Eur J Cancer Clin Oncol, Vol. 21, No. 3, pp. 307-316, 1985.
Printed in Great Britain.

0277-5379/85$3.00+0.00

Characterization of an Extracellular Matrix-degrading Protease Derived from a Highly Metastatic Tumor Cell Line*

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Abstract—*A proteolytic activity associated with the microsomal fraction of L-5178Y/Esb tumor cells has been characterized. The enzyme has a molecular weight of 80-90 kD as determined by affinity-labelling with [^{3}H]DFP and SDS-gel electrophoresis. It cleaves ester substrates at the carboxyl position of lysine and arginine and can activate the proenzyme plasminogen. The enzyme is found to be associated with the plasma membranes of high and low metastatic tumor cell lines and is shed in high-molecular-weight form mainly by the high metastatic variant. The pH optimum for esterase and protease activities was 7.5-8.5. Although similar to trypsin in substrate specificity, the enzyme was not inhibited by lima-bean trypsin inhibitor but was inhibited by DFP, PMSF, aprotinin and leupeptin. Partially purified preparations of the protease can alone degrade ^{125}I-labelled endothelial cell extracellular matrix, pointing at the putative role of this enzyme in tumor invasion.*

INTRODUCTION

TRANSFER of malignant tumor cells from bloodstream to surrounding tissues is thought to occur in three principal stages: (i) attachment of tumor cells to capillary endothelial cells; (ii) degradation of the interstitial basal lamina; and (iii) transport from the invasion site to the growth site.

There is now considerable evidence that hydrolytic enzymes secreted or present at the surface of malignant tumor cells greatly influence their growth properties and invasive potential [1]. Cell-surface proteases, for instance, have been shown to degrade basement membranes and their extracellular matrix (ECM) [2-4]. Some enzymes often prefer a particular substrate, such as collagen [5-7], or alternatively degrade a wide range of substrates, including several components of the basement membrane. Broad specificity proteases of the latter type can usually activate latent enzymes such as plasminogen [8] and thus amplify their effect by initiating enzyme cascades [9-12]. Activation of serum plasminogen could thus lead to fibrinolysis and further damage of the basal lamina. In this way malignant tumors may utilize normal regulatory systems to promote their own growth.

Electron microscopic studies on tissue sections show local dissolution of the basement membrane in contact with metastatic tumor cells [13]. In some systems inhibitors of proteolytic enzymes block tumor cell penetration of ECM *in vitro* [14] or inhibit formation of metastatic foci *in vivo* [15].

In vitro studies using isolated endothelial cell extracellular matrix show local destruction by cultured metastatic tumor cells [2, 16]. Recent studies in the L-5178Y lymphoma system suggest that heparan sulfate degrading enzymes are

Accepted 30 July 1984.
*This work was supported by the Deutsche Forschungsgemeinschaft (SFB 136 and Project: Schi 46/4-1) and a DKFZ Israel cooperation program.

Abbreviations: DFP, diisopropylfluorophosphate; DTAF, dichlorotriazinylaminofluoresceine; ECM, extracellular matrix; PBS, phosphate-buffered saline; PA, plasminogen activator; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; pNA, *para*-nitroanilide; TLCK, *p*-tosyl-L-lysine chloromethylketone; TPCK, L-1-tosylamido-L-phenylethylchloromethylketone; TCA, trichloroacetic acid; SDS, Sodium dodecylsulfate.

required for the disruption of *in vitro* generated extracellular matrix by the highly metastatic subline L-5178Y/ES, abbreviated ESb [17]. In this study we have investigated and characterized another degradative enzyme of the Eb/ESb tumor system, namely a neutral protease which can also degrade distinct components of ECM.

MATERIALS AND METHODS

Cell culture of tumor cell lines and preparation of tumor cell lysates

Eb tumor cells, a subline of the methylcholanthrene-induced DBA/2 T-lymphoma L-5178Y, and ESb, a spontaneous highly metastatic variant thereof [18–20], were grown in RPMI 1640 medium (Grand Island Biological Co., Grand Island, NY) with 10% fetal calf serum, 2×10^{-5} M 2-mercaptoethanol (2-ME), 7 mM L-glutamine and antibiotics.

Before lysis the cells were washed twice in PBS and finally lysed using 0.01 M Tris buffer, pH 7.5, containing 1% Triton X-100 ('lysis-buffer'). After 1 hr on ice the lysates were cleared from particulate material by centrifugation in an Eppendorf tabletop centrifuge at 10,000 *g* and the clear supernatant was taken for determination of proteolytic activity.

To study the release of proteolytic activity the cells were grown in RPMI 1640, L-glutamine (7 nM), 2-ME, HEPES buffer (10 mM) and antibiotics.

Assays for protease activity

Plasminogen activator (PA) activity was measured using a modified photometric assay as originally described by Overwien *et al.* [21]. In short, 150 μl of cell lysate were mixed with 25 μl of substrate solution, consisting of 0.04 U/ml freshly thawed human plasminogen (No. P-1517, SIGMA, München, BRD) in 0.01 M Tris buffer, pH 7.5, containing 1% Triton X-100. After 18 hr at 37°C the absorption at 405 nm was directly read from the 96-well flat-bottomed microtiter plates using a multiscan apparatus (Titertek-Multiskan, Flow Laboratories). Controls were cell lysates with chromogenic substrate alone (no plasminogen).

To measure the direct cleavage of chromogenic substrates these were dissolved in Tris buffer at pH 7.5 or 8.0 at 2 mg/ml. Of this substrate solution, 20 μl were added to protease containing samples. After 18 or 24 hr at 37°C the absorbance at 405 nm was determined.

The standardization of the assay systems for (a) activation of plasminogen and (b) for direct cleavage of substrate No. 2288 has been performed with (i) urokinase (EC 3.4.21.31; Sigma Biochemicals; code No. U-1627), (ii) bovine pancreatic trypsin (EC 3.4.21.4; Sigma Biochemicals; code No. T-8642) and (iii) cell lysates of ESb cells. For the direct cleavage of substrate No. 2288 we found a linear relationship between protease activity and optical density at 405 nm in the range of 0.0020–0.6900 optical density when using substrate concentrations as given above.

Cell fractionation

Tumor cells were disrupted by the nitrogen-cavitation method and the subcellular components were then isolated by differential centrifugation. Afterwards the quality of the separation was controlled by marker enzyme analysis. The whole process followed conventional methods [22–24] and will be described in detail elsewhere for the Eb/ESb tumor system.

Column chromatography

For gel filtration, serum-free supernatants were concentrated by ammonium sulfate precipitation at 80% saturation. The precipitated material was redissolved in degassed 0.01 M Tris buffer, pH 8.0 ('running buffer'). Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) was equilibrated in running buffer. Two milliliters of the concentrated supernatant were layered on top of a column (1.2 × 60 cm) and eluted at 4°C with running buffer at a flow rate of 4 ml/hr. The protein content of 25 fractions was determined by absorbance at 280 nm. The column was calibrated with the molecular weight standards: catalase (230 kD), thyroglobulin (680 kD), dextran blue and DNP-alanine.

Labelling with ([^{3}H]DFP)

Five milligrams of plasmamembranes or of partially purified proteolytic activity were incubated with 75 μCi of [^{3}H]DFP (1.3-[^{3}H]DFP, NET-605 New England Nuclear, Boston, MA) in 0.1 M Tris buffer, pH 8.0, under permanent shaking for 18 hr. Afterwards the material was cooled in ice and precipitated with ice-cold TCA at a final concentration of 10%. The precipitate was washed twice with methanol/acetone (50:50), dried, resuspended in SDS electrophoresis sample buffer and electrophoresed on a 10% polyacrylamide slab gel. Marker proteins were labelled with DTAF (Sigma, D-8266) as described elsewhere [25] and identified after the run using a u.v.-light source. The gel was sliced into 2-mm pieces, treated with protosol/water (90:10), suspended in scintillation fluid and counted.

Production of extracellular matrix using bovine corneal endothelial cells and its degradation after labelling with ^{125}I

Bovine corneal endothelial cells were isolated from steer eyes and kept in culture as described

previously [17, 26]. Cells were routinely kept in Dulbecco's modified Eagle's medium (DMEM, H-16) supplemented with 5% fetal calf serum and gentamycin (50 μg/ml) at 37°C in a 10% CO_2 humidified atmosphere. Corneal endothelial cells were dissociated with 0.05% trypsin/0.02% versene solution and plated at an initial density of 4×10^4 cells per well in a 24-well tissue culture plate (Falcon No. 3524, Oxnard, CA) and kept in growth medium (see above). FGF was added to 100 ng/ml every other day until the cells were nearly confluent. The cultures were incubated for an additional 6-8 days in the absence of growth factor. To prepare the cell-free ECM the cultures were washed once with phosphate-buffered saline (PBS) and exposed (30 min of gentle shaking at room temperature) to 0.05% Triton X-100 in PBS (v/v). The cell layer was dissolved, leaving the underlying ECM intact and firmly attached to the entire tissue culture dish. Remaining nuclei and cytoskeletal particles were removed by a 2- to 3-min exposure to 0.025 N NH_4OH followed by four washes in PBS.

The ECM fixed to the dishes was then labelled following the lactoperoxidase method of Teng and Chen [27].

Fifty microcuries of ^{125}I, together with lactoperoxidase (0.04 mg), glucose (0.9 mg) and glucose oxidase (0.1 U) in 1 ml PBS, were added to each well. After 15 min at room temperature the wells were washed 5 times in Ca^{2+}- and Mg^{2+}-free PBS containing 0.5 mM PMSF.

Partially purified proteolytic activity, intact cells or cellular subfractions were added to these wells. Aliquots of the supernatants were removed at certain times, dried on glass-fiber filters, rinsed first for 30 min in ice-cold 10% TCA and twice in ice-cold 5% TCA, dried and counted.

RESULTS

Proteolytic activities associated with high and low metastatic sublines of the L-5178Y lymphoma

In an attempt to identify degradative proteolytic enzymes associated with the low metastatic T lymphoma L-5178Y/Eb (Eb) and its highly metastatic variant L-5178Y/ESb (ESb) we tested lysates of these cell lines for PA activity. The extreme sensitivity of this two-step enzymatic reaction allows the detection of minute amounts of proteolytic activity. As shown in Fig. 1, cell lysates of both ESb and Eb tumor cells contain PA activity. It was also found that higher concentrations of cell lysate were capable of cleaving chromogenic substrate in the absence of plasminogen.

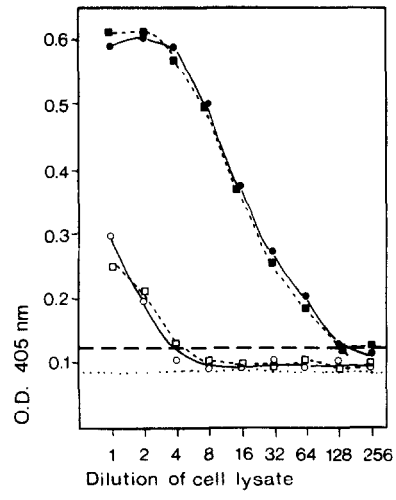

Fig. 1. Plasminogen activator activity in Eb and ESb tumor cell lysates. 1 × 10⁷ ESb or Eb tumor cells were lysed in 1 ml *of lysisbuffer.* 150 μl *of this lysate were diluted by successive* 1:2 *dilutions in a flat-bottomed microtiter plate. After addition of substrate solution the reaction was run for* 24 hr *at* 37°C. *Optical density at* 405 nm *was directly determined from the plate. The data are expressed as mean value from six replicate determinations. (■——■; ●——●) Eb or ESb cell lysate together with plasminogen and chromogenic substrate No.* 2251. *(□——□; ○——○) Eb of ESb cell lysate together with chromogenic substrate No.* 2251 *only. (■; □) Eb cell lysate. (●; ○) ESb cell lysate.*

Substrate specificity of the tumor-derived protease(s)

It is known that plasminogen can be activated by a variety of different proteolytic enzymes, such as trypsin, alpha-chymotrypsin, kallikrein, thrombin, urokinase and even a streptokinase-plasmin complex [8]. The observation that high concentrations of cell lysate cause direct cleavage of chromogenic substrate prompted us to study the cleavage specificity using a panel of defined chromogenic substrates without the intermediate step of plasmin generation.

ESb tumor cell lysates were tested on a panel of defined low-molecular-weight chromogenic substrates (Table 1). At pH 7.5 substrates with lysine or arginine carrying the pNA group were cleaved most rapidly. In this respect the enzyme appears similar to trypsin in its substrate specificity. The substrate Ile-Pro-Arg-pNA (No. 2288) was most rapidly cleaved and thus was chosen as the standard for further studies.

pH-optimum of the tumor-associated protease

ESb-derived microsomes (see also below) were incubated with substrate No. 2288 at various pH values. The maximum cleavage rate was obtained at pH 7.5-8.5 (Fig. 2). These results suggest that the activity is not of lysosomal origin, since lysosomal proteases normally function best in acidic conditions [28-30]. A pH-optimum of around 8.0 is also required for optimal degrada-

Table 1. Relative activity of cell lysate on chromogenic substrates at pH 7.5

Amino acid composition of chromogenic substrate	(code No.)	Absorbance at 405 nm ($\times 10^3$)	% relative activity
Pro-pNA	(2519)	0	0
Trp-pNA	(2532)	0	0
Glu-Pro-Val-pNA	(2484)	0	0
Phe-pNA	(2545)	0	0
Ala-pNA	(2483)	0	0
Met-pNA	(2327)	0	0
Arg-Pro-Tyr-pNA	(2586)	0	0
Val-Leu-Lys-pNA	(2251)	157	81.8
Ile-Glu(-OR)-Gly-Arg-pNA	(2222)	20	10.4
-Gly-Arg-pNA	(2322)	6	3.1
Pro-Phe-Arg-pNA	(2302)	102	53.1
Val-Leu-Arg-pNA	(2266)	182	94.8
Ile-Pro-Arg-pNA	(2288)	192	100.0

3×10^7 ESb cells were lysed in 3 ml lysis buffer. 100 ml of this lysate were co-incubated with 50 ml of substrate stock solution (containing 2 mg/ml of the respective chromogenic substrate in 0.1 M Tris buffer, pH 8.0). As low controls served (i) chromogenic substrate with lysis buffer only, and (ii) cell lysate with 0.1 Tris buffer only. The low control values were subtracted from the data obtained from duplicate determinations in presence of cell lysate and chromogenic substrate. The reaction was run for 24 hr at 37°C and pH 8.0.

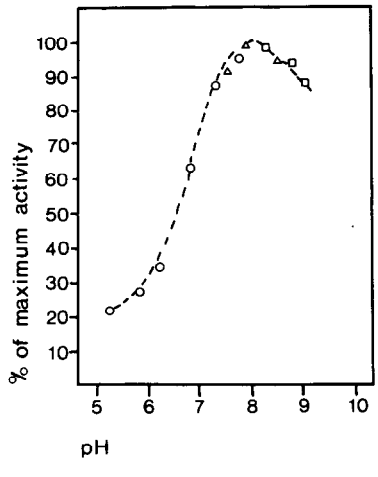

Fig. 2. pH-optimum of the tumor-derived plasma membrane-associated proteolytic activity. Plasma membranes (100 μg per value) from ESb tumor cells were mixed with 0.1 mg of chromogenic substrate No. 2288 in 1 ml of the respective buffer: (○) 0.1 M phosphate buffer, (△) 0.1 M Tris buffer and (□) 0.1 M borate buffer. The enzyme reaction was run for 18 hr at 37°C. The data are expressed as '% of maximum activity', which ws obtained using 0.1 M Tris buffer, pH 8.0 (optical density at 405 nm: 0.257).

tion of ECM (see below and Fig. 7C). When partially purified protease obtained from conditioned media (see below) was tested for its pH-optimum we observed an identical pH dependence (data not shown).

Subcellular distribution of proteolytic activities in high and low metastatic tumor sublines

Subcellular fractions of Eb- and ESb-type tumor cells were obtained by standard methods involving nitrogen cavitation and differential centrifugation. Marker enzyme analysis, shown in Table 2, indicates that the various subfractions had been sufficiently purified. An aliquot of each fraction (5 mg protein) was mixed with 1 ml of 0.1 M Tris buffer, pH 8.0, and 50 μl of substrate No. 2288 (2 mg/ml). After 24 hr incubation at 37°C the particulate material was pelleted at 10,000 *g* for 10 min and the absorbance of the supernatant at 405 nm was determined.

The two cellular subfractions which contained most of the proteolytic activity were (i) the mitochondrial/lysosomal granules and (ii) the microsomes, which are enriched from the cell's plasma membrane (see marker enzymes; Table 2). Both high and low metastatic sublines possessed approximately equal levels of specific activity. Although most proteases of lysosomal origin have maximum activity at acid pH, some are also active at neutral pH [30]. When testing the pH-dependency of esterase activity in the lysosomal fraction we found activity over a broad pH range, with one major peak at pH 5 and a minor peak (about 30% of total activity) at pH 8 (data not shown). In contrast, the microsome fraction showed only one peak of enzyme activity (see Fig. 2).

Release of proteolytic activity in vitro

Eb- and ESb-type tumor cells were cultured in serum-free medium as described under Materials and Methods. After defined time periods (Fig. 3) the cell viability in the cultures and the proteolytic activity in the cell free supernatants was determined. As can be seen from Fig. 3b, both tumor cell lines could be cultured without serum for at least 24 hr without significant loss of viability.

Table 2. Localization of proteolytic activity and marker enzyme activities in cellular subfractions of Eb and ESb tumor cells

Cellular subfraction	Absorbance at 405 nm (× 10^4 mg protein)	% of total proteolytic activity	DNA*	SDH†	β′-Glucosidase†	LDH†	5′Nucleotidase†
			Eb				
Homogenate	20.3	100	146.5	4.3	17.5	211.4	22.5
Nuclei	27.11	16	375.7	2.1	12.0	49.1	11.9
Mitochondria/ lysosomal granules	163.87	32	189.9	30.4	78.4	49.7	15.6
Cytosol	22.50	17	4.7	0	4.5	452.3	8.7
Microsomes	96.43	35	9.8	0	8.3	1.1	67.5
			Esb				
Homogenate	29.27	100	160.4	4.0	13.6	182.4	22.8
Nuclei	30.81	15	358.3	1.5	8.0	48.0	13.6
Mitochondria/ Lysosomal granules	166.85	33	286.5	29.3	66.2	43.2	17.4
Cytosol	70.83	14	8.0	0	4.5	436.8	8.7
Microsomes	116.67	38	14.8	0	9.1	1.05	83.7

5 mg of protein of the respective cellular subfractions were incubated with 0.1 mg of the chromogenic substrate No. 2288 in 1 ml Tris buffer, pH 8.0. The enzymatic reaction was run for 18 hr at 37°C. Afterwards the reaction mixture was spun in an Eppoendorf table-top centrifuge at 10,000 **g** and the absorption at 405 nm was determined from the clear supernatants.

*μg/mg protein.

†μmol/mg protein/min.

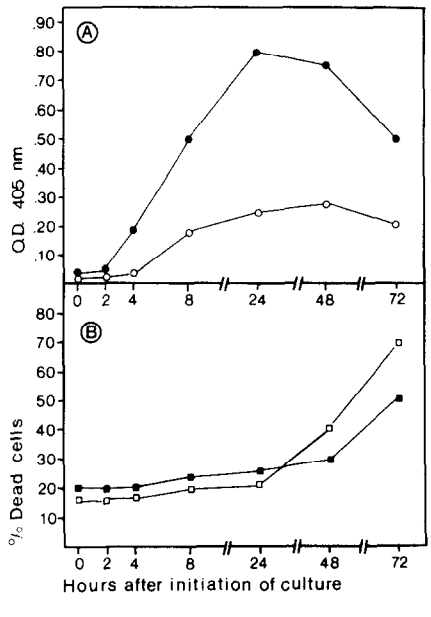

Fig. 3. Release of proteolytic activity by Eb and Esb tumor cells in vitro. *ESb (●/■) or Eb (○/□) tumor cells were cultured at an initial density of* 5 × 10^5 cells/ml *in medium supplemented with* 2-*ME* (5 × 10^{-5} M *final concentration), HEPES buffer* (10 mM), L-*glutamine* (4 mM) *and penicillin-streptomycin* (200 U/ml). *After various intervals of time, as indicated in the abscissa, supernatant was collected from the cultures and tested for proteolytic activity as described in Materials and Methods (A). The enzyme reaction was run for* 18 hr *at* 37°C. *Viability was determined by trypan blue dye exclusion (B).*

The proteolytic activity in the supernatant increased as a function of the time of culture. The highly metastatic ESb cells released about 3-4 times as much proteolytic activity as the low metastatic lymphoma cell line (Fig. 3A).

Partial purification of protease released by ESb tumor cells into serum-free culture medium

Seven hundred and fifty milliliters of serum-free medium conditioned by ESb tumor cells were concentrated by treatment with ammonium sulfate at 80% saturation and chromatographed using Sepharose CL-4B. The elution profile is shown in Fig. 4. The fractions were tested for optical density at 280 nm and for proteolytic activity on either substrate 2288 or on labelled extracellular matrix (ECM). The proteolytic activity eluted as two major peaks. The first one in the position of the void volume was associated with particulate material and consisted presumably of plasma membrane particles which were shed into the culture medium and were not disrupted by treatment with ammonium sulfate.

Proteolytic activity was also eluted in the size range of 10^6 D. The specific activity of the enzyme was enriched approximately 30-fold compared with the ammonium sulfate concentrate.

It was also found that the fractions which contained proteolytic activity on chromogenic substrate also caused degradation of ^{125}I-labelled subendothelial extracellular matrix (Fig. 4).

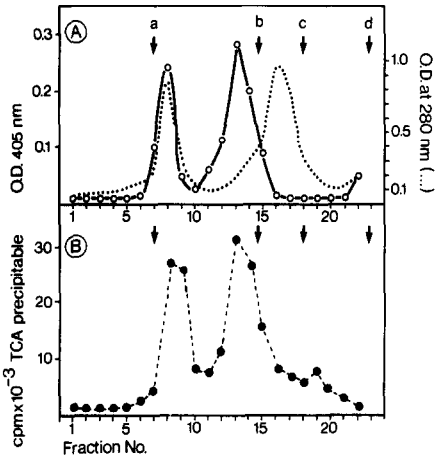

Fig. 4. Partial purification of proteolytic activity from conditioned medium using molecular-sieve chromatography. 750 ml of serum-free medium conditioned by ESb tumor cells were concentrated by ammonium sulfate precipitation resulting in 10 ml concentrate with a total of 270 mg protein. 2 ml thereof were chromatographed using a Sepharose CL-4B column. The fractions obtained were tested for cleavage of chromogenic substrate No. 2288 (O——O; part A) or optical density at 2.80 nm (· · · · ·; part A). The fractions were also assayed for degradation of ^{125}I-labelled ECM (part B). Molecular weight standards were: thyroglobulin (b; 680 kD), catalase (c; 230 kD), dextran blue (a) and phenol red (d).

Inhibition studies

A classification of the partially purified protease was attempted by means of defined protease inhibitors. Aliquots of the pooled active fractions (Nos. 12–14) were pretreated with various high-or low-molecular-weight inhibitors and tested for residual proteolytic activity on substrate No. 2288. As can be seen from Fig. 5, the proteolytic activity is inhibited in a dose-dependent manner by increasing amounts of DFP and PMSF, suggesting a serine residue at the active site which became alkylated by these agents. Furthermore, inhibition by TLCK and leupeptin but not by TPCK suggests that the enzyme is trypsin-like. Finally, inhibition studies with the high-molecular-weight inhibitors aprotinin (inhibition) and lima-bean trypsin inhibitor (no inhibition) indicates that the protease is different from normal pancreatic trypsin since this enzyme is equally well counteracted by both inhibitors (Fig. 5K, L).

Affinity labelling of tumor-derived protease with [^{3}H]DFP and analysis by SDS-PAGE

Irreversible binding of DFP to serine residues at the active site permits radioactive affinity labelling of the protease. Plasma membranes (Fig. 6A) and partially purified protease from serum-free supernatants (Fig. 6B) were labelled with 75 μCi of [^{3}H]DFP overnight at room temperature. The TCA precipitated material was washed twice in methanol/acetone and separated using a 10% polyacrylamide slab gel under non-reducing conditions. The gel was then sliced into 2-mm pieces, treated with protosol and the radioactivity determined after addition of scintillation fluid.

As can be seen from Fig. 6, both the plasma membrane-associated protease (6A) and the partially purified protease activity (6B) were found to contain one major ^{3}H-labelled component with an apparent molecular weight of 84,000 D. Under reducing conditions the apparent molecular weight was not significantly changed (not shown). We thus conclude that the

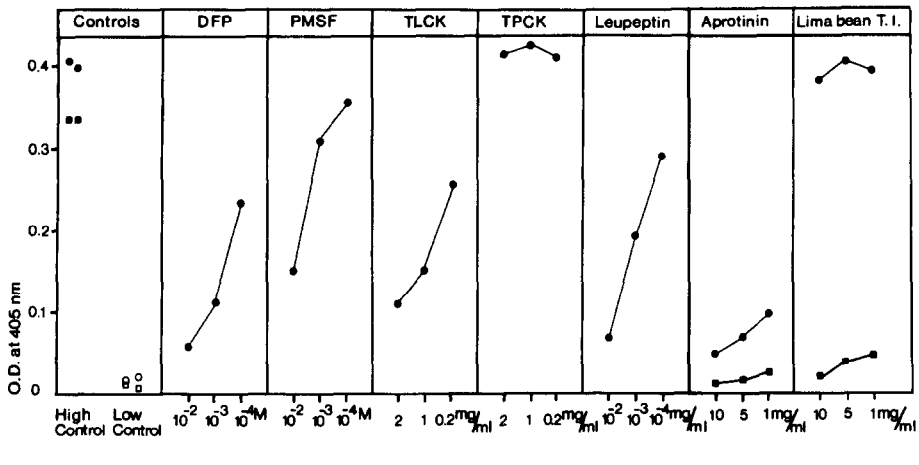

Fig. 5. Inhibition studies using partially purified proteolytic activity after gel filtration. Partially purified proteolytic activity obtained after gel filtration (20 μg per value) or bovine pancreatic trypsin (2 μg per value) were reacted in 0.01 M Tris buffer at pH 8.0 for 6 hr with various enzyme inhibitors. Then 0.1 mg of the chromogenic substrate No. 2288 were added to this mixture. After 18 hr (in the case of the ESb-derived enzyme) or 2 hr (in the case of pancreatic trypsin) at 37°C the absorbance at 405 nm was determined. Controls were: high control: enzyme and chromogenic substrate (●;■); low control: enzyme only (O;□). The results obtained with the Esb-derived enzyme are depicted as circles (●,O; ●——●) and the results obtained with trypsin as quadrats (■; □; ■——■).

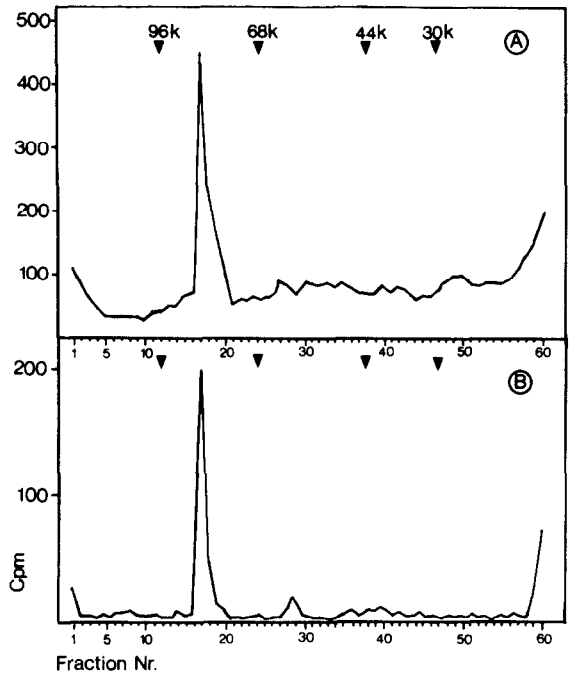

Fig. 6. Affinity labelling of proteolytic activity with [^{3}H]*DFP. Plasma membranes prepared from ESb tumor cells (A) or partially purified proteolytic activity obtained after gel filtration of medium conditioned by ESb tumor cells (B) were reacted with* [^{3}H]*DFP as described above. The chromatography was performed overnight at* 11 mA *using a* 10% *polyacrylamide slab gel. Marker proteins were labelled with DTAF and their position was identified using a u.v.-light source. Marker proteins were: phosphorylase-B* (96 kD), *bovine serum albumin* (68 kD), *ovalbumin* (44 kD) *and carboanhydrase* (30 kD).

membrane-bound and shed enzymes have a similar active subunit size.

Degradation of labelled ECM by intact tumor cells and by partially purified protease

As shown in Fig. 7A, intact tumor cells release ^{125}I-labelled TCA precipitable fragments from labelled ECM in a time-dependent fashion. It was possible to partially inhibit the degradation by addition of the inhibitor leupeptin, which is relatively non-toxic to cells and did not affect viability at the concentrations used. Leupeptin and DFP both strongly inhibited matrix degradation by partially purified enzyme (Fig. 7B). The pH-optimum for degradation of ^{125}I-labelled ECM by partially purified enzyme was found to be 7.5–8.5 (Fig. 7C).

DISCUSSION

As a further step in understanding the metastatic behavior of ESb tumor cells we have looked for hydrolytic enzyme activities expressed by this cell line. In this paper we describe the isolation and partial characterization of a proteolytic activity from these cells. The enzyme is trypsin-like with respect to its substrate specificity, but is clearly different to pancreatic trypsin in its pattern of inhibition. The enzyme is present in large amounts at the plasma membrane and also occurs in the serum-free supernates of cultured tumor cells. Although high (ESb) and low (Eb) metastatic sublines of the tumor do not differ in

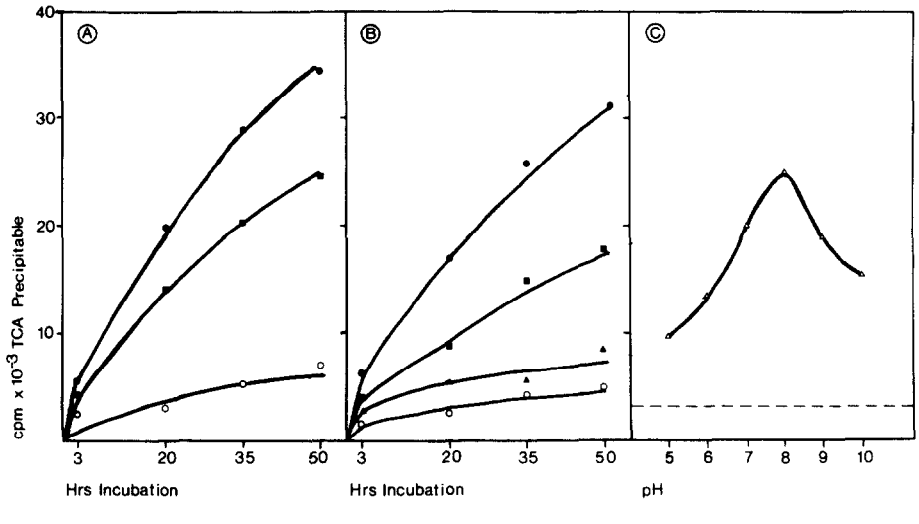

Fig. 7. Degradation of radiolabelled ECM. ECM labelled with ^{125}I *was subjected to degradation by intact cells (A) or partially purified proteolytic activity obtained after gel filtration (B). After various intervals of time an aliquot of the supernatants was removed, precipitated with TCA and counted. Part A shows: incubation of* [^{125}I]*ECM with cells only* (●), *with cells together with* 0.1 mg/ml *leupeptin* (■) *or with serum-free medium only* (○). *Part B shows: degradation by partially purified protease* (●), *by partially purified protease in the presence of* 0.1 mg/ml *leupeptin* (■) *and partially purified protease in the presence of* 10^{-2}M *DFP* (△). *Low control was obtained by incubation with* 0.1 M *Tris buffer, pH* 8.0 (○). *Part C shows the degradation of labelled ECM by partially purified protease at different pH values.*

the amount of cell-associated enzyme, the high metastatic line releases 3–4 times as much activity into the medium as does the low metastatic line.

The activity is released in the form of high-molecular-weight complexes of around 10^6 D which are precipitated by ammonium sulfate. Little is known at present about the composition of these complexes. High-molecular-weight proteolytic activity could be dissociated by the detergent SDS and separated from other components by gel electrophoresis. Affinity labelling of the enzyme with [^{3}H]DFP clearly shows that both the cell-bound and released enzymes have a subunit size of about 84 kD. Isolation of proteolytic activities in complex form has been shown in other systems [31] and may represent a way of anchoring the enzyme to a matrix, such as the plasma membrane. SDS was found to dissociate the complex, giving an active subunit size of 84 kD. Affinity-labelling experiments show that [^{3}H]DFP labels proteins of similar size from plasma membrane and released material, strongly suggesting that the labelled components are the same in both sources. Preliminary results now show that esterase activity can be recovered from SDS-gels in the 80,000- to 90,000-D region (not shown). We have also found (data not shown) that enzyme activity can be partially set free from the membranes by treatment with high salt concentrations, suggesting that the protease is associated with but not integrated into the membrane.

Broad-specificity proteases have been isolated from the membranes or culture supernatants of different cell types [3, 4, 32–35]. The detection method is usually the PA assay. In this case we have shown esterase activity by direct cleavage of chromogenic substrates and protease activity by destruction of [^{125}I]ECM. Clearly, the enzyme is able to function without exogenous proenzymes present. Another tumor-associated protease capable of degrading ECM was described by Liotta and co-workers [5, 6]. Although this enzyme differs from the enzyme described here in substrate specificity, the latter enzyme appears as an inactive form of collagenase with a molecular weight of about 80 kD and requires activation by proteolytic cleavage to express collagenolytic activity. This activation can be carried out by either plasminogen activators directly or via plasmin [7].

Further, recent reports describe the isolation of a trypsin-like serine protease from the plasma membranes of the rat carcinosarcoma Walker 259 [35]. The [^{3}H]DFP-labelled subunit of this enzyme has a molecular weight of 23 kD and the enzyme can be absorbed to and desorbed from soybean trypsin inhibitor coupled to Sepharose 4B. The fact that we did not succeed with that kind of purification in case of the Esb-derived protease and the fact that the [^{3}H]DFP-labelled subunit has a molecular weight of about 84 kD speak against the identity of the two proteases.

Another class of serine proteases that has to be discussed in this context are the so-called plasminogen activators which have also been analyzed in murine cells. Multiple molecular weight forms of plasminogen activators have been found [36], with a distribution differing between various cell lines. Recent findings indicate that two immunologically distinctive forms of plasminogen activators exist: (i) an M_r 70-kD type (usually designated tissue-type PA) and (ii) an M_r 50-kD type (often designated urokinase-type PA) [37, 38]. As to the identity of the enzyme described in this communication, it is obvious that the urokinase type (M_r 50 kD) is excluded by the difference in size. There may, however, be a relation between the described protease and tissue-type PA. The fact that we can detect the enzyme without addition of exogenous plasminogen points at a possible role of this protease beyond activation of plasminogen. Furthermore, it is known that tissue-type PA requires fibrin as a cofactor in order to detect the proteolytic activity [39]. In the present system there was no fibrin present during the protease assays, which argues against the protease to be a classical tissue-type PA-activator. Future studies using specific antisera, monoclonal antibodies or DNA technology might resolve the problem of identity of the here-described tumor-associated protease and its relation to already known enzymes, such as tissue-type PA.

We speculate here that although the ability to produce proteolytic enzymes does not necessarily make a cell invasive, it is probably a requirement for metastasis. Thus blocking of protease activity by DFP or leupeptin *in vitro* prevents matrix degradation by soluble enzyme. The observation that leupeptin is less effective in inhibiting matrix degradation by whole ESb cells suggests that the inhibitor cannot reach the site of proteolytic activity. The large amounts of protease released by ESb cells into culture supernates, if retained in the space between tumor cell and matrix, would give rise to a high local enzyme concentration. Furthermore, enzyme confined to such a space would be protected from circulating proteolytic enzyme inhibitors. The pH-optima of both esterase and protease activities (7.5–8.5) indicate that the enzyme functions well at physiological pH values experienced at the cell boundary. Taken together, these three factors would lead to efficient local breakdown of extracellular matrices in contact with the tumor cell.

We present evidence here for a tumor-associated protease which can function independently of other enzymes. It is not clear, however, whether other enzymes may be recruited *in vivo* to degrade basement membranes. If, as we speculate, degradation occurs at sites of cell-matrix contact, then such supplementary enzymes must be present on the same invasive tumor cell. We have recently shown that ESb cells carry a glycolytic activity which can degrade sulfated proteoglycans, another major component of ECM [17].

It is important to stress, however, that each tumor system should be considered separate with regard to membrane enzyme activities. Clearly, not all metastatic tumor cells express the same enzymes and the repertoire of enzymes available at the cellular surface may influence the location of secondary tumors. The relationships between enzymes on different cell types and their localization during the invasive process in complex *in vitro* systems (such as three-dimensional organ isolates) [40] or *in vivo* will be investigated by immunochemical studies using either specific antisera or monoclonal antibodies.

Acknowledgements—The expert technical assistance of Mrs Karin Reimbold is gratefully acknowledged.

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