Properties of a Plasma Membrane-associated Cathepsin B-like Cysteine Proteinase in Metastatic B16 Melanoma Variants

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ABSTRACT

Activities of a cathepsin B-like cysteine proteinase have previously been observed to correlate with the malignancy of several animal and human tumors. Plasma membrane fractions of some of these tumors have been found to be enriched in cathepsin B-like activity. We have determined the subcellular distribution of this enzyme and three additional lysosomal hydrolases (cathepsin H, β-hexosaminidase, and β-glucuronidase) in normal murine liver and six metastatic variants of the B16 melanoma. The tissues were fractionated initially by differential centrifugation followed by Percoll density gradient centrifugation of the light mitochondrial fraction. Two fractions were obtained: an L-2 fraction enriched in all four lysosomal hydrolases; and an L-1 fraction enriched in a marker enzyme for the plasma membrane. Cathepsin B-like and β-hexosaminidase activities, but not the other hydrolase activities, were also found to be enriched in the L-1 fractions of the metastatic B16 tumors.

We explored the nature of the association of the cathepsin B-like activity with the plasma membrane using fractions from the spontaneously metastatic B16 amelanotic melanoma. Activity could not be dissociated from the plasma membrane fraction by washing with a physiological salt solution suggesting that it was not adsorbed to this fraction nonspecifically, nor could it be displaced by mannose 6-phosphate or other sugars which compete for binding to the known lysosomal receptors. High salt concentrations, low concentrations of the mild detergent saponin, mild acidification, or phosphatidylinositol-specific phospholipase C did not elute the cathepsin B-like activity. However, activity was eluted by exposure to 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate, a detergent used in the purification of integral membrane proteins. The B16 amelanotic melanoma plasma membrane-associated cathepsin B-like activity had a slightly higher pH optimum and was resistant to inactivation by neutral pH and to inhibition by three low molecular weight inhibitors of cysteine proteinases. The Kᵢ values for inhibition by leupeptin and steptin A were 20-fold higher. The presence of a cathepsin B-like cysteine proteinase at the surface of metastatic tumor cells, particularly in a form which can retain activity at physiological pH and retain activity in the presence of extracellular proteinase inhibitors, may contribute to the focal dissolution of the extracellular matrix observed at sites of contact with invading tumor cells.

INTRODUCTION

Many different proteolytic enzymes have been implicated in tumor cell invasion and metastasis (1–3). In our laboratory we have been interested in one of these, CB, the activity of which has been correlated with the malignancy or metastatic potential of human tumors or animal tumor models, respectively (3–11). Cathepsin B is normally considered to be a lysosomal cysteine proteinase. However, activity (primarily latent) similar to that of cathepsin B has been measured in the media of tumor cells and explants in culture (5, 7), in the ascites fluid of women with ovarian carcinomas (12), and in the media of mammary gland explants (13). In addition, several laboratories including our own have reported that in animal and human tumor cells CB activity is found in association with the plasma membrane (8, 10, 14–17).

Lysosomal enzymes are processed and transported to the lysosomes via receptor-dependent pathways such as the mannose 6-phosphate pathway (18). This intracellular sorting has been extensively studied in fibroblasts for certain lysosomal enzymes not including cathepsin B. Defects in these transport systems have been identified in fibroblasts from patients with lysosomal storage diseases (19) and in some murine tumor cell lines (20, 21). In both cases the defects result in increased secretion of lysosomal enzymes. In I-cell fibroblasts β-hexosaminidase in addition to being secreted was found to be localized in small vesicles associated with the plasma membrane (22).

In three murine melanomas we have found an association of CB and β-hexosaminidase with the plasma membrane which accords with increased metastatic potential (10). The presence of these activities at the membrane of metastatic tumor cells suggests that these enzymes could participate in local dissolution of the extracellular matrices during the invasive stages of the metastatic cascade. These two enzymes working in concert might lead to an enhanced dissolution similar to that observed for trypsin digestion of aortic basement membrane subsequent to treatment with β-hexosaminidase (23). In the present study we have extended our investigations to determine the subcellular distributions of CB, β-hexosaminidase, and two additional lysosomal hydrolases (as well as of marker enzymes for other subcellular organelles) in six metastatic variants of the murine B16 melanoma. In addition we have determined the nature of the association of CB with the plasma membrane fraction of B16a tumor cells and compared the properties of this CB to those of lysosomal cathepsin B in the same cells.

MATERIALS AND METHODS

Reagents. Percoll was purchased from Sigma (St. Louis, MO). Substrates for assay of cysteine proteinases (see below) were obtained from Enzyme Systems Products (Livermore, CA) and for assay of glycosidases from Research Products International (Elk Grove Village, IL). Phosphatidylinositol-specific phospholipase C was a gift of Dr. M. G. Low (Oklahoma Medical Research Foundation). All other chemicals were of reagent grade and were obtained from commercial sources.

Tissues. Metastatic variants of the murine B16 melanoma (B16-BL6, B16-B15b, B16-013) were provided through the courtesy of Dr. Garth L. Nicolson of the M. D. Anderson Hospital and Tumor Institute, Houston, TX. An amelanotic variant of the murine B16 melanoma...
and the B16-F1 and B16-F10 metastatic variants were obtained from the Division of Cancer Treatment (National Cancer Institute) human and animal tumor bank. All the tumor lines were frozen down in liquid N₂ immediately upon receipt. The tumor lines were propagated in vivo by s.c. injection of cells from frozen stocks or cellular brei from s.c. tumors into the left axillary region of syngeneic male (female for B16-013) C57BL/6J mice. In order to maintain their metastatic phenotypes, the tumor lines were routinely restarted from liquid N₂ frozen stocks after six isotransplant generations in vivo. The livers were obtained from male C57BL/6J mice without tumors.

Metastasis Studies. The metastatic potential of the B16 variants was verified by methods described previously (24). Briefly, viable tumor cells were isolated from s.c. tumors by collagenase digestion and centrifugal elutriation (9). Tumor cells were counted on a Model ZBI Coulter Counter. Viability was determined by trypan blue dye exclusion. Cell aliquots were injected either into the tail vein or s.c. into the left axillary region of syngeneic male (female for B16-013) mice. At 21 or 28 days post-tumor cell injection the mice were sacrificed by cervical dislocation. The lungs were removed and fixed in Bouin’s solution. Macroscopic pulmonary metastases were counted using a dissecting microscope. A minimum of 12 mice were used per tumor line per metastasis study. These experiments have been repeated from 3 to 100 times for the 6 melanoma lines over a period of 3 years to ensure that each tumor line maintains its metastatic phenotype.

Subcellular Fractionation. A 5-g pool of normal livers or s.c. tumors (weight range, 0.8–2.5 g) was minced in ice cold homogenization buffer (250 mM sucrose-25 mM MES-1 mM EDTA, pH 6.5). All further manipulations were performed at 4°C. The minced tissue was homogenized using 2 × 5 strokes in a Potter-Elvehjem homogenizer at maximum speed (1200 rpm) separated by an intermediate cooling period in an ice slurry. The homogenate was filtered through four layers of cheesecloth and the volume adjusted to 10% (w/v) with the homogenization buffer.

The homogenate was centrifuged at 500 × g for 11 min to yield a nuclear pellet; the pellet was washed in 20 ml of buffer and recentrifuged. The two nuclear supernatants were pooled and centrifuged at 7000 × g for 10 min to yield a heavy mitochondrial pellet; this pellet was washed and recentrifuged. The supernatants were then centrifuged at 15,000 × g for 19 min to yield a light mitochondrial pellet. The light mitochondrial pellet was also washed and recentrifuged.

The final light mitochondrial pellet was further purified by density gradient centrifugation on 30% isosmotic Percoll prepared in homogenization buffer. The self-forming gradient was generated by centrifugation for 16 min at 60,000 × g. Fractions were collected in one of two ways: (a) two visible bands (L-1 and L-2) were aspirated with a Pasteur pipet; or (b) 0.4-m1 fractions were collected from the bottom of the tube using a peristaltic pump. In the latter case the homogenization buffer used was 250 mM sucrose-25 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid-1 mM EDTA, pH 7.3. Since purified cathepsin B is inactivated above pH 7 (25) and most Percoll fractionations are performed at neutral pH (26), we evaluated the subcellular distribution of cathepsin B using homogenization buffers at both pH 6.5 and pH 7.3. The distribution of CB was identical (data not shown; Ref. 10). Density marker beads were centrifuged in parallel gradients to determine density. The L-1 and L-2 bands (1.045 and 1.97 g/ml, respectively) collected by aspiration were separated from the Percoll medium after diluting in the homogenization buffer and recentrifuging at 100,000 × g for 55 min. Fractions were resuspended in or diluted with homogenization buffer, quick frozen, and stored at −70°C for enzyme assays.

Triton X-100 (0.1% (v/v) final concentration) was added to all aliquots except those for assay of Na⁺,K⁺-ATPase. Fractions were used immediately (without freezing) for comparison of properties of the L-1 and L-2 fractions.

Enzyme Assays. Cathepsin B (EC 3.4.22.1) was assayed at pH 6.2 using either carbobenzyloxyarginylarginyl-4-methoxy-β-naphthylamide (10) or carbobenzyloxyarginylarginyl-7-amino-4-methylcoumarin as substrate. In the former case Vₘₐₓ was obtained from Lineweaver and Burk plots of initial velocities of enzyme reactions; corrections were made for quenching by the sample and for nonenzymatic hydrolysis of substrate. In the latter case inhibition experiments were performed in an open microcuvet in a Perkin-Elmer 650-10 LC spectrophotometer fitted with a temperature-controlled cuvet holder and a chart recorder. The progress of the reaction was followed at 25°C by monitoring the release of 7-amino-4-methylcoumarin with excitation at 383 nm and emission at 455 nm. The initial volume of the incubation mixture was 500 μl and the inhibitor was added in 5- to 10-μl volumes unless indicated differently.

Cathepsin H (EC 3.4.22.–) was assayed as described previously (10, 27) at pH 6.8 using L-arginyl-4-methoxy-β-naphthylamide as substrate. Two lysosomal glycosidases [β-hexosaminidase (EC 3.2.1.30) and β-GLU (EC 3.2.1.31)] were determined using 4-methylumbelliferyl substrates as described previously (28). Activity of ouabain-sensitive (Na⁺/K⁺-activated) ATPase (EC 3.6.1.3) was determined according to the method of Jorgensen (29). Glucose-6-phosphatase was assayed as described by Aronson and Touster (30). Galactosyltransferase was assayed according to the procedure of Brew et al. (31). Protein was determined by the Bradford procedure (32) using bovine serum albumin as standard. Concentration of reaction products was derived from standard curves by linear regression analysis.

pH Stability. The inactivation of plasma membrane-associated CB and lysosomal cathepsin B at neutral pH was determined by preincubating freshly isolated samples at pH 7.3 and 37°C for 5 to 15 min and then assaying at pH 6.2 as described above.

pH Optimum. The pH optimum of plasma membrane-associated CB and lysosomal cathepsin B was determined by performing the assay in MES and piperazine-N,N'-bis(ethanesulfonic acid) buffers over the pH ranges 5.4 to 7.0 and 6.9 to 7.4, respectively, and incubating for 5, 25, and 45 min to assure linearity. The assay was modified from that described previously (10); 400 μl of the corresponding buffer and 400 μl of 1 N HCI stop solution were used.

Effect of Mild Acidification. The effect of mild acidification on the elution of plasma membrane-associated CB activity was determined in the presence of 25 μg/ml of saponin. This concentration of the detergent has been shown to form stable holes in plasma membrane (33). The sample (homogenization) buffer was removed by centrifuging for 15 min at 60,000 × g; the pellet was resuspended in homogenization buffer titrated to pH 3.0 and incubated for 15 min at 25°C. The controls were incubated at pH 6.5 and without saponin. The samples were centrifuged for 60 min at 160,000 × g, the pellets were resuspended in the homogenization buffer, and Triton X-100 was added to a final concentration of 0.1%. The pellets and the supernatants were assayed for CB activity as described.

Dissociation of CB from Plasma Membrane Fractions. For the solubilization (or elution) of plasma membrane-associated CB, fresh samples (sample buffers removed as described above) were preincubated at 4°C or 25°C in the presence of the corresponding solubilizing or eluting agent for the periods indicated. Solubilizing or eluting agents were brought to the designated concentrations in homogenization buffer. The pellets were then isolated by centrifugation (160,000 × g for 60 min), resuspended in homogenization buffer in the presence of 0.1% Triton X-100, and assayed, along with the supernatants, for CB activity as described. To test dissociation by phosphatidylinsitol-specific phospholipase C, samples were incubated for 40 min at 37°C in 25 mM Tris-acetate-12.5 mM MES-125 mM sucrose-0.5 mM EDTA, pH 6.8.

Isolation of Inhibitors. Endogenous low molecular weight cysteine proteinase inhibitors (stefins) were isolated from human tissues (normal and tumor) according to published procedures for isolation from human liver (34).

RESULTS

B16 Metastatic Variants. Variants have been developed from a parent B16 melanotic melanoma by in vivo and in vitro procedures. For the present study we used variants with low (B16-F1) or high (B16-F10) potential for colonization of lung (35), high potential for colonization of ovary (B16-013) (36) or...
brain (B16-B15b) (37) or a high potential for local tissue invasion (B16-BL6) (38). In addition we used an amelanotic variant of the B16 melanoma (B16a) which has high potential for colonization of the lung and for spontaneous metastasis to the lung from a s.c. tumor (24). Nakajima et al. (39) have previously established that the ability of several of these lines to colonize the lung correlates with their ability to degrade heparan sulfate in the rank order B16-BL6 > B16-F10 > B16-F1 > B16-013. We confirmed the lung colonization potential of the variants, e.g., 21 days post-tail vein injection of 3.75 × 10⁶ viable tumor cells into 10 mice the median and range of lung colonies for the B16-F1, B16-F10, and B16a variants were 25 (range, 8–44), 64 (range, 28–92), and 54 (range, 15–127), respectively. In contrast only the B16a line was observed to produce spontaneous pulmonary metastases at 28 days post-s.c. injection of 10⁶ viable tumor cells (median, 20; range, 0–64).

Differential Centrifugation. The subcellular distribution of marker enzymes was analyzed initially after separation into four fractions (nuclear, heavy mitochondrial, and supernatant) by differential centrifugation. The recovery of protein ranged from 79 to 103%; recovery of enzyme activities ranged from 70 to 108%. The distribution of three lysosomal hydrolases and of Na⁺,K⁺-ATPase, galactosyltransferase, and glucose-6-phosphatase in normal murine liver and the highly metastatic B16a tumor is shown in Tables 1 and 2, respectively. The degree of enrichment in each fraction is represented by the RSA, i.e., the ratio of the specific activity in that fraction to the specific activity in the homogenate, with a RSA greater than one indicating an enrichment in that fraction.

CB activity in the liver was distributed primarily in the heavy mitochondrial fraction (RSA = 5). In contrast, in the metastatic B16a tumor the activity was distributed primarily in the light mitochondrial fraction (RSA = 4; compare Tables 1 and 2). The ratio of CB activity in the light mitochondrial fraction to that in the heavy mitochondrial fraction was 0.4 for liver and 1.8 for B16a tumors. The subcellular distributions of cathepsin H, another lysosomal cysteine protease, and two lysosomal glycosidases, β-hexosaminidase and β-GLU, were also evaluated (Tables 1 and 2; data for β-GLU not shown). The subcellular distribution of β-hexosaminidase activities in the two tissues exhibited a change similar to but less dramatic than seen for the CB activities. In the liver, the ratio of β-hexosaminidase activity in the light mitochondrial fraction to that in the heavy mitochondrial fraction was 0.3 and in the B16a fraction it was 0.9. In contrast, cathepsin H and β-GLU were equally distributed between the heavy and light mitochondrial fractions of the liver and B16a tumor. The ratios of their activities in the light mitochondrial fraction to those in the heavy mitochondrial fraction in liver were 0.8 and 0.7 for cathepsin H and β-GLU, respectively; the ratios in the B16a tumor were 1.0 and 1.1, respectively.

Table 1 Distribution of marker enzymes and protein in subcellular fractions of normal murine liver

<table>
<thead>
<tr>
<th>Subcellular fraction</th>
<th>Homogenate</th>
<th>N</th>
<th>H</th>
<th>L</th>
<th>S</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (mg/ml)</td>
<td>19.1</td>
<td>23.9</td>
<td>7.9</td>
<td>4.7</td>
<td>8.2</td>
<td>97.5</td>
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<tr>
<td>Cathepsin B</td>
<td>5.5</td>
<td>5.2</td>
<td>29.1</td>
<td>12.8</td>
<td>0.1</td>
<td>88.8</td>
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<tr>
<td>Specific activity</td>
<td>1.0</td>
<td>0.9</td>
<td>5.3</td>
<td>2.3</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>β-Hexosaminidase</td>
<td>1.56 ± 0.10</td>
<td>1.31 ± 0.01</td>
<td>6.70 ± 1.00</td>
<td>2.30 ± 0.14</td>
<td>0.65 ± 0.01</td>
<td>89.3</td>
</tr>
<tr>
<td>Specific activity</td>
<td>1.0</td>
<td>0.84</td>
<td>4.3</td>
<td>1.50</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Cathepsin H</td>
<td>1.25</td>
<td>0.82</td>
<td>3.39</td>
<td>2.62</td>
<td>0.40</td>
<td>80.8</td>
</tr>
<tr>
<td>Specific activity</td>
<td>1.0</td>
<td>0.7</td>
<td>2.7</td>
<td>2.1</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Na⁺,K⁺-ATPase</td>
<td>16.9 ± 4.3</td>
<td>37.8 ± 0.7</td>
<td>35.9 ± 3.3</td>
<td>40.3 ± 4.5</td>
<td>4.7 ± 0.5</td>
<td>102.1</td>
</tr>
<tr>
<td>Specific activity</td>
<td>1.0</td>
<td>2.2</td>
<td>2.12</td>
<td>2.4</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Glucose-6-phosphatase</td>
<td>93.8 ± 1.2</td>
<td>112.8 ± 2.0</td>
<td>131.3 ± 1.0</td>
<td>343.4 ± 6.5</td>
<td>70.9 ± 1.7</td>
<td>107.6</td>
</tr>
<tr>
<td>Specific activity</td>
<td>1.0</td>
<td>1.2</td>
<td>1.4</td>
<td>3.7</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Galactosyltransferase</td>
<td>23.0 ± 3.8</td>
<td>13.0 ± 1.6</td>
<td>47.3 ± 4.4</td>
<td>49.8 ± 9.0</td>
<td>13.1 ± 2.9</td>
<td>70.1</td>
</tr>
<tr>
<td>Specific activity</td>
<td>1.0</td>
<td>0.6</td>
<td>2.1</td>
<td>2.2</td>
<td>0.6</td>
<td></td>
</tr>
</tbody>
</table>

The relative distributions of marker enzymes for three membranous components of the cell [the plasma membrane (Na⁺,K⁺-ATPase), the endoplasmic reticulum (glucose-6-phosphatase), and the Golgi apparatus (galactosyltransferase)] were enhanced [i.e., their RSA's were >1] in light mitochondrial fractions of both tissues. In the B16a tumor a greater proportion of the activities of two lysosomal hydrolases (CB and β-hexosaminidase) sedimented in the light mitochondrial fraction with the membrane marker enzymes than did so in the liver. Experimental manipulations to increase our yield of Na⁺,K⁺-ATPase activity in the light mitochondrial fraction of liver were unsuccessful (data not shown). Therefore the same methodology was used for fractionation of liver and all six B16 melanoma variants.

Percoll Density Gradient Centrifugation. By fractionation of the postnuclear supernatant on a Percoll density gradient two subpopulations of lysosomes can be obtained (26). We attempted to further subdivide the lysosomes by starting with the light mitochondrial fraction (presumably containing only the less dense primary lysosomes). Therefore light mitochondrial fractions obtained from the seven tissues were subjected to density gradient centrifugation on a self-generated gradient of light mitochondrial markers.

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PROPERTIES OF TUMOR MEMBRANE CYSTEINE PROTEINASE

Table 2 Distribution of marker enzymes and protein in subcellular fractions of murine B16a tumors

A 5-g pool of tumors was homogenized and subjected to differential centrifugation as described in "Materials and Methods." For details see legend to Table 1 and the text.

<table>
<thead>
<tr>
<th>Subcellular fraction</th>
<th>Homogenate</th>
<th>N</th>
<th>H</th>
<th>L</th>
<th>S</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (mg/ml)</td>
<td>10.1</td>
<td>6.7</td>
<td>5.3</td>
<td>2.4</td>
<td>4.5</td>
<td>78.6</td>
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<tr>
<td>Cathepsin B Specific activity</td>
<td>10.9</td>
<td>6.8</td>
<td>25.3</td>
<td>46.0</td>
<td>2.6</td>
<td>95.8</td>
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<td>RSA</td>
<td>1.0</td>
<td>0.6</td>
<td>2.3</td>
<td>4.2</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>β-Hexosaminidase Specific activity</td>
<td>5.7 ± 0.1</td>
<td>15.9 ± 0.1</td>
<td>9.8 ± 0.2</td>
<td>9.2 ± 0.2</td>
<td>3.3 ± 0.1</td>
<td>85.3</td>
</tr>
<tr>
<td>RSA</td>
<td>1.0</td>
<td>2.8</td>
<td>1.7</td>
<td>1.6</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>Cathepsin H Specific activity</td>
<td>0.32</td>
<td>0.22</td>
<td>0.72</td>
<td>0.69</td>
<td>0.15</td>
<td>83.3</td>
</tr>
<tr>
<td>RSA</td>
<td>1.0</td>
<td>0.7</td>
<td>2.3</td>
<td>2.2</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Na+,K+-ATPase Specific activity</td>
<td>16.3 ± 0.0</td>
<td>34.9 ± 2.1</td>
<td>29.2 ± 3.2</td>
<td>58.0 ± 7.9</td>
<td>7.8 ± 0.5</td>
<td>98.8</td>
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<tr>
<td>RSA</td>
<td>1.0</td>
<td>2.1</td>
<td>1.8</td>
<td>3.6</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Glucose-6-phosphatase Specific activity</td>
<td>4.3 ± 0.9</td>
<td>9.7 ± 0.6</td>
<td>7.8 ± 0.03</td>
<td>9.3 ± 0.0</td>
<td>2.3 ± 0.5</td>
<td>94.1</td>
</tr>
<tr>
<td>RSA</td>
<td>1.0</td>
<td>2.3</td>
<td>1.8</td>
<td>2.2</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Galactosyltransferase Specific activity</td>
<td>133 ± 5</td>
<td>N.D.</td>
<td>256 ± 23</td>
<td>286 ± 23</td>
<td>35 ± 3</td>
<td>83.0</td>
</tr>
<tr>
<td>RSA</td>
<td>1.0</td>
<td></td>
<td>1.91</td>
<td>2.15</td>
<td>0.25</td>
<td></td>
</tr>
</tbody>
</table>

isooosmotic 30% Percoll. Two visible bands resulted. The upper band was designated L-1, had a density of 1.045 g/ml, and was located just below the meniscus; the lower band was designated L-2, had a density of 1.07 g/ml, and was located near the bottom of the tube. The highest specific activities and RSAs of Na+,K+-ATPase were found in the L-1 fraction in all tissues examined (Fig. 1A), thus indicating that the L-1 fractions were enriched in plasma membrane. The separation of plasma membrane from the melanoma variants was more successful than that from the normal liver (Fig. 1A). In order to make valid comparisons among the various tissues we used identical fractionation techniques for all seven tissues. Measurement of galactosyltransferase activity indicated that the Golgi apparatus cosedimented with the plasma membrane of both normal liver and the highly metastatic B16a tumor since the highest specific activities and RSAs of galactosyltransferase were found in the

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Fig. 1. Marker enzyme activity in L-1 (B) and L-2 (C) subfractions isolated by Percoll density gradient centrifugation of murine liver and six melanoma variants. A, specific activity (top) and RSA (bottom) of Na+,K+-ATPase. An RSA = 1.00 is indicated by the dashed line. B, same as A for CB activity. C, same as A for β-hexosaminidase activity.
L-1 fraction (data not shown). In contrast, the endoplasmic reticulum cosedimented with the plasma membrane fraction of normal liver but with the lysosomal fraction of the B16a tumor; i.e., the highest specific activities and RSAs of the glucose-6-phosphatase were found in the L-1 fraction of normal liver and in the L-2 fraction of the B16a tumor (data not shown).

The highest specific activities and RSAs of the four acid hydrolases were found in the L-2 fractions of the seven tissues (CB and β-hexosaminidase, Fig. 1, B and C; cathepsin H and β-GLU, data not shown), thus indicating that the L-2 fractions were enriched in lysosomes. The RSAs of CB in the L-1 or plasma membrane fractions of five of the six metastatic B16 melanoma variants were greater than one (range, 1.9–6.6), suggesting an alteration in the subcellular distribution of CB in metastatic tumors. For the three additional acid hydrolases RSA values in the L-1 fraction of B16-F1 and B16a tumors were 0.1 and 0.7 (cathepsin H), 1.2 and 1.3 (β-GLU), and 1.7 and 1.5 (β-hexosaminidase), respectively. These values suggest that the subcellular distributions of cathepsin H and β-GLU were not altered in metastatic tumors but that of β-hexosaminidase may have been partially altered.

The distribution of lysosomal and other marker enzymes within the Percoll gradient was also evaluated by successive collection of fractions (0.4 ml) from the bottom of the tube. Elution profiles for liver and the highly metastatic B16a tumor are depicted in Fig. 2. An alteration in the distribution of the activities of CB and β-hexosaminidase from the bottom of the gradient (location of L-2 fraction) to the top of the gradient (location of L-1 fraction) was observed in the B16a tumor. Similar alterations in the distributions of cathepsin H, β-GLU, and Na⁺,K⁺-ATPase activities or protein were not observed (Fig. 2 or data not shown; also see above).

Nature of the Association of CB with Plasma Membrane in Tumor Cells. CB could be associated with the tumor cell plasma membrane in one of four ways: there may be a nonspecific adsorption of the enzyme to the membrane during tissue homogenization and/or isolation of the plasma membrane fraction; the enzyme may be a peripheral membrane protein loosely associated with the membrane; the enzyme may be attached to a receptor such as the mannose 6-phosphate receptor commonly used for intracellular transport of lysosomal enzymes to the lysosomes; or, finally, the enzyme may be an integral membrane protein embedded in the lipid bilayer. To investigate the nature of this association, the L-1 or plasma membrane fraction was subjected to several treatments.

To test whether CB was nonspecifically adsorbed to the L-1 fraction, the fraction was washed with a physiological salt solution (0.15 M potassium acetate). One hundred % of the CB activity was retained in the plasma membrane pellet indicating that CB was not nonspecifically adsorbed. Since peripheral membrane proteins can be dissociated using high salt concentrations or low concentrations of mild detergents, we tested whether CB could be dissociated from the plasma membrane fraction by exposure to 0.5 or 1.0 M NaCl or to saponin (25 μg/ml). Saponin is a mild detergent which has been used for the characterization of membrane-associated proteins and to permeabilize the plasma membrane (via formation of stable holes (31)). Saponin was ineffective (>98% retention of CB activity). Eighty-two % of the CB activity was retained in the plasma membrane pellet after exposure to 0.5 M NaCl and 74% after exposure to 1.0 M NaCl. In addition, the high salt concentration was observed to inhibit CB activity, an observation we and others (40) have also made for purified cathepsin B. Unfortunately, two additional extraction methods [saponin (10 μg/ml)-sodium sulfate and sodium carbonate (pH 11.5)] used to dissociate peripheral membrane proteins (41) could not be tested for their ability to elute CB activity from the plasma membrane fraction since CB is irreversibly inactivated at the alkaline pH of the latter (42) and inhibited at the high salt concentration of the former (present study; Ref. 40).

As indicated above mannose 6-phosphate receptors mediate the selective targeting of newly synthesized lysosomal enzymes to lysosomes (43). This is apparently true for β-hexosaminidase in normal liver since 71% of β-hexosaminidase activity can be dissociated from intracellular membranes by exposure to 10 mM mannose 6-phosphate (44). In contrast, in liver from patients with I-cell disease β-hexosaminidase seems to be transported by alternate pathways with binding proteins for fucose, mannose, and perhaps galactose (44). Therefore, to test whether plasma membrane-associated CB was still coupled to such binding proteins, fresh samples of B16a tumor plasma membrane were incubated for 45 min at 25°C in the presence of 10 mM mannose 6-phosphate, mannose, fucose, or galactose. Following centrifugation the supernatants and the pellets were assayed for CB activity. Mannose eluted 7.8% of the CB activity; the other sugars eluted 5.9–7.2% of CB activity but the buffer control also eluted 6.0% of the CB activity. Therefore, these results do not provide evidence for CB in the L-1 fraction being coupled to binding proteins for these sugars. We tested for the possibility that CB might be anchored to the membrane by covalent attachment to phosphatidylinositol. However, CB activity could not be eluted from the plasma membrane fraction by treatment with phosphatidylinositol-specific phospholipase C. The nonsolubilized CB activities after exposure to 0.15 and 15.0 μg/ml phosphatidylinositol-specific phospholipase C were


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97.8 ± 5.2 and 86.6 ± 6.1% of buffer control CB activities. Thus CB does not appear to be anchored in the membrane via phosphatidylinositol. To test that CB might be bound to yet unidentified receptors we exposed the plasma membrane fractions to acid pH for a brief period (i.e., analogous to the conditions used for elution from affinity columns). Ninety-seven % of the CB activity was retained in the pellets, providing no evidence that CB in the B16a L-1 fraction was bound to an unidentified receptor. In addition, these results suggest that the association of CB with this fraction is with the plasma membrane and not with the Golgi; i.e., a lysosomal enzyme associated with the Golgi should be bound to a receptor.

We further tested the association of CB with the L-1 fraction by treating the fraction with 2 mM (0.1%) Chaps, a detergent without a net charge which is often used for the isolation of integral membrane proteins (45). After exposure to Chaps the samples were centrifuged and CB activity was measured in the supernatant and the pellet. Both L-1 and L-2 fractions were exposed to Chaps. A 5-fold increase in the CB activity of the L-1 supernatant was observed (Fig. 3). A minor increase (1.2-fold) was observed in cathepsin B activity in the L-2 (lysosomal) supernatant, presumably due to disruption of the lysosomes by the detergent. These data are highly indicative that CB is an integral membrane protein in B16a tumors. Further evidence supporting this contention is that a substantial amount of CB could be eluted from homogenates of B16a tumors by three successive treatments with 1-butanol.

Butanol has been shown previously to elute integral membrane proteins from tumor cell plasma membranes (46).

**pH Optima and Stability of Plasma Membrane-associated (L-1) CB and Lysosomal (L-2) Cathepsin B.** Cathepsin B purified from a number of human tissues including tumors has been shown to have a pH optimum against synthetic substrates similar to that of liver cathepsin B, i.e., pH 6.2, and to be inactivated above pH 7.0 (42), unless the activity is measured in a continuous assay at 25°C (47). In order for CB localized in the plasma membrane of metastatic tumors to play a role in focal dissolution of extracellular matrices during tumor invasion, stability and activity of the membrane CB at neutral to slightly acid pH would be optimal. CB activity in the plasma membrane vesicles shed by murine 15091A mammary adenocarcinoma cells has been shown to have increased stability (15).

The pH optima of plasma membrane-associated CB and lysosomal cathepsin B were determined using a series of MES and piperazine-N,N’-bis(ethanesulfonic acid) buffers over the pH ranges 5.4–7.0 and 6.9–7.4, respectively. The pH optimum of the plasma membrane-associated CB was slightly higher (pH 6.7) than that of lysosomal cathepsin B (pH 6.2). Mort et al. (12) have reported that a latent CB released from tumors also has higher pH optimum. Resistance to inactivation at neutral pH was assessed by incubating the L-1 and L-2 fractions at pH 7.3 for time periods up to 15 min and then assaying activity at pH 6.2 as described in “Materials and Methods.” There was a 77% decrease in lysosomal cathepsin B activity over 15 min but only a 38% decrease in plasma membrane-associated CB activity over the same period (Fig. 4).

**Effects of Inhibitors on Plasma Membrane-associated (L-1) CB Activity and Lysosomal (L-2) Cathepsin B Activity.** Localization of CB within a membrane microenvironment such as the plasma membrane of tumors could make this proteinase less susceptible to inhibition as has been demonstrated for a trypsin-like neutral protease on the surface of Ehrlich ascites tumor cells (48). To test this hypothesis we have compared the response of the plasma membrane-associated CB and lysosomal cathepsin B to three inhibitors. For these studies neither enzyme was solubilized with detergent, either Triton X-100 or Chaps. The first inhibitor tested was E-64, a specific active site titrant for cysteine proteinases (49). The titration of activity in the two fractions indicated that the enzyme in both fractions (L-1 and L-2) had the same number of active sites per unit of protein (x intercept). However, the CB associated with the plasma membrane was 33% more active in the presence of 1–40 pm E-64 than was lysosomal cathepsin B (Fig. 5). The second inhibitor was the tetrapeptide leupeptin. Leupeptin is a selective inhibitor of cysteine proteinases which at higher concentrations also inhibits serine proteinases (50). Leupeptin was less effective against the plasma membrane-associated CB (K_1 = 860 pm) than against lysosomal cathepsin B (K_1 = 44 pm) as shown in Fig. 6A. Recently we have purified the endogenous low molecular weight cysteine proteinase inhibitors (stefins) from normal human liver and human malignant sarcoma (51). Sarcoma stefin A is 10-fold less effective in inhibiting purified liver cathepsin B than is liver stefin A (51). We tested sarcoma stefin A for inhibition of activity and found that it was 20-fold less effective against the plasma membrane-associated CB (K_1 = 900
a-glucosidase, and β-hexosaminidase are localized extracellularly or in small vesicles close to the plasma membrane yet β-glucocerebrosidase exhibits the same lysosomal localization seen in control fibroblasts (22). In cell fractionation studies β-hexosaminidase activity was found to be present in less dense fractions of the I-cell fibroblasts than of the control fibroblasts (52). We therefore speculate that the dissociation we observed in the subcellular distribution of lysosomal enzymes in the B16 variants may correspond to that observed in I-cell fibroblasts.

The biochemical and cell fractionation techniques used in the present study cannot definitively localize CB or β-hexosaminidase to either the plasma membrane or plasma membrane-associated vesicles. Immunohistochemical studies at the electron microscopic level will be required for localization of these two enzyme proteins. However, only by biochemical measurements for CB and β-hexosaminidase can we determine whether these enzymes have activity in plasma membrane fractions. Establishing whether active forms of CB and β-hexosaminidase are present at the surface of metastatic tumors is of obvious import to a role(s) for these hydrolases in tumor metastasis. If both enzymes can be localized to the same plasma membrane-associated vesicles, this could establish a local microenvironment in which proteolytic degradation of the extracellular matrix is increased by concomitant degradation of carbohydrate (23). We have observed that dissolution of the basement membrane by B16a tumor cells arrested in the pulmonary microvasculature occurs at many focal sites (53).

Since plasma membrane fractions isolated by differential and density gradient centrifugation are subject to contamination by other organelles, the CB and β-hexosaminidase activities we measured in these fractions might reflect such contamination. The high percentages of both CB and β-hexosaminidase activities present in the tumor L-1 fractions (i.e., in the B16a 37 and 32%, respectively) suggest that the activities were not simply due to contamination. By comparison, in the liver the percentages in the L-1 fraction were 4 and 6%, respectively. Although use of liver as a normal tissue is not optimal, the lack of availability of normal melanocytes precluded their use and the extensive literature on liver cathepsin B led to our choice of liver as the normal control tissue. In addition, we have also established that CB activity in a murine hepatoma is associated with the L-1 fraction (10). Further evidence supporting a membrane association is that CB activity has also been found in association with plasma membrane vesicles shed into the culture medium by murine 15091A adenocarcinoma cells (15) and in association with plasma membrane fractions adherent to beads after lysis of rat anaplastic sarcoma cells attached to these beads (8). In addition, Baici and Knopfel (11) have reported that in preliminary experiments CB protein has been localized to the plasma membrane of rabbit V2 carcinoma cells using avidin-biotin-peroxidase immunohistochemistry, at the light microscopic level.

We determined that the enrichment of CB in the plasma membrane fractions was not due to nonspecific adsorption, to a loose peripheral association, to covalent linkage to phosphatidylinositol, or to CB being bound to any of the known receptors for lysosomal enzymes. The association of CB with the tumor plasma membrane fraction could be disrupted only with detergent or butanol, suggesting that CB may be an integral membrane protein in metastatic tumors. Our results do not yet rule out the possibility that CB may be bound to an unidentified protein in the plasma membrane.
CB might possess proteolytic activity when free enzyme would
sociated could be of importance because membrane-associated
bility of carbobenzyloxyphenylalanylalanyldiazomethylketone, another specific low molecular weight inhibitor
cytosine proteinases, to inhibit CB in shed membrane vesicles (15) substantiate this premise. In addition to an apparent re-
duced binding to inhibitors, CB activity associated with the
tumor cell plasma membrane fragment seems to have enhanced stability at physiological pH as demonstrated by its resistance to inactivation at pH 7.3 (Fig. 4; 3, 15). Mort et al. (12) and Baici and Knopfel (11) have demonstrated that higher molecular weight forms of CB released from tumors also have en-
hanced resistance to inactivation at neutral to slightly alkaline pH. The ability of these enzymes to be inhibited by low molec-
ular weight cysteine proteinase inhibitors has not been evalu-
ated. However, higher molecular weight forms of tumor CB
react only with the high molecular weight proteinase inhibitor α2-macroglobulin after they have been destabilized, i.e., are no
longer resistant to inactivation by pH > 7.0 (55). Mort and Recklies (55) have suggested that the high molecular weight CB
forms are precursors of lysosomal cathepsin B.

We have observed multifocal dissolution in vivo at sites where the B16a membrane comes in direct contact with the basement
membrane (53). A form of CB in the plasma membrane of metastatic tumors that in this microenvironment, i.e., mem-
brane-associated, is resistant to inactivation at neutral pH and to inhibition by extracellular inhibitors could facilitate this
multifocal dissolution and thereby tumor invasion. The altera-
tion(s) in the intracellular transport of cathepsin B in tumor cells which result in its association with the plasma membrane
are not known. However, deficiencies in the receptors for the
mannose 6-phosphate pathway which targets lysosomal en-
zymes to lysosomes have been reported in several tumor cell
lines (20). Such deficiencies in a Morris hepatoma line result
in the secretion of 30% of newly synthesized cathepsin C, a
lysosomal exopeptidase (21). We speculate that the association
of 37% of CB activity with the plasma membrane fraction of B16a tumor cells (10) may reflect similar deficiencies in man-
nose 6-phosphate receptors.

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