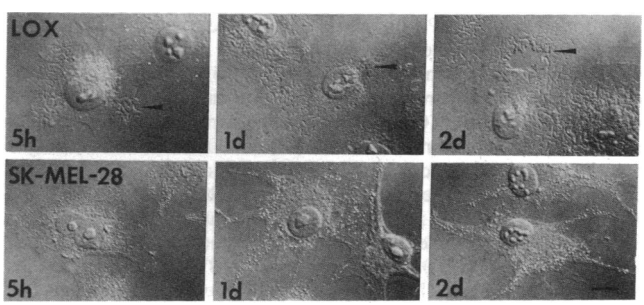

FIG. 1. Visualization of invasion sites of two human melanoma cell lines, LOX and SK-MEL28, into glutaraldehyde-crosslinked gelatin films. DIC images of cell cultures show surface indentations on the film formed under LOX cells (arrowheads) but not SK-MEL28 cells at 5 hr, 1 day, and 2 days. (Bar = 50 μm.)

negative staining band with apparent molecular mass of 170 kDa was present in the detergent-soluble membrane fraction of LOX cells but not of SK-MEL28 cells. The membranes recovered from culture media conditioned with LOX also had the 170-kDa gelatinase, but it was not found with SK-MEL28 (Fig. 2*A*) and other tumor cells. The 170-kDa protease was also found in the membranes from nuclear fractions, but it was found neither in cytosol and media fractions from LOX cells nor in any fraction from SK-MEL28 (Fig. 2*A*). To demonstrate the effectiveness of gelatin zymography in detecting proteases, we tested decreasing amounts of total protein (60, 30, 15, 7.5, and 3.8 μg) from LOX membranes that had been solubilized in Laemmli SDS sample buffer without 2-mercaptoethanol reduction by gelatin zymography (Fig. 2*B*) and conventional SDS/PAGE.

The 170-kDa gelatinase in LOX membranes cannot completely degrade fibronectin, fibrin, or casein, as shown by substrate zymography (Fig. 3). Using fibrin overlay gels in the presence and absence of plasminogen to determine the activity of plasminogen activator and fibrin-degrading enzymes, respectively, we have found a 55-kDa plasminogen activator in the membrane, and both the 55-kDa plasminogen activator and a 110-kDa fibrin-degrading protease in the cytosol of LOX cells (Fig. 3). In addition, a low molecular mass (55-kDa) gelatinase, which was sensitive to the metalloprotease inhibitor EDTA, was found in various amounts from different preparations, most abundantly in the cytosol fraction of tumor cells (Fig. 3). These proteases, including the 55-kDa metallogelatinase and the 55-kDa plasminogen activator, were also found in the membranes of some tumor cells such as DLD1 that are negative in the *in vitro* invasion assay (not shown).

The 170-kDa gelatinase represents the major protease in the membrane of LOX cells and can be partially purified by Sephacryl S-200 gel filtration and WGA-agarose affinity chromatography (Fig. 4 *A* and *B*). Approximately 3 units of activity of the 170-kDa protease was obtained with Sephacryl S-200 gel filtration (Fig. 4*A*), contained in 10 μg of protein.

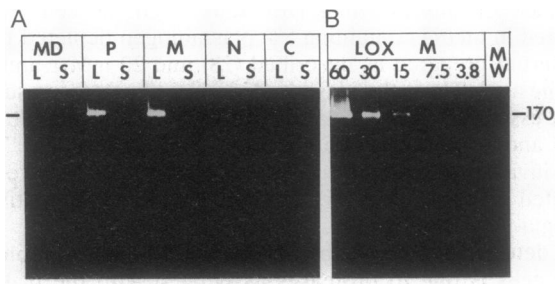

FIG. 2. Identification of a 170-kDa protease in subcellular fractions of LOX and SK-MEL28 cells. (*A*) Gelatin zymogram of various subcellular fractions from LOX (L) and SK-MEL28 (S) cells. These fractions include concentrated serum-free conditioned medium (MD), octyl glucoside detergent extract of the 100,000 × *g* pellet from conditioned medium (P), detergent-soluble membrane fraction (M), detergent-soluble nuclear fraction (N), and cytosol fraction (C), which were prepared in solutions containing 5 mM EDTA. Approximately 30 μg of total proteins was applied to each lane and subjected to gelatin zymography. Only the membrane derived from conditioned medium (P) and membrane fraction (M) of LOX cells show a major negatively stained band with apparent molecular mass of 170 kDa that represents complete digestion of immobilized gelatin by the protease. The 170-kDa protease is also present in a small amount in the membranes from cellular fragments in nuclear fraction (N) but not in the medium (MD) and the cytosol (C). (*B*) Enzymatic activities of the membrane from LOX cells. The detergent-soluble membrane fraction of LOX cells (M) was serially diluted and analyzed on a gelatin zymogram. Each lane contains the amount of total protein as indicated in μg. The gelatin zymogram shows that in the lane containing 30 μg of total protein a prominent band at 170 kDa is apparent. MW indicates the lane containing molecular mass standards.

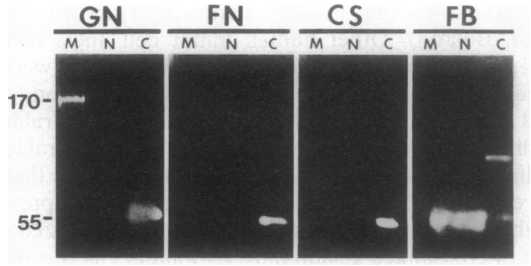

FIG. 3. Association of a 170-kDa gelatinase and a 55-kDa plasminogen activator with LOX membranes. The detergent-soluble membrane fraction (M), detergent-soluble nuclear fraction (N), and cytosol fraction (C) were used to produce zymograms on gelatin (GN), human plasma fibronectin (FN), and casein (CS) substrate. In addition, these cellular extracts were also analyzed for plasminogen-dependent proteolysis by using fibrin gel overlay in the presence of plasminogen (indicated as FB at the right) and the absence of plasminogen (not shown). Approximately 30 μg of total protein was loaded on each lane. Only the membrane fraction showed the 170-kDa protease on the gelatin gel. However, the 170-kDa band was not found in other fractions on the gelatin gel or in any of the fractions on fibronectin, casein, and fibrin gels. In this experiment a 55-kDa metalloprotease (sensitive to EDTA) was found in the cytosol fraction which can degrade gelatin, fibronectin, and casein. In the fibrin overlay gel, a 55-kDa plasminogen activator was identified in association with the membrane of detergent-soluble membrane fraction and detergent-soluble nuclear fraction. The 55-kDa plasminogen activator, however, was not active in the fibrin gel lacking plasminogen (not shown). In addition, a 110-kDa fibrin-degrading protease was identified in the cytosol fraction.

One unit of activity is defined as the amount of activity giving the same density on a zymogram as the band shown in the 30-μg lane of Fig. 2*B*, which was derived from 30 μg of protein from the total membrane extract. Thus, we achieved an approximately 3-fold increase in specific activity. The material recovered from the WGA column was enriched in activity of the 170-kDa protein an additional 10-fold (Fig. 4*B*). These combined procedures produced an overall 30-fold enrichment of the 170-kDa protease compared with the octyl glucoside-soluble membrane extract as determined by measurements of total protein and enzymatic activity on gelatin zymograms. A low molecular mass (55-kDa) metalloprotease was removed by passing the LOX membrane extracts over Sephacryl S-200 and WGA columns (Fig. 4 *A* and *B*).

The 170-kDa gelatinase was also found in a Triton X-114 extract of the LOX cell layer (Fig. 4*C*). The 170-kDa gelatinase was partitioned into the detergent phase of the Triton X-114 extracts (Fig. 4*C*), producing a 10-fold enrichment compared with the total Triton X-114 detergent extract of the LOX cell layer. The 170-kDa protease obtained by binding to WGA could also be partitioned into the detergent phase of the Triton X-114 extracts (not shown). These data suggest that the protease is an integral membrane protein. The 170-kDa gelatinase from WGA-binding proteins of LOX cell membranes bound to an organomercurial adsorbent (not shown), suggesting a role as a cysteine protease (29).

The 170-kDa protein was active when the samples were incubated at room temperature and at 37°C, whereas it was inactivated after incubation at 55°C for 10 min or at 100°C for 2 min (Fig. 5*A*). The gelatinase activity of 170-kDa protein was enhanced slightly by preincubation with 2-mercaptoethanol, and its apparent molecular mass remained at 170 kDa under the reducing conditions (Fig. 5*A*). As shown in Fig. 5*B*, the 170-kDa gelatinase from LOX cells was active in the range from pH 6 to pH 8.4, with its optimum at pH 7. To classify the 170-kDa protease, we examined the effects of various protease inhibitors specific for serine, aspartate, cysteine, or metalloproteases (30) on its gelatin-degrading activity. Fig. 5*C* shows the sensitivity of the 170-kDa gelat-

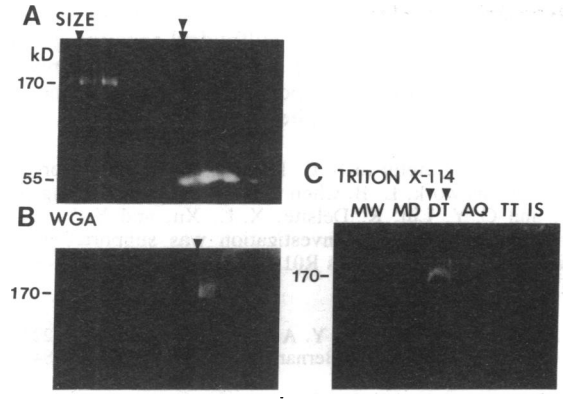

FIG. 4. Partial purification of the 170-kDa protease as assayed on gelatin zymograms. (*A*) Sephacryl S-200 gel filtration of the detergent-soluble membrane fraction from LOX cells. Each fraction was 2.4 ml and fraction 12 (single arrowhead) was the onset of the void volume. Approximately 10 μg of total protein from each fraction was loaded in each lane of the gelatin zymogram, and the 170-kDa protease was detected from fractions 12 to 14 in an approximate 3-fold increase in specific activity over the starting extract. This procedure separates the 170-kDa protease from the 55-kDa metalloprotease (right double arrowheads). The 55-kDa band was not observed in preparations with NaN_3 and EDTA. (*B*) WGA-agarose affinity chromatography of the 170-kDa protease. Fractions 12–14 from the Sephacryl S-200 gel filtration were pooled and analyzed by WGA-agarose affinity chromatography. The WGA-binding proteins were eluted with 0.5 M *N*-acetyl-D-glucosamine. Approximately 1 μg of total protein from each fraction was loaded in each lane of the gelatin zymogram, and the 170-kDa protease was detected from fractions 13 (arrowhead) to 15. The 170-kDa enzyme was enriched 10-fold in specific activity over that of the material loaded onto the column. (*C*) Triton X-114 phase partitioning of the detergent-soluble material recovered from LOX cells. Molecular mass markers were loaded in the first lane (MW). Subsequent lanes contained concentrated serum-free conditioned medium (MD), the detergent phase (DT) and aqueous phase (AQ) of the Triton X-114 detergent extract of the LOX cell layer, Triton X-114 detergent extract of the LOX cell layer (TT), and the insoluble material from the Triton X-114 detergent extract of the LOX cell layer (IS). Approximately 30 μg of total protein was applied to each lane and subjected to gelatin zymography. The 170-kDa protease was partitioned into the detergent phase of the Triton X-114 extracts (DT, double arrowheads), concentrating the 170-kDa activity 10-fold over that of the total Triton X-114 detergent extract of the LOX cell layer (TT).

inase to various protease inhibitors. Inhibitors of the aspartate, serine, or metalloproteases, including pepstatin (0.03 mM), benzamidine (10 mM), EDTA (5 mM), and 1,10-phenanthroline (2 mM), had no discernible effect on the activity of the 170-kDa protein. However, treatment of the sample with a mixture of EDTA (2 mM) and DTT (2 mM), which inhibits metal-dependent proteases and activates cysteine proteases (30), significantly enhanced the activity of the 170-kDa protein. The 170-kDa gelatinase was inhibited by 1 mM NEM or 1 mM PMSF, but PMSF-inhibitable activity could be recovered by adding 10 mM DTT (Fig. 5*C*, lane PMSF+DTT). The 170-kDa protease could also be inhibited by 0.2 mM $HgCl_2$, and the $HgCl_2$-inhibitable activity could be recovered by adding 5 mM cysteine (data not shown). This inhibition data together with the data on binding of the 170-kDa protein to the organomercurial absorbent show that the activity of the protease involves a sulfhydryl group.

DISCUSSION

In this report, we correlate the morphologically defined invasiveness of tumor cells with the presence of specific membrane-associated proteases. We have assessed the invasiveness of human tumor cells by using crosslinked gelatin

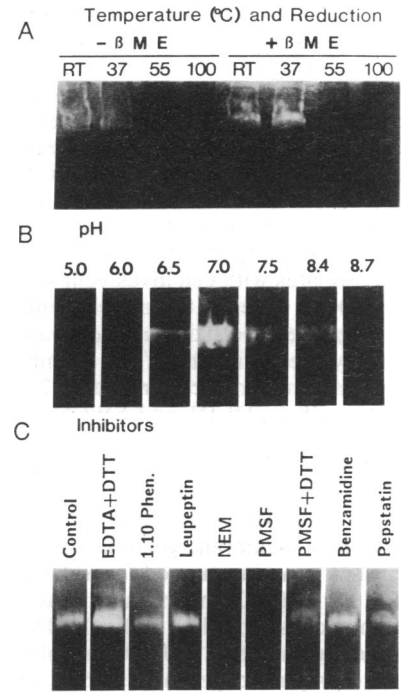

FIG. 5. Characterization of the 170-kDa protease on gelatin zymograms. (*A*) Effects of heating and the reducing agent 2-mercaptoethanol on the activity of 170-kDa protein. Approximately 60 μg of total protein was mixed with Laemmli sample buffer with (+βME) or without (−βME) 2.5% (vol/vol) 2-mercaptoethanol and incubated at various temperatures as indicated before being subjected to gelatin zymography. RT, room temperature (22°C) for 1 hr; 37, 37°C for 1 hr; 55, 55°C for 10 min; 100, 100°C for 2 min. The 170-kDa gelatinase was active in the samples incubated at room temperature and at 37°C, whereas it was inactivated after incubation at 55°C for 10 min or 100°C for 2 min. The 170-kDa activity was slightly enhanced by 2-mercaptoethanol, but without significant change in the electrophoretic mobility. (*B*) Optimal pH of the 170-kDa gelatinase. Slices of gelatin gels containing the 170-kDa protease were prepared and incubated in HBSS buffered at the indicated pH. The 170-kDa gelatinase was active in the range from pH 6 to pH 8.4, with its optimal pH at 7.0. (*C*) Inhibitor specificities of the 170-kDa protease. Prior to electrophoresis, the detergent-soluble membrane fractions from LOX cells were treated with various protease inhibitors. Concentrations of inhibitors were as follows: EDTA, 2 mM; DTT, 2 mM; 1,10-phenanthroline, 2 mM; leupeptin, 0.1 mM; NEM, 1 mM; PMSF, 1 mM; benzamidine, 10 mM; and pepstatin, 0.03 mM. In the lane marked PMSF+DTT, sample was preincubated with 1 mM PMSF, and after electrophoresis the gel slice was incubated in HBSS containing 10 mM DTT. The activity of the 170-kDa protease was enhanced by incubating with a mixture of 2 mM EDTA and 2 mM DTT. The 170-kDa protease was inhibited by NEM or PMSF, but PMSF inhibition could be partially reversed by incubating with DTT.

film culture (2), and we have identified an invasive human melanoma cell line LOX (21) that shows a stable invasive phenotype in degrading crosslinked gelatin film *in vitro*. Melanoma line SK-MEL28 (22) and 32 other human tumor cell lines were negative in this *in vitro* invasion assay. Cell fractionation resolved three neutral proteases, a sulfhydryl-sensitive 170-kDa gelatinase, a 55-kDa metallogelatinase, and a plasminogen activator, in association with the membrane of LOX cells. Interestingly, the metallogelatinase and plasminogen activator were found in other tumor cells which were noninvasive *in vitro* (A.A., R. Delsite, and W.-T.C., unpublished results). The activity of 170-kDa membrane-associated protease is specific for the LOX cells, which may account for the invasiveness of these cells. Although the precise role of the 170-kDa protease in the activation of ECM degradation remains to be explored, we postulate that with its unique appearance on the LOX membrane it not only directly

degrades the ECM but also may initiate a cascade of proteolysis at the critical sites of contact between cells and the substratum (2, 19, 31–35).

Recently, using a combination of the *in vitro* invasion assay and cell fractionation, we found that another human melanoma line, RPMI, was less invasive and expressed less of the 170-kDa membrane gelatinase than the LOX cells. In addition, the 170-kDa protease was coisolated with the ECM contact-enriched membrane of LOX (S. C. Mueller, W. Monsky, and W.-T.C., unpublished results). Thus, the 170-kDa protease correlates with cell invasiveness in our *in vitro* invasion assay. Other types of tumor cells which have been tested and are negative in both the *in vitro* invasion assay and gelatinase activity measurement of the membrane fraction include esophageal squamous carcinoma TE2, colon adenocarcinoma cells such as DLD1 and SW620, bladder carcinoma EJ cells, pancreatic adenocarcinoma Capan-1 and Capan-2, fibrosarcoma cells HT1080, and simian virus 40-transformed lung fibroblasts WI-38 VA13. At this point, we cannot exclude the possibility that these tumor cells may express the 170-kDa membrane gelatinase or other secreted metallogelatinases in an inactivated form. Using the current approach to study 170-kDa membrane protease in a large number of tumor cells would be difficult. The use of antibodies directed against the 170-kDa membrane protease should facilitate its purification and studies on its potential role as a specific marker for tumor cell invasiveness.

The 170-kDa protease may be an integral membrane protease since it has been found in the detergent phase, but not in the aqueous phase, of the Triton X-114-solubilized membrane fraction of LOX cells. The 170-kDa protease is also present in the octyl glucoside-solubilized membrane fraction of LOX cells but not in the cytosol fraction or in supernate from conditioned medium. Its activity was also retained in membrane fractions after freezing and thawing and extraction with high-salt buffers. Additionally, the 170-kDa protease was found in the detergent extracts of 100,000 × *g* pellets derived from conditioned media, suggesting that the 170-kDa protease is released into the medium in membrane vesicles (23).

At this point, it is not certain whether the 170-kDa protease is similar to lysosomal cathepsin cysteine proteases. Studies with inhibitors suggest that it is a distinct enzyme from another membrane-associated protease, a cathepsin B-like activity previously identified in tumor cells (4, 5). The 170-kDa protease is a WGA-binding glycoprotein, as are many integral membrane proteins, but cathepsin B is not (29, 30). It is also different from known cysteine proteases such as cathepsin B, L, or H in terms of molecular weight, optimal pH, and inhibitor specificity. It is a high molecular weight protease as determined by SDS/PAGE zymography and molecular sieve chromatography on Sephacryl S-200. This proteolytic activity is active at neutral pH, and it is enhanced by EDTA and DTT. In comparison with the prototype cysteine protease cathepsin B (4, 5, 29, 30), the 170-kDa gelatinase was insensitive to 0.1 mM leupeptin. However, the 170-kDa protease was sensitive to other inhibitors, such as NEM, $HgCl_2$, and PMSF, that bind the sulfhydryl group of amino acid residues of the proteases. The inhibition by $HgCl_2$ and PMSF could be reversed by DTT and cysteine. This peculiar inhibitor profile of the 170-kDa protease makes it problematic to classify it as a cysteine protease. Earlier studies on direct thioether linkage of labeled azaserine to the sulfhydryl group of a cysteine residue of formylglycinamide ribonucleotide amidotransferase (36) have demonstrated cysteine at the active site of this enzyme. This direct approach is needed to determine if the active site of the 170-kDa protein involves cysteine. Here, we suggest that the 170-kDa gelatinase is a previously unknown sulfhydryl-sensitive protease present in the membranes of invasive cells. This protease warrants further study as a possible marker of tumor cells expressing a very invasive phenotype.

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1. Jones, P. A. & DeClerck, Y. A. (1980) *Cancer Res.* **40,** 3222–3227.
2. Chen, W.-T., Olden, K., Bernard, B. A. & Chu, F.-F. (1984) *J. Cell Biol.* **98,** 1546–1555.
3. Dano, K., Behrendt, N., Lund, L. R., Ronne, E., Pollanen, J., Salonen, E. M., Stephens, R. W., Tapiovaara, H. & Vaheri, A. (1989) *Cancer Metastasis*, eds. Schirrmacher, V. & Schwartz-Albiez, R. (Springer, Berlin), pp. 98–107.
4. Poole, A. R., Tiltman, K. J., Recklies, A. D. & Stoker, T. A. (1980) *Nature (London)* **273,** 545–547.
5. Sloane, B. F., Rozhin, J., Johnson, K., Taylor, H., Crissman, J. D. & Honn, K. V. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2483–2487.
6. Liotta, L. A., Abe, S., Robey, P. G. & Martin, G. R. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 2268–2272.
7. Nakajima, M., Irimura, T., DiFerrante, N. & Nicolson, G. L. (1984) *J. Biol. Chem.* **259,** 2283–2290.
8. Almenoff, J. & Orlowski, M. (1983) *Biochemistry* **22,** 590–599.
9. Malfroy, B., Schofield, P., Kuang, W. J., Seeburg, P. H., Mason, A. J. & Henzel, W. J. (1987) *Biochem. Biophys. Res. Commun.* **144,** 59–66.
10. Tanaka, K., Nakamura, T. & Ichihara, A. (1986) *J. Biol. Chem.* **261,** 2610–2615.
11. Poste, G., Doll, J., Hart, I. & Fidler, I. J. (1980) *Cancer Res.* **40,** 1636–1644.
12. Yavelow, J., Caggana, M. & Beck, K. A. (1987) *Cancer Res.* **47,** 1598–1601.
13. Zucker, S., Wieman, J. M., Lysik, R. M., Wilkie, D., Ramamurthy, N. S., Golub, L. M. & Lane, B. (1987) *Cancer Res.* **47,** 1608–1614.
14. Shipp, M. A., Vijayaraghavan, J., Schmidt, E. V., Masteller, E. L., D'Adamio, L., Hersh, L. B. & Reinherz, E. L. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 297–301.
15. Quigley, J. P. (1976) *J. Cell Biol.* **71,** 472–486.
16. Ossowski, L. & Reich, E. (1983) *Cell* **35,** 611–619.
17. Chen, L. B. & Buchanan, J. M. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 1132–1136.
18. Chen, W.-T., Chen, J.-M., Parsons, S. J. & Parsons, J. T. (1985) *Nature (London)* **316,** 156–158.
19. Chen, W.-T. (1989) *J. Exp. Zool.* **251,** 167–185.
20. Chen, J.-M. & Chen, W.-T. (1987) *Cell* **48,** 193–203.
21. Fodstad, O., Aamdal, S., Mcmenamin, M., Nesland, J. M. & Pihl, A. (1988) *Int. J. Cancer* **41,** 442–449.
22. Carey, T. E., Takahashi, T., Resnick, L. A., Oettgen, H. F. & Old, L. J. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 3278–3282.
23. Zucker, S., Wieman, J. M., Lysik, R. M., Wilkie, D. P., Ramamurthy, N. & Lane, B. (1987) *Biochim. Biophys. Acta* **924,** 225–237.
24. Bordier, C. (1981) *J. Biol. Chem.* **256,** 1604–1607.
25. Heussen, C. & Dowdle, E. B. (1980) *Anal. Biochem.* **102,** 196–202.
26. Granelli-Piperno, A. & Reich, E. (1978) *J. Exp. Med.* **148,** 223–234.
27. Kelly, T., Molony, L. & Burridge, K. (1987) *J. Biol. Chem.* **262,** 17189–17199.
28. Deutsch, D. G. & Mertz, E. T. (1970) *Science* **170,** 1095–1096.
29. Barrett, A. J. (1973) *Biochem. J.* **131,** 809–822.
30. Barrett, A. J. (1977) in *Proteinases in Mammalian Cells and Tissues*, ed. Barrett, A. J. (Elsevier, Amsterdam), pp. 181–208.
31. Hebert, C. A. & Baker, J. B. (1988) *J. Cell Biol.* **106,** 1241–1247.
32. Pollanen, J., Hedman, K., Nielsen, L. S., Dano, K. & Vaheri, A. (1988) *J. Cell Biol.* **106,** 87–95.
33. Plow, E. F., Freany, D. E., Plescia, J. & Miles, L. A. (1986) *J. Cell Biol.* **103,** 2411–2420.
34. Stoppelli, M. P., Tacchetti, C., Cubellis, M. V., Hearing, V. J., Cassani, G., Appella, E. & Blasi, F. (1986) *Cell* **45,** 675–684.
35. Stephens, R. W., Pollanen, J., Tapiovaara, H., Leung, K. C., Sim, P. S., Salonen, E. M., Ronne, E., Behrendt, N., Dano, K. & Vaheri, A. (1989) *J. Cell Biol.* **108,** 1987–1995.
36. Dawid, I. B., French, T. C. & Buchanan, J. M. (1963) *J. Biol. Chem.* **238,** 2178–2185.