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A membrane-associated cysteine protease inhibitor from murine hepatoma

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A cysteine protease inhibitor was purified from total membrane fractions of an invasive murine hepatoma, Hepa cl 9. On gel filtration under non-reducing conditions the purified inhibitor was eluted in a single peak of M_r 10–15 kDa, but resolved as two bands at 14 and 70 kDa on SDS-PAGE under reducing conditions. By isoelectric focusing, the inhibitor ran at an isoelectric point of 4.75. Immunoblotting studies using the enhanced chemiluminescence technique indicated no crossreactivity with monoclonal antibodies to stefin B and cystatin C or with a polyclonal antibody to low M_r kininogen. In contrast, the 14 kDa and 70 kDa bands both crossreacted with a polyclonal antibody to stefin A, suggesting that the cysteine protease inhibitor associated with Hepa cl 9 membranes may be a modified form of stefin A.

Cysteine protease inhibitor; Cystatin; Stefin; Tumor

1. INTRODUCTION

Increased activity of cysteine proteases in tumors has been shown to correspond in some cases to decreased regulation by cystatins, protein inhibitors of cysteine proteases [1–3]. The cystatin superfamily consists of tight-binding cysteine protease inhibitors (CPI)s, often referred to as pseudo-irreversible, and has been subdivided into three families: stefins, cystatins, and kininogens (for review, see [4,5]). The stefins are primarily intracellular cytosolic proteins, whereas the cystatins are secreted. Stefins are further subdivided into stefins A with acidic pIs and stefins B with neutral pIs. The pIs for cystatin C are in the alkaline range. Hiwasa et al. [6] have suggested that p21, the membrane-associated c-Ha-*ras* gene product, is a member of the cystatin superfamily. Since we had observed CPI activity in membrane fractions of tumors [3], we have purified this CPI from total membrane fractions of an invasive murine hepatoma, for comparison with other cystatins.

2. MATERIALS AND METHODS

2.1. *Materials*

CHAPS (3-[(3-cholamidopropyl)-dimethylamino]-1-propane-sulfonate), E-64 (L-*trans*-epoxysuccinyl-leucylamido[4-guanidino]butane), and Brij-35 were purchased from Sigma (St. Louis, MO, USA). Polyclonal and monoclonal antibodies against human cystatin C as well as recombinant human cystatin C were gifts from Dr. M. Abrahamson, University of Lund, Lund, Sweden. Polyclonal antibody against rat epidermal stefin A was a gift from Dr. Fukuyama, University of California, San Francisco, CA. Antiserum to human low M_r kininogen was a gift from Dr. S. Olson, Henry Ford Hospital, Detroit, MI, USA. Human stefins A and B as well as monoclonal antibody against human liver stefin B were gifts from Dr. V. Turk, Josef Stefan Institute, Ljubljana, Slovenia.

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2.2. *Tissue*

The highly invasive hepatoma (Hepa cl 9) was propagated in syngeneic male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) according to our published procedure [7]. Briefly, tumor brei were injected subcutaneously at the left axillary region. The mice were sacrificed and tumors harvested when tumor weight reached approximately 5 g.

2.3. *Purification*

2.3.1. *Preparation of crude membrane fractions*

Approximately 100 g of Hepa cl 9 was homogenized in 80 ml of dispersion solution (0.1 M NaH_2PO_4, 2 mM EDTA, 0.25 M sucrose, 1 mM benzamidine, pH 7.4) in a Tekmar homogenizer (Tekmar Co., Cincinnati, OH) at room temperature. The homogenate was centrifuged in a Beckman L7-55 ultracentrifuge at 35,000 rpm for 1 h at 4°C utilizing a Beckman Type 35 rotor. The membrane pellet was washed three times and resuspended in the above buffer containing 0.5% CHAPS and stirred overnight at 4°C. The membrane extract was then centrifuged as above. The supernatant was saved as the crude membrane fraction and used for subsequent purification steps.

2.3.2. Alkaline precipitation

The crude membrane fraction was adjusted to pH 11.5 with 4 N NaOH and incubated at room temperature for 2 h. The pH was subsequently adjusted to 6.5 with 4 N HCl and the suspension centrifuged in a Beckman J2-21 centrifuge at 2,860 × *g* for 30 min at 4°C and the pellet discarded.

2.3.3. Acetone precipitation

An equal volume of prechilled acetone (−10°C) was added gradually to the above supernatant and the precipitate removed by centrifugation at 2,860 × *g* as above. To the supernatant was added a second volume of chilled acetone and the resulting precipitate recovered. The pellet was resuspended in 15 ml of 50 mM NaH_2PO_4, pH 6.5, 1 mM EDTA, 0.5 M NaCl, containing 0.1% Brij-35, and incubated overnight

at 4°C with constant stirring. Insoluble proteins were removed by centrifugation and the supernatant recovered.

2.3.4. Chromatography on a papain affinity column

Papain affinity resin was prepared as described previously [2,8]. The resin was added to the final supernatant from the acetone precipitation step and the suspension incubated overnight at 4°C and subsequently transferred to a 5-ml column. The affinity column was equilibrated with 70 ml of 50 mM NaH_2PO_4, pH 6.5, 1 mM EDTA, 0.5 M NaCl and 0.1% Brij-35. Non-specifically bound proteins were eluted from the column in 50 mM NaH_2PO_4, pH 6.5, 1 mM EDTA, 1 M NaCl, and 0.1% Brij-35 (buffer A) and discarded. The membrane-associated CPI activity was eluted from the column in 10 mM NaH_2PO_4, pH 11.5, 1 mM EDTA, 0.5 M NaCl, and 0.1% Brij-35 (buffer B). Individual fractions were assayed for inhibitory activity against papain after incubation at 100°C for 5 min to uncouple any papain-inhibitor complexes and to inactivate residual papain activity that co-eluted from the column. Fractions were pooled and then concentrated by pressure dialysis.

2.3.5. Gel filtration chromatography

The concentrated CPI activity from affinity chromatography was applied to two Superose 12 gel filtration columns in tandem in 0.05 M Tris, pH 7.5, 0.15 M NaCl. Fractions of 0.5 ml were collected and assayed for inhibitory activity against papain.

2.4. *Isoelectric focusing*

Isoelectric focusing was performed on a precast Phast System (Pharmacia, Piscataway, NJ) gel, pH 4.0–6.5, and silver-stained according to the manufacturer's procedure.

2.5. *SDS-PAGE and immunoblot analysis*

Electrophoresis was performed in 12% polyacrylamide gels in the buffer system described by Laemmli [11]. Proteins were transferred electrophoretically to nitrocellulose membranes in a Transphor electroblotting apparatus (Hoefer Scientific, San Francisco, CA). The immunoblot procedure was performed according to the method of BioRad (Richmond, CA), using powdered milk as blocking agent. The blots were developed according to Amersham's ECL detection method (Amersham, Arlington Heights, IL, USA). Multiple probing of the same blots with different antibodies was performed according to the procedure of Amersham.

2.6. *CPI activity*

Inhibitory activity against the plant cysteine protease papain (titrated against E-64) was determined in a stopped assay according to our published protocols [2].

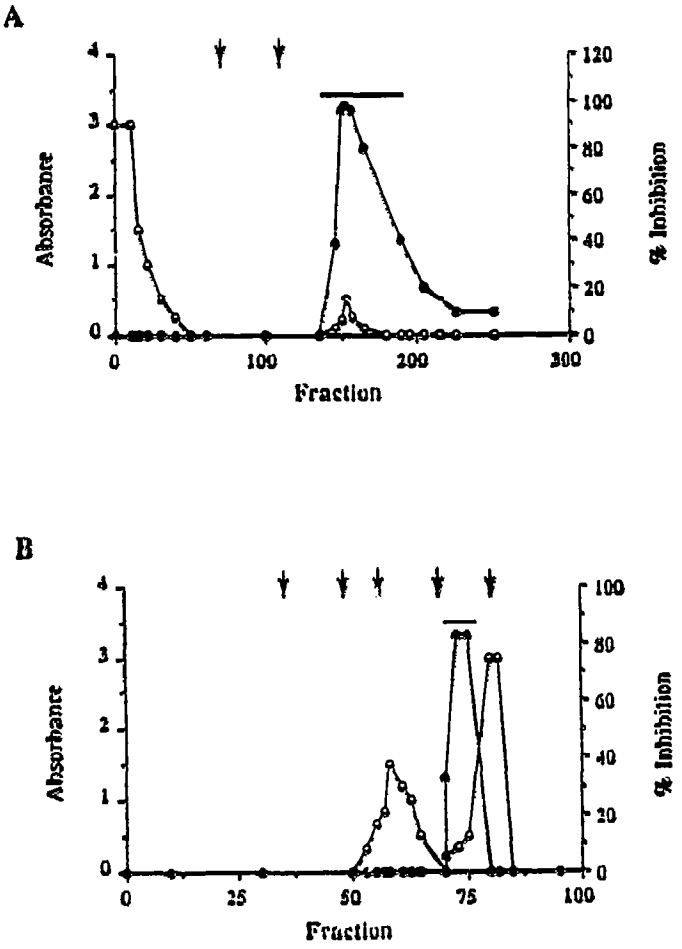

Fig. 1. Affinity (A) and gel filtration chromatography (B) of the membrane-associated CPI. The arrows in panel (A) depict the point of application of buffers A and B, respectively (see section 2). Arrows in panel (B) depict point of elution of M_r standards: thyroglobulin (670,000), γ-globulin (158,000), ovalbumin (44,000), myoglobin (17,000), vitamin B-12 (1,350), respectively. ●, % inhibitory activity; ○, absorbance at 280 nm. The fractions pooled are indicated by horizontal bars.

RESULTS

We purified a CPI from the total membrane fraction of an invasive murine hepatoma, Hepa cl 9, by a modification of techniques used for isolation of soluble low M_r cystatins from human tumors [2]. The total membrane fraction of normal murine liver did not contain CPI activity. Results of a typical purification of the

Table I

Results of a typical purification of membrane-associated cysteine protease inhibitor from murine hepatoma, Hepa cl 9

	Protein (mg)	Total Activity (IU)	Yield (%)	Specific Activity (IU·mg protein^{-1})	Purification (-fold)
Homogenate	1,143	1.92	(100)	0.002	1
Alkaline precipitation	297	1.82	95	0.006	3
Acetone precipitation	48	0.24	13	0.005	2.5
Affinity chromatography	0.36	0.09	5	0.244	122
Gel filtration chromatography	0.04	0.11	6	2.70	1,350

One inhibitory unit (IU) is defined as the amount of inhibitor preparation which totally inhibited one activity unit of papain (itself titrated against E-64) with one activity unit representing the release of 500 μmol of NHMec.

membrane-associated CPI from Hepa cl 9 are depicted in Table I. Affinity chromatography on a papain Sepharose column resulted in a single peak corresponding to CPI activity (Fig. 1A). This affinity step resulted in an ~50-fold increase in purification of the membrane-associated CPI, an increase in purification-fold similar to that observed previously with soluble cystatins [2,10]. The affinity fractions containing CPI activity were pooled and subjected to gel filtration chromatography (see Section 2). A single peak of CPI activity, representing a protein of M_r 10–15 kDa, was eluted from the gel filtration column (Fig. 1B). The specific activity of the membrane-associated CPI against papain (Table I) was much lower than that of the cytosolic tumor stefins [2].

The immunological relationship of the membrane-associated CPI to cystatins was determined in immunoblots using antibodies to stefin A, stefin B, cystatin C and low-M_r kininogen. The membrane-associated CPI did not crossreact with monoclonal antibodies against cystatin C or stefin B or a polyclonal antibody against low-M_r kininogen (Figs. 2 and 3). However, two crossreactive bands, running at 14 kDa and 70 kDa, were detected by a polyclonal antibody to rat epidermal stefin A (Fig. 4).

Since the membrane-associated CPI crossreacted with a stefin A antibody, we compared the isoelectric point of the membrane-associated CPI with that of human stefin A by isoelectric focusing (Fig. 5). The control human stefin A ran as several isoforms, the two predominant isoforms having pIs of 4.70 and 4.85. The predominant isoform of the membrane-associated CPI had an isoelectric point of 4.75.

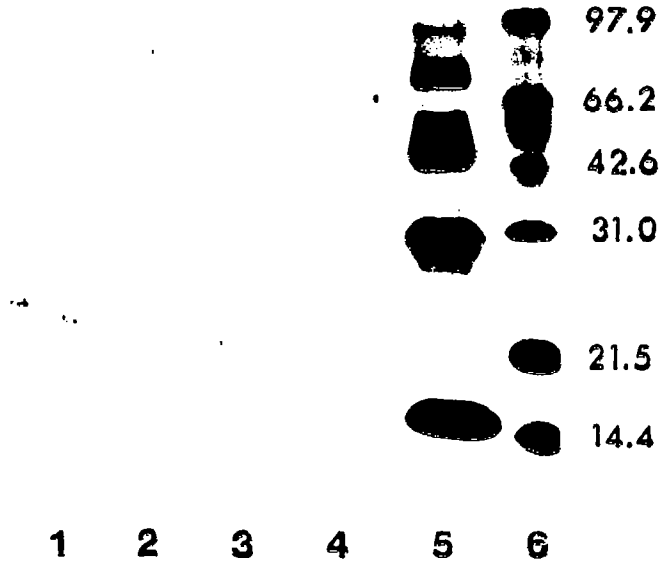

Fig. 2. Immunoblot analysis of the membrane-associated CPI with a monoclonal antibody against cystatin C. Lanes represent: 1, human stefin A (11 μg); 2 and 3, human stefin B (4 μg and 25 μg); 4, purified membrane-associated CPI (4 μg); 5, recombinant cystatin C (12.5 μg) appearing as multimeric aggregates; 6, prestained protein M_r standards.

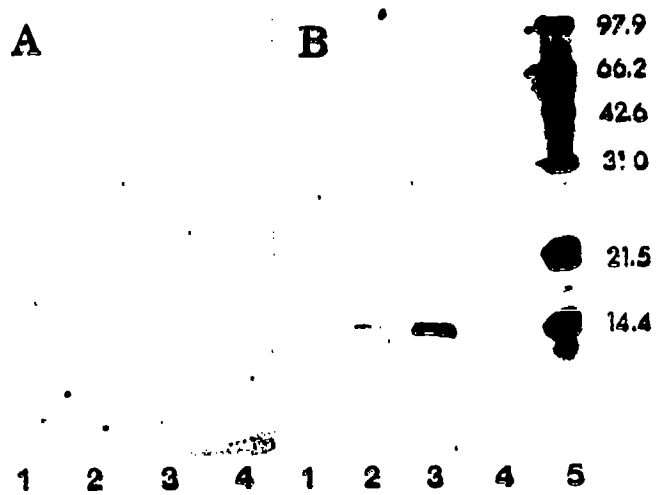

Fig. 3. Immunoblot analysis of the membrane-associated CPI. The same blot was analyzed sequentially with polyclonal antibodies to human cystatin C (not illustrated) and low M_r kininogen (panel A) and a monoclonal antibody to human stefin B (panel B). Lanes represent: 1, purified membrane-associated CPI from Hepa cl 9 (4 μg); 2 and 3, human stefin B (4 μg and 25 μg, respectively); 4, human stefin A (11 μg); 5, prestained protein M_r standards.

4. DISCUSSION

The CPI purified from the total membrane fraction of the highly invasive murine hepatoma, Hepa cl 9, appears to be a modified form of stefin A. Two bands corresponding to M_r 14 and 70 kDa were immunoreactive with a stefin A antibody, yet only a single peak corresponding to 10–15 kDa on gel filtration had inhibitory activity against papain. In addition, only a single isoform of membrane CPI could be resolved by isoelec-

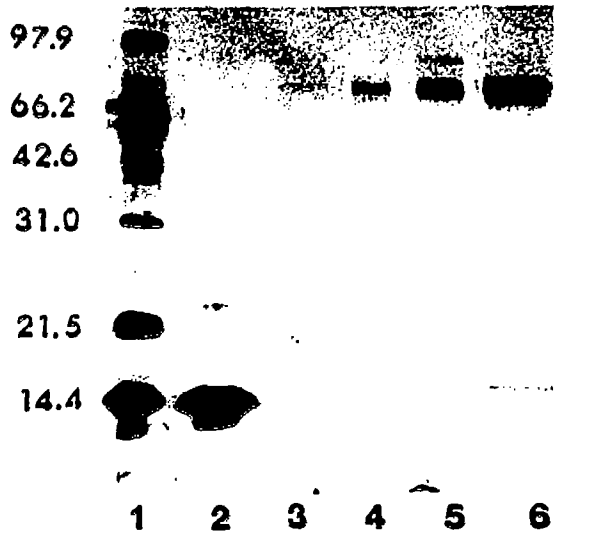

Fig. 4. Immunoblot analysis of the membrane-associated CPI with a polyclonal antibody against rat epidermal stefin A. Lanes represent: 1, prestained protein M_r standards; 2, human stefin A (11 μg); 3, human stefin B (4 μg); 4–6, purified membrane-associated CPI (2 μg, 4 μg, and 10 μg, respectively).

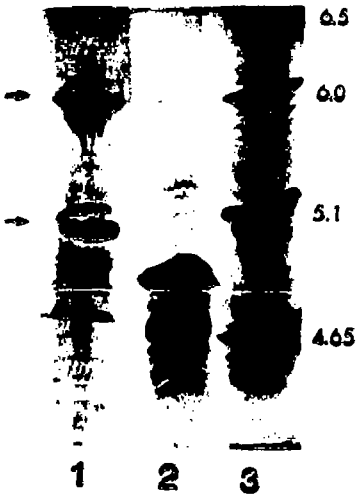

Fig. 5. Isoelectric focusing of the membrane-associated CPI. Lanes represent: 1, purified membrane-associated CPI from Hepa cl 9 (0.12 μg); 2, human stefin A (0.11 μg); 3, isoelectric focusing protein standards. Arrows depict the positions of sample application.

tric focusing. These observations suggest that the 70 kDa species of membrane CPI may be an aggregate of the 14 kDa species. Aggregates have been reported for other cystatins: by ourselves [2] and others [10,11] for stefin B and cystatin C [Lah, personal communication]. The immunoblot depicted in Fig. 2 of the present study also illustrates the formation of aggregates by recombinant cystatin C. In contrast to the membrane CPI purified from hepatoma, the recombinant *ras* gene product, reported by Hiwasa et al. [6,12,13] to have CPI activity, is a membrane-associated protein of 21 kDa. Proteins with CPI activity in the M_r range of the *ras* gene products were not detected in the hepatoma membranes. The nature of the modification(s) in stefin A responsible for its association with membranes in the Hepa cl 9 tumor have not been determined. However, a structural alteration in stefin A has been suggested to be responsible for the reduced inhibitory capability (increased K_i) against papain and cathepsins B and L) of stefin A purified from the cytosolic fraction of human sarcoma [2]. Possible associations of stefin A with the membrane were not assessed in the previous study.

The localization of proteolytic enzymes with respect to their endogenous inhibitors as well as the absolute levels of proteolytic enzymes and inhibitors appear to be critical to the ability of tumor cells to invade through basement membrane. This is true for cysteine proteases and cystatins [3] (for review, see [14]), for metalloproteases and tissue inhibitors of metalloproteases (for review, see [15,16]) and for the plasminogen activator urokinase and plasminogen activator inhibitors (for review, see [17]). Whether the membrane-associated cystatin described here plays a role in the invasiveness of the Hepa cl 9 cells will require further study.

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