ABSTRACT

The Bowman Birk protease inhibitor (BBI) has been shown to be an effective suppressor of carcinogenesis in vivo and in vitro. In this report we demonstrate that normal human fibroblasts and Bloom cells contain a BBI-inhibitable proteolytic activity. The enzyme cleaves gelatin, has a molecular mass of 43 kDa, and is located in the cytosol. This activity has maximal activity at pH 8 and was inhibited by diisopropylfluorophosphate but was not affected by EDTA or 1,10-phenanthroline, indicating that this enzyme is a serine protease. We have reported previously that a similar BBI-inhibitable activity is present in C3H/10T1/2 mouse embryobryo fibroblast cells. Our results suggest that a common "target enzyme" of the BBI is present in mouse and human cells.

INTRODUCTION

Protease inhibitors have been shown to be highly effective suppressors of carcinogenesis in vitro and in several animal cancer models in vivo (1-12). It is probable that protease inhibitors also suppress human carcinogenesis because human populations consuming high levels of legumes, which are rich in protease inhibitor activity, have lower incidences of breast, colon, pancreatic, and prostate cancers (13-16). Although the precise mechanism(s) by which these compounds exert their anticarcinogenic effects have not been elucidated, we have hypothesized that protease inhibitors block carcinogenesis by inhibiting cellular enzymes involved in the induction and/or expression of the transformed phenotype (17-19).

We have extensively studied the anticarcinogenic activity of the soybean-derived BBI(1, 4, 8-12). The BBI is an 8000-Da protein which contains distinct trypsin and chymotrypsin inhibitory sites (20, 21). BBI has been shown to suppress dimethylhydrazine-induced colon and liver carcinogenesis in mice as well as dimethylbenz(a)anthracene-induced check pouch carcinogenesis and methylcholanthrene-induced lung carcinogenesis (8-11). The BBI reduces radiation and chemical carcinogen-induced transformation in vitro (1, 22). This compound has also been shown to reduce the incidence of spontaneous chromosomal abnormalities in Bloom cells (22). Additionally, the BBI has been shown to be internalized by C3H/10T1/2 cells (2, 23). Major goals of our research efforts are to determine the interaction of anticarcinogenic protease inhibitors with mammalian cells and identify cellular proteases which are inhibited by these compounds. To date, no BBI-inhibitable proteases have been identified in human cells. In the current report, we demonstrate that 1522 human fibroblasts contain a BBI-inhibitable proteolytic activity.
this technique, we identified several bands of protease activity with masses ranging from 50-70 kDa (Fig. 1). Treatment of the cell homogenate preparations with trypsin resulted in the formation of a new band of protease activity; this enzyme had a mass of about 43 kDa (Fig. 1, lane 2). This activity was not observed in samples which were stored overnight at 4°C or incubated at 37°C for 1 h in the absence or presence of 5 mM urea. To determine the intracellular localization of this protease activity, subcellular fractionation experiments were performed. Log phase 1522 cells were scraped from the culture dishes and homogenized in a Dounce homogenizer. Each subcellular fraction was isolated by differential centrifugation. Marker enzyme activity was determined in each fraction (Table 1). Unactivated and trypsin-activated samples from each subcellular fraction were analyzed for protease activity on gelatin-containing zymograms. No protease activity in this molecular mass range was observed in the 1,000 and 10,000 x g pellets, while a small amount of activity was observed in the 100,000 x g pellet; the bulk of the 43-kDa proteolytic activity was present in the 100,000 x g supernatant fraction (Fig. 2). These results indicate that the majority of this enzyme activity is located in the cytosol. We have not found this protease activity in conditioned medium obtained from these cells, indicating that this protease is not secreted (data not shown).

We characterized this proteolytic activity using several protease inhibitors with well-characterized mechanisms of inhibition. For these studies, cell homogenates were run on gelatin zymograms; the zymograms were incubated in reaction buffer containing the desired enzyme inhibitor. The 43-kDa activity was inhibited with DFP but was unaffected by EDTA (Fig. 3). These results indicate that this activity is a serine protease (29).

In contrast, the proteases present in unactivated cell homogenate preparations (Fig. 1, lane 1) were inhibited by EDTA but not effected by DFP (Fig. 3), indicating that these enzymes are metalloproteases (28). The 43-kDa proteolytic activity was also inhibited by BBI (Fig. 3). The effect of other compounds on the 43-kDa proteolytic activity are summarized in Table 2. To determine the pH optima of this activity, the zymograms were incubated in buffers of increasing pH; maximal protease activity was observed at pH 8 (Fig. 4).

The fact that the 43-kDa protease activity requires trypsin activation to be converted into an active protease could suggest that this protease is processed from a larger precursor or is complexed with an endogenous inhibitor. To further address this problem, cell homogenates were trypsin activated, treated with DFP, and then run on the gelatin zymograms. Under these conditions, we observed no inhibition of the 43-kDa proteolytic activity but complete inhibition of trypsin (Fig. 1). However, as discussed above, the 43-kDa activity is inhibited when zymograms containing trypsin-activated cell homogenates are incubated in reaction buffer containing DFP (Fig. 3). These results indicate that, in solution, the active site of this protease is inaccessible to DFP. We hypothesize that when the samples are run on the zymogram, the protease and inhibitor separate, thus allowing DFP to covalently bind to the serine residue (29) in the active site of the enzyme.

If this protease were complexed with an inhibitor, then the size of the protease-inhibitor complex should be larger than the active protease. We estimated the size of the “unactivated” gelatinase by passing cell homogenates over a Bio Gel P-100 gel filtration column and assaying each fraction for protease activity. The unactivated enzyme elutes from the column with an apparent mass of 70–80 kDa (Fig. 5).

We also determined whether a similar BBI-inhibitable proteolytic activity is present in other cells. Bloom cells were found to contain a trypsin-activatable proteolytic activity with the same mass as observed in 1522 cells, which was inhibited by the BBI and DFP but was not affected by EDTA (Fig. 3). We have reported previously that C3H/10T1/2 cells also contain a protease activity in this molecular mass range which is inhibited by the BBI. Comparison of trypsin-activated 1522 and C3H/10T1/2 cell homogenates revealed that the protease derived from human cells has a slightly faster mobility than the mouse-derived enzyme on the gelatin-containing zymograms (Fig. 3) but appears to be similar to the proteolytic activity in 1522 and Bloom cells with respect to protease inhibitor sensitivity (Fig. 3).

**DISCUSSION**

In this report, we have demonstrated that logarithmically growing 1522 human fibroblasts contain a 43-kDa proteolytic activity which is inhibited by the BBI. Our subcellular fractionation experiments revealed that the bulk of this activity is present in the 100,000 x g supernatant fraction, indicating that this proteolytic activity is present in the cytosol. This protease activity in AG1522 human fibroblast subcellular fractions

<table>
<thead>
<tr>
<th>Subcellular fraction</th>
<th>β-Glu</th>
<th>LDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1k x g pellet</td>
<td>7.21</td>
<td>3.07</td>
</tr>
<tr>
<td>10k x g pellet</td>
<td>9.61</td>
<td>4.66</td>
</tr>
<tr>
<td>100k x g supernatant</td>
<td>0.39</td>
<td>1.70</td>
</tr>
</tbody>
</table>

β-Glu activity is expressed as ΔA405nm/12 h/mg protein. Numbers in parentheses, total amount of enzyme activity present in the sample expressed as the specific activity x total protein present in the sample (ΔA405nm/12 h).

LDH activity is expressed as ΔA540nm/min/mg protein. Numbers in parentheses, total amount of enzyme activity present in the sample expressed as the specific activity x total protein present in the sample (ΔA540nm/min).

**Table 1** Marker enzyme activity in AG1522 human fibroblast subcellular fractions

In this report, we have demonstrated that logarithmically growing 1522 human fibroblasts contain a 43-kDa proteolytic activity which is inhibited by the BBI. Our subcellular fractionation experiments revealed that the bulk of this activity is present in the 100,000 x g supernatant fraction, indicating that this proteolytic activity is present in the cytosol. This protease activity in AG1522 human fibroblast subcellular fractions

**Fig. 1.** Trypsin activation of proteolytic activity. 1522 cell homogenates were untreated or activated with trypsin and then run on gelatin-containing zymograms. Lane 1, unactivated cell homogenate; lane 2, trypsin-activated homogenate; lane 3, cell homogenate activated with trypsin and treated with DFP (5 mM) prior to being run on the zymogram; lane 4, trypsin control; lane 5, trypsin treated with DFP (5 mM) prior to being run on the zymogram; lane 6, molecular mass markers; and right ordinate, molecular mass in kDa; arrow, position of the 43-kDa protease.
had maximal activity at pH 8 and was potently inhibited by DFP (Fig. 3), a highly selective inhibitor of serine proteases (29).

An intriguing observation from these studies was that this enzyme was "activated" with trypsin. Two possibilities could explain this finding: (a) this proteolytic activity is complexed with an endogenous inhibitor or (b) this activity is processed from a larger proenzyme into a smaller, mature form which is proteolytically active. If this proteolytic activity was complexed with an endogenous inhibitor, then the protease-inhibitor complex must form a complex which is stable in the presence of SDS. A fibroblast-derived urokinase inhibitor (UK-1) has been described which forms an SDS-stable complex with urokinase (30). Trypsin could selectively cleave the inhibitor; when this material is run on the zymogram, the protease and inhibitor are separated and the protease is active. Further support that the 43-kDa protease is complexed with an inhibitor comes from our inhibition studies with DFP. Our observation that DFP will not inhibit the 43-kDa enzyme in trypsin-activated samples prior to being run on the zymogram (Fig. 1) may suggest that the inhibitor is still complexed with this protease. In the SDS-containing zymogram, the protease and inhibitor are separated and the protease becomes sensitive to DFP inhibition. If this proteolytic activity were processed by trypsin from an inactive (zymogen) to an active form, then one would expect DFP to inhibit protease activity in trypsin-activated samples before they are run on the zymogram. These results indicate that the active site of this protease is inaccessible to DFP in solution. Denaturation and size fractionation of the complex on the zymogram separates the endogenous inhibitor from the protease, thus allowing DFP to covalently bind to the serine residue in the active site of the enzyme. Our gel filtration studies suggest that the unactivated protease complex has a mass of 70–80 kDa. If the size of the endogenous inhibitor of this proteolytic activity were 30 kDa, this would result in a protease-inhibitor complex in the appropriate size range (43 + 30 = 73 kDa). Indeed, protease inhibitors in this size range have been identified (27).

In these studies, we observed that Bloom cell homogenates contain a BBI-inhibitable proteolytic activity with the same mobility as that found in 1522 cells. Bloom syndrome is an autosomal recessive genetic disease; cells obtained from individuals afflicted with this disease show an enhanced state of chromosomal instability. The relatively high level of chromosomal aberrations and sister chromatid exchanges observed in Bloom cells are thought to predispose these individuals to an increased risk of developing cancer. Work in our laboratory has shown that the BBI can reduce the incidence of chromosomal aberrations in Bloom cells (22), suggesting that a protease may be involved in the genetic instability observed in these cells.
The 43-kDa proteolytic activity in 1522 and Bloom cells is similar to a protease we have described in C3H/10T1/2 mouse embryo fibroblast cells.3 These results suggest that this proteolytic activity is a potential target enzyme of the BBI. Our previous studies have indicated that a limited number of proteins from mouse or human fibroblast cells will specifically bind to a BBI affinity column (19).

The 43-kDa proteolytic activity was not affected by EDTA or 1,10-phenanthroline, indicating that this activity is not a metalloproteinase and hence distinct from collagenases (such as stromelysin), which are potently inhibited by EDTA and other chelators of metal ions (31). This enzyme is similar to a BBI affinity column (19).

### Table 2. Effect of different compounds on proteolytic activity in AG1522 cells

<table>
<thead>
<tr>
<th>Compound*</th>
<th>Effect on proteolytic activity†</th>
<th>Type of protease activity‡</th>
<th>Inhibited by BBI</th>
<th>Fraction Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethyl sulfoxide (1%)</td>
<td>None</td>
<td>Thiol</td>
<td>Partial</td>
<td>21.5</td>
</tr>
<tr>
<td>N-ethylmaleimide (5 mM)</td>
<td>None</td>
<td>Thiol</td>
<td>Complete</td>
<td>43</td>
</tr>
<tr>
<td>Iodoacetamide (5 mM)</td>
<td>None</td>
<td>Thiol</td>
<td>Partial</td>
<td>68</td>
</tr>
<tr>
<td>DFP (5 mM)</td>
<td>Inhibit</td>
<td>Serine</td>
<td>Complete</td>
<td>0.00</td>
</tr>
<tr>
<td>EDTA (20 mM)</td>
<td>None</td>
<td>Serine/thiol</td>
<td>Complete</td>
<td>0.01</td>
</tr>
<tr>
<td>Antipain (10 μg/ml)</td>
<td>Inhibit</td>
<td>Serine</td>
<td>None</td>
<td>0.02</td>
</tr>
<tr>
<td>BBI (25 μg/ml)</td>
<td>Inhibit</td>
<td>Serine</td>
<td>None</td>
<td>0.03</td>
</tr>
<tr>
<td>Soybean trypsin inhibitor (25 μg/ml)</td>
<td>Partial</td>
<td>Serine</td>
<td>None</td>
<td>0.04</td>
</tr>
<tr>
<td>Chymostatin (10 μg/ml)</td>
<td>Inhibit</td>
<td>Serine</td>
<td>None</td>
<td>0.05</td>
</tr>
<tr>
<td>1,10-Phenanthroline (0.5 mM)</td>
<td>None</td>
<td>Metallo</td>
<td>None</td>
<td>0.06</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, concentrations of the indicated reagent present in the incubation buffer.
† Effect on proteolytic activity: none, no inhibition; inhibit, complete inhibition of proteolytic activity; partial, >50% inhibition of activity.
‡ Proteolytic activity was inhibited when the zymogram was incubated in reaction buffer containing DFP. If samples were trypsin activated, treated with DFP, and then run on the zymograms, no inhibition of proteolytic activity was observed.

![Protease Activity as a Function of pH](image)

Fig. 4. Proteolytic activity in 1522 cells as a function of pH. 1522 cell homogenates were activated with trypsin and then run on gelatin-containing zymograms. The amount of proteolytic activity present was determined by densitometrically scanning zymograms. Enzyme activity is expressed in arbitrary units and was determined by calculating the area under the curve obtained from each densitometric tracing. Maximal enzyme activity was observed at pH 8.

![Gel filtration analysis of proteolytic activity](image)

Fig. 5. Gel filtration analysis of proteolytic activity. Cell homogenates were prepared from growing cells and passed over 1 ml DEAE resin in 10 mM Tris (pH 8); bound proteins were eluted with 10 mM Tris, pH 8.0-0.2 M NaCl. The DEAE-purified material was subsequently passed over a BioGel P-100 column (1 x 50 cm) in 50 mM Tris, pH 7.1-100 mM NaCl collecting 0.7-ml fractions. Each fraction was activated with trypsin and analyzed for protease activity on gelatin-containing zymograms. Arrows, positions where molecular mass standards elute from the column; Void, void volume, blue dextran (approximately 107 kDa); 68, bovine serum albumin (68 kDa); 43, ovalbumin (43 kDa); 21.5, soybean trypsin inhibitor (21.5 kDa). Cross-hatched box, position where the trypsin-activatable proteolytic activity elutes from the column.

urokinase by virtue of its molecular mass and inhibition by DFP (32). However, the 43-kDa proteolytic activity differs from urokinase in that it efficiently cleaves gelatin and is not secreted into the growth medium (32).

We have proposed that protease inhibitors suppress malignant transformation by inhibiting cellular enzymes involved in the induction and/or expression of the transformed phenotype (17–19). An intriguing finding is that we have identified similar proteolytic activities in human-derived (1522 and Bloom) and mouse-derived (C3H/10T1/2) cells. These results suggest that this protease is a potential common "target" enzyme of the anticarcinogenic BBI. The fact that these proteolytic enzymes require trypsin "activation" to be active on the zymograms implies that the functioning of these proteases is stringently controlled inside the cell. We have reported that low concentrations of certain protease inhibitors are highly effective at suppressing carcinogenesis (3, 12); these results may indicate that very small amounts of this BBI-inhibitable protease are active in the cells. At the present time, the function that these enzymes play in the oncogenic transformation of mammalian cells is not known and will require further study.

ACKNOWLEDGMENTS

We thank Dr. John Biaglow for the use of his equipment and Dr. Edward Hughes and Dr. Ann R. Kennedy for helpful discussion. We also thank Dr. Maryann Flick for assistance with the densitometric tracings.

REFERENCES


Human Fibroblasts Contain a Proteolytic Activity Which Is Inhibited by the Bowman-Birk Protease Inhibitor

Paul C. Billings, Joan M. Habres, David C. Liao, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/51/20/5539

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/51/20/5539.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.