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The major component of a large, intracellular proteinase accumulated by inhibitors is a complex of α_2-macroglobulin and thrombin

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A large, intracellular proteinase accumulated by inhibitors (PABI) was found in cultured mammalian cells as a large, multicatalytic proteinase with a greatly elevated concentration in the presence of small peptide proteinase inhibitors (Tsuji and Kurachi (1989) J. Biol. Chem. 264, 16093). Electron microscopic analysis showed that the tertiary structure of PABI highly resembled that of α_2-macroglobulin complexed with a proteinase(s). Isolation of the anti-PABI cross-reacting material from calf serum added to the culture media of baby hamster kidney cells further supported that the primary component of PABI was α_2-macroglobulin. Immunoblot analyses and the substrate specificity of PABI indicated that the major proteinase component contained in PABI was thrombin. When α_2-macroglobulin was added to the PABI-depleted serum, a significant accumulation or a degradation of the intracellular α_2-macroglobulin was observed in the presence or absence of leupeptin, respectively. Similarly, when thrombin was added to the PABI-depleted fetal calf serum supplemented with fresh α_2-macroglobulin, a significant amount of intracellular thrombin was found only in the presence of leupeptin. These results indicate that the major component of the intracellular PABI molecules is a complex of α_2-macroglobulin with thrombin which is internalized from the culture media. Intracellular accumulation of PABI, therefore, is a phenomenon primarily relevant to the culture cells. Whether or not PABI is also generated in certain physiological or pathological conditions requires further study.

Introduction

PABI is a large, multicatalytic intracellular proteinase of about 700 kDa which was recently characterized [1]. When a small proteinase inhibitor, such as leupeptin, was added to the culture media of mammalian cells, the concentration of PABI increased 100–200-fold above the basal level. This unique accumulation was considered to be due to greatly reduced levels of degradation in the presence of such a proteinase inhibitor. PABI showed a rather complex substrate specificity, with obvious similarity to that of trypsin and chymotrypsin, but not to those of plasmin, elastase or cyteine proteinases. PABI was a multimer of a major subunit of 84 kDa and was not activated by ATP. These properties were unique to PABI and clearly different from several other known, large proteinases [2–9]. No specific biological role has yet been determined for PABI. Proteosome, one of large multicatalytic proteinases is apparently involved in ubiquitin-independent protein degradation. It may also be involved in other important functions, such as a stabilizing effect on mRNAs [10,11]. Proteosome, 7700 kDa in size, is composed of multisubunits with a molecular mass of 22–33 kDa. These proteinases are constitutive components of cells and can be activated by polylysine, SDS, ATP or heparin. These proteinases, however, are not known to accumulate intracellularly in the presence of small proteinase inhibitors. Recently, an ATP activated, high-molecular-weight proteinase isolated from muscle was reported to be a cysteine proteinase-α_1-macroglobulin complex [12].

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In this paper, we describe the purification of anti-PABI cross-reacting material from plasma and high resolution electron microscopic analysis to show that PABI is unexpectedly a complex of α_2-macroglobulin, primarily with thrombin. We also describe experiments which show that α_2-macroglobulin and thrombin are incorporated into cultured cells and significantly accumulate in the presence of leupeptin.

Materials and Methods

Materials

Purified human α_2-macroglobulin and trypsin (2 × crystalline) were purchased from Sigma (St. Louis, MO). Purified bovine α_2-macroglobulin was obtained from Boehringer-Mannheim (F.R.G.). Goat anti-rabbit IgG conjugated with alkaline phosphatase and prestained SDS-PAGE standards (phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme) were from Bio-Rad Laboratories. Human thrombin (specific activity, 4.05 NIH units/μg) and anti-human prothrombin antibodies were kindly provided by Dr. Jules A. Shafer at the Department of Biochemistry of this campus. The thrombin preparation contained α-thrombin as the major component besides its nicked derivatives (β- and γ-thrombin). Newborn calf serum was from Whittaker Bioproducts (Walkersville, MD). 4-chloro-1-naphthol was purchased from Sigma. The other reagents used were of the highest analytical grade available.

Preparation of antiserum and immunoblot analysis

Preparation of antiserum against purified PABI was previously described [1]. Immunoblot analysis of proteins was carried out as follows. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemli [13]. The proteins separated were then electrophoretically transferred to the nitrocellulose paper at a setting of 30 V for 16 h according to the method of Towbin et al. [14]. Immunodetection of PABI on nitrocellulose paper was carried out as described previously [1]. Protein concentration was determined by the method of Bradford [15]. Double immunodiffusion analysis was performed at room temperature using 1% agarose in 50 mM phosphate-buffered saline (PBS) (pH 7.4), by the method of Ouchterlony [16]. After immunodiffusion, agarose gel was washed with PBS and then stained with Coomassie brilliant blue R-250.

Purification of cross-reacting protein with anti-PABI

All purification procedures were performed at 4°C unless otherwise stated. Serum from newborn calf (100 ml) was incubated with poly(ethyleneglycol) (PEG) 6000. The precipitate obtained with 4–18% (w/v) PEG concentration was dissolved with 25 mM Tris-HCl (pH 7.5) dialyzed against the same buffer overnight and applied to a DEAE-cellulose column (2.0 × 24 cm), equilibrated with 25 mM Tris-HCl (pH 7.5). The column was extensively washed with the same buffer and eluted with 1 l of a linear gradient buffer solution formed of 0 and 0.4 M NaCl at a flow rate of 60 ml/h. Fractions of 6 ml were collected and were assayed by immunoblot analysis. Fractions containing immunoreactivity were pooled, concentrated by ultracentrifugation (18700 × g, 20 h in a Beckman centrifuge) and the pellet was dissolved in 25 mM Tris-HCl (pH 7.5) and applied to a hydroxy apatite column (2.0 × 18 cm) equilibrated with 25 mM Tris-HCl (pH 7.5). The column was extensively washed with the same buffer and eluted with a linear gradient of 0 to 0.3 M sodium phosphate (pH 7.4). The immunoreactive fractions were concentrated by ultrafiltration (Amicon, XM-100 filter) and applied to a Sepharcryl S-300 (superfine) column (2.0 × 93 cm) equilibrated with 25 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl. The column was eluted with the same buffer at a flow rate of 15 ml/h. Fractions of 2.0 ml were collected and the immunoreactive fractions were pooled and concentrated by ultrafiltration.

Cell culture

BHK cells were cultured in Eagle's minimum essential media (MEM) (Gibco) supplemented with streptomycin, penicillin and 10% fetal calf serum in a 5% CO_2 incubator at 37°C.

Immunoblot analysis

Immunoblot analysis was carried out as previously described [1] with minor modifications. Cell extracts (7.5 μg as protein), media (7.5 μg as protein) or purified human α-thrombin (2.5 μg) were dissolved in gel loading buffer (62.5 mM Tris-HCl (pH 6.8, containing 10% glycerol, 2% SDS and 10% β-mercaptoethanol) and heated at 100°C for 5 min. The samples were then subjected to SDS-polyacrylamide gel (12%) electrophoresis employing a mini-gel apparatus (Bio-Rad). Prestained protein standards were used as size markers. The electrophoresed proteins were transferred to a nitrocellulose filter [14]. The blotted filters were incubated with 3% gelatin in 50 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl (TBS) at 37°C for 30 min. The filters were then incubated with the anti-PABI antiserum [1] at 1:500 dilution, followed by incubation with goat anti-rabbit IgG conjugated with horseradish peroxidase according to manufacturer's instructions. The filter blotted with human thrombin was incubated with partially purified rabbit antibodies, anti-PABI antibodies or rabbit anti-human prothrombin antibodies at a 1:1000 dilution, following the procedures described above. The filters were then incubated with TBS containing 4-chloro-1-napthol (0.5 mg/ml) for 30 min at

room temperature. The anti-human prothrombin employed in the experiments cross-reacted with bovine prothrombin.

Analysis of α_2-macroglobulin uptake by BHK cells

PABI depleted serum was prepared as follows. Fetal calf serum (2.5 ml) was added with a half volume of anti-PABI serum and incubated at 37°C for 2 h followed by continued incubation at 4°C overnight. The serum was then centrifuged at 3500 rpm for 15 min. The supernatant obtained was used as α_2-macroglobulin-depleted FCS. BHK cells grown to near confluency in 10 cm dishes were briefly treated with 2 ml of 0.25% (v/w) trypsin in 10 mM sodium phosphate/saline buffer (pH 7.4) (PBS buffer) containing 0.5 mM EDTA. After discarding the trypsin solution, fresh PBS buffer (2 ml) was added to harvest the cells. BHK cells were plated in 6 cm dishes at about 15% confluency in cultured in 3.0 ml MEM with and without 60 μg/ml leupeptin, supplemented either with (1) 10% FCS only, (2) 10% PABI-depleted FCS or (3) 10% PABI-depleted FCS containing 0.75 mg bovine α_2-macroglobulin. Cells were grown for 48 h to about 80% confluency and harvested by scraping after washing cells three times with 10 mM sodium phosphate saline (pH 7.4). The harvested cells were briefly centrifuged and stored as a pellet at −20°C until use. In a separate experiment, BHK cells were grown for 48 h in media with and without leupeptin supplemented with, (1) 10% PABI-depleted FCS preincubated with 0.75 mg α_2-macroglobulin for 30 min at room temperature or (2) 10% PABI-depleted FCS preincubated with 0.75 mg α_2-macroglobulin and 0.09 mg thrombin for 30 min at room temperature.

For immunoblot analysis, the frozen pellets were thawed and vigorously mixed by vortexing with 0.2 ml of ice-cold 20 mM Tris-HCl (pH 7.5) containing 1 mM $CaCl_2$. The homogenate was then centrifuged at 12000 rpm for 2 min and the supernatants obtained were used as cell extract for Western blot analysis. An aliquot (15 μl) of media diluted with the loading buffer to give the final protein concentration of 0.5 μg/ml was also subjected to Western blot analysis.

Electron microscopic analysis of PABI

Sample preparation. A frozen stock solution of PABI (75 μg/ml in 25 mM Tris and 5 mM $CaCl_2$) was thawed and 1 μl was diluted in 50 μl of triethanolamine buffer (25 mM triethanolamine, 5 mM $CaCl_2$ and 0.02% NaAzide) and fixed for 1 h on ice with 1% glutaraldehyde. A drop of the fixed PABI solution (3 μl) was applied to a glow discharged thin carbon film supported on a 'holey' carbon-coated EM grid. The sample was allowed to adsorb for 1 min, washed several times with glass distilled water, stained with 1% uranyl acetate, blotted and allowed to air dry.

Microscopy and densitometry. The specimen was examined in a Zeiss EM902 microscope in bright field mode using the no-loss electrons at a magnification of 35000 × and a defocus of 0.6 μm. Micrographs were digitized using a Photometrics Star I camera system with a Micro Nikkor f = 55 mm lens to produce a final pixel size of 0.52 nm. Images were transferred from a Northgate 386PC compatible computer to a VAXstation 3500.

Image analysis. Images of PABI were interactively selected on the VAXstation using the SPIDER software system [17]. A reference particle was selected and centered by angular and translation correlation with a low-pass filtered image of a rectangle. Other particles were rotationally aligned with the low-pass filtered reference particle using the autocorrelation function. Translational alignment was done by cross-correlation. A series of similar looking aligned particles was averaged to form a new reference which was used in a second alignment pass. Correspondence analysis [18] was performed on the aligned images to select a subset to participate in the final average. The similar images were divided into two subaverages and a phase residual calculation on their Fourier transforms provided information on the resolution (3.3 nm) of the final average image [19].

Results

Purification and identification of anti-PABI cross-reacting material from bovine plasma

As shown in Fig. 1 (left panel), anti-PABI antibody [1] strongly cross-reacted with calf plasma and weakly, but clearly, with human plasma. It did not cross-react with rat plasma. Immunoblot analysis showed a band of molecular weight of about 180000 for both calf plasma and human plasma with much higher intensity for calf plasma sample (Fig. 2). Fig. 3 shows the elution profile

Fig. 1. Double immunodiffusion analysis. Human, rat and calf serum (left) and purified immunoreactive protein, calf serum and purified α_2-macroglobulin from calf and human (right) were tested against antiserum to PABI. Center well (A): 50 μl of antiserum to PABI; well 1, 5 μl of human plasma; well 2, 5 μl of rat plasma; well 3, 5 μl of calf serum; well 4, 20 μg of purified APCRM; well 5, 20 μg of purified α_2-macroglobulin from calf plasma; and well 6, 20 μg of purified α_2-macroglobulin from human plasma.

with three peaks from sephacryl S-300 with immunoblot analysis of elution fractions. The front peak which contains the major band of about 180000 was observed as cross-reacting material. A few minor bands of lower molecular weights were also observed. The anti-PABI cross-reactivity material (APCRM) was purified to more than 95% homogeneity (Fig. 4). About 60 mg of the purified APCRM was obtained from 100 ml of newborn calf serum. The yield was estimated to be about 30%. The purified APCRM was then tested for its identity with plasma α_2-macroglobulin. Cross-reactivity of anti-PABI with purified bovine α_2-macroglobulin is shown in Fig. 1 (right panel). The precipitine line clearly fused with that of purified APCRM (wells 4 and 5) strongly suggesting the identity of APCRM with bovine α_2-macroglobulin. The precipitine line of purified human α_2-macroglobulin fused neither with that of purified bovine α_2-macroglobulin nor with that of APCRM in calf plasma. When the purified APCRM and the purified bovine α_2-macroglobulin were digested with trypsin, essentially identical fragmented peptide patterns were observed (Fig. 4). These results indicate that the major component of PABI is bovine α_2-macroglobulin.

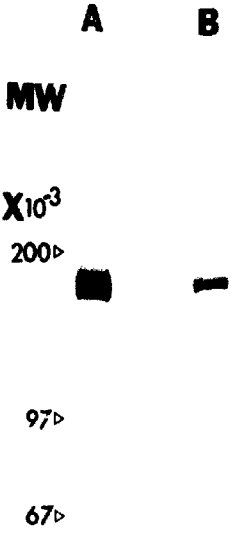

Fig. 2. Immunoblot analysis of PABI in calf serum and human plasma. 1 µl of plasma added with 4 µl of the loading buffer was treated in boiling water for 5 min before application to the SDS-polyacrylamide gel (4–15% gradient gel). Protein bands separated were visualized by immunostaining with anti-PABI serum. Lane A, calf serum; and lane B, human serum.

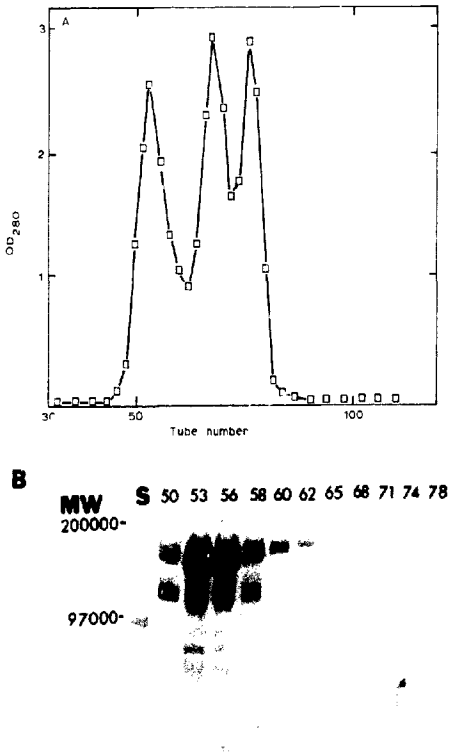

Fig. 3. Gel filtration of the immunoreactive protein with antiserum against PABI on Sephacryl S-300. The eluates from hydroxy apatite were applied to the column (2×93 cm). The column was then eluted at a flow rate of 15 ml/h with 25 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl. 5 µl of fractions were applied to SDS-polyacrylamide gel (4–15% gradient gel) and immunoblot analysis was carried out. (A) protein elution profile; (B) immunoblot analysis with anti-PABI serum.

but not other proteins such as α_1-macroglobulin and pregnancy zone protein.

Electron microscopic analysis of PABI

Results of electron microscopic analysis and computer image analysis are shown in Fig. 5. Panel A shows the electron microscopic image (side view) of PABI. Computer image analysis revealed the H shaped structure of PABI. This structure was found to be highly similar to the basic model determined for tetrameric human α_2-macroglobulin complexed with either chymotrypsin, trypsin or thrombin which was described as a padlock shape, H shape or cyrillic character shape [20–23]. The ninety degree rotated image (end view) was also very similar to that of human α_2-macroglobulin-chymotrypsin complex previously reported [22]. Because the shape of the α_2-macroglobulin molecule is unique,

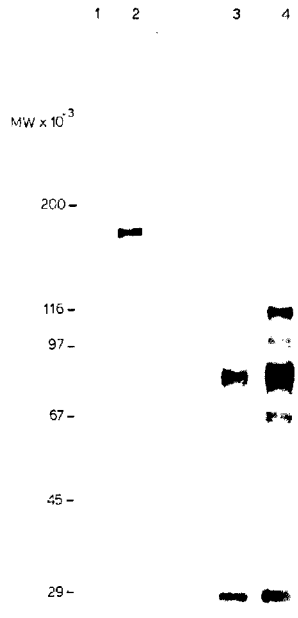

Fig. 4. SDS-polyacrylamide gel electrophoresis of the purified immunoreactive protein, α_2-macroglobulin and their tryptic peptides. Polyacrylamide gel employed was for a linear gradient gel formed of 4–15%. Purified α_2-macroglobulin from calf plasma, before (lane 1) and after (lane 3) digestion with trypsin (molar ration 1:1 at 37°C for 1 h) and the immuno reactive proteins (APCRM) before (lane 2) and after (lane 4) digestion with trypsin (molar ration 1:1). Proteins were detected by silver staining.

these observations strongly suggest that PABI is identical to a complex of tetrameric α_2-macroglobulin and a proteinase(s).

Immunoblot analysis of PABI

The results of immunoblot analysis of human thrombin with anti-PABI antibody as well as anti-prothrombin antibody are shown in Fig. 6. In this immunoblot analysis, both anti-PABI and anti-prothrombin detected the B-chain of human α-thrombin (33 kDa band in this gel) and its nicked derivatives (bands of about 13, 22 and 19 kDa) [24]. A-chain of α-thrombin (62 amino acid residues in length) ran out of the gel and is not shown in this picture. These results strongly suggest that at least one type of the proteinases complexed with α_2-macroglobulin in PABI is thrombin. Antiplasmin failed to cross-react with PABI (data not shown), agreeing with the previous observation that PABI does not have plasmin-like substrate specificity [1]. Other proteinases with chymotrypsin-like substrate specificity which may also be complexed with α_2-macroglobulin have yet to be identified.

α_2-macroglobulin uptake by BHK cells

Fig. 7 shows the presence and absence of α_2-macroglobulin (bands a and b) in the culture media of BHK cells under various conditions. When the intact serum was supplemented to the media, the presence or absence of leupeptin did not make any apparent difference on

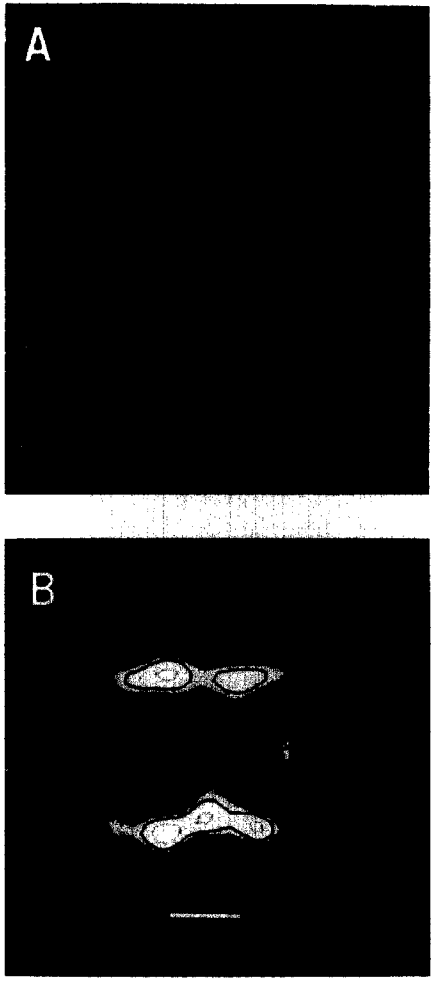

Fig. 5. Electron Microscopic analysis of PABI. (A) Electron micrograph of PABI macromolecules negatively stained with uranyl acetate and imaged in bright field, no energy-loss mode with a Zeiss EM902 microscope. PABI have the H-like structure characteristic of α_2-macroglobulin complexed with proteinase. Magnification: 248000×. (B) Average of aligned PABI images after low-pass filtration to 3.3 nm resolution. Contour lines delineate regions of similar stain exclusion. Scale bar is 10 nm.

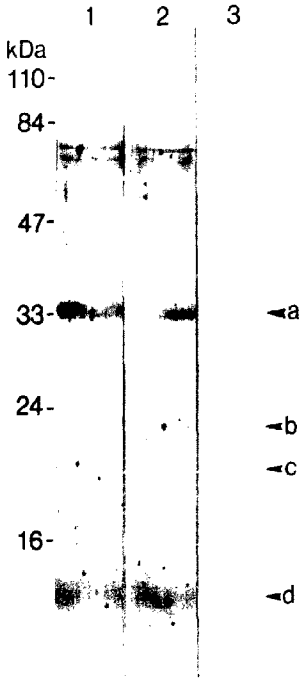

Fig. 6. Immunoblot analysis of thrombin with anti-prothrombin and anti-PABI antibodies. Human thrombin samples were electrophoresed in a 12% polyacrylamide gel in a reduced condition, and blotted to a filter as described in the text. All lanes contain 2.5 μg of α-thrombin. Lane 1, stained with anti-thrombin antibody as the first antibody; lane 2, stained with anti-PABI antibody; and lane 3, stained with anti-hepsin antibody (control). Blotted filter for lane 2 was incubated longer for color development (15 min) than for the filter of lane 1 (10 min) to clearly show the stained bands. Sizes (kDa) of the protein standards are shown on the left. Band a corresponds to B-chain of α-thrombin, while bands b, c and d correspond to derivatives of nicked B-chain in the thrombin preparation.

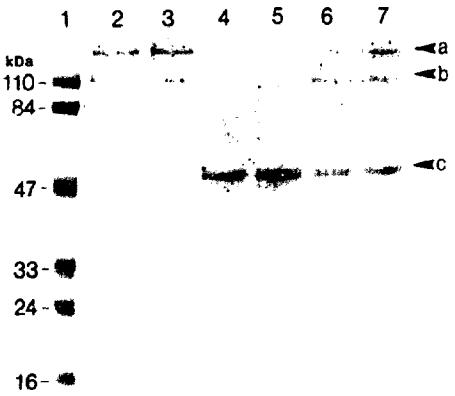

Fig. 7. Immunoblot analysis of BHK cell culture media with anti-PABI antibodies. Protein samples were electrophoresed in a 12% polyacrylamide gel in a reduced condition, blotted to a filter and analyzed with anti-PABI antibodies as described in the text. Sizes (kDa) of the prestained standard proteins (Lane 1) are shown on the left. Lanes 2–7 contain 7.5 μg of BHK proteins. Lanes 2 and 3 contain proteins from cells grown in media supplemented with 10% calf fetal serum; lanes 4 and 5 contain proteins from cells grown in media supplemented with 10% PABI-depleted serum; lanes 6 and 7 contain proteins from cells grown in media supplemented with 10% PABI-depleted serum and purified bovine α_2-macroglobulin (final 0.25 mg/ml). Culture media for cells in lanes 3, 5 and 7 was supplemented with leupeptin. Bands shown with arrows a and b correspond to α_2-macroglobulin. Band c corresponds to fragment derived from reduced rabbit IgGs stained with goat anti-rabbit IgG.

the α_2-macroglobulin concentration in the media (lanes 1 and 2). α_2-Macroglobulin-depleted serum showed only a trace amount of α_2-macroglobulin in the media (lanes 4 and 5 of Fig. 7), while the media supplemented with purified bovine α_2-macroglobulin again shows α_2-macroglobulin bands (lanes 6 and 7) as intense as in lanes 1 and 2. These results indicate that the amount of anti-PABI antibodies employed to deplete serum α_2-macroglobulin is just enough to deplete the endogenous serum α_2-macroglobulin, and not in excess to further bind the supplemented purified α_2-macroglobulin. Band C in lanes 4, 5, 6 and 7 in Fig. 7 correspond to a fragment derived from rabbit anti-PABI IgGs added to the media in order to deplete serum α_2-macroglobulin.

When intracellular PABI was analyzed (Fig. 8), much more elevated α_2-macroglobulin levels (represented by bands a, b and c of lanes 3 and 7 in Fig. 8) were observed in the presence of leupeptin compared to those in the absence of leupeptin (lanes 2 and 6). In the absence of leupeptin, apparent intracellular degradation products of α_2-macroglobulin (band e) were observed at

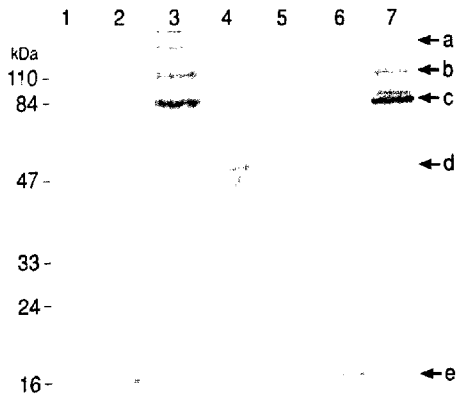

Fig. 8. Immunoblot analysis of BHK cell extracts with anti-PABI antibodies. Proteins of the cells were analyzed with anti-PABI antibodies. All other experimental conditions and numbering are same as in Fig. 7. Bands a, b, c, d and e correspond to the fragments derived from α_2-macroglobulin.

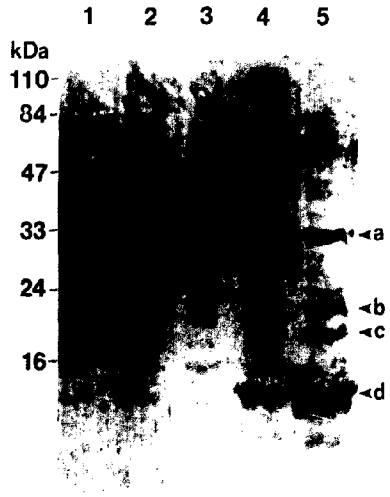

Fig. 9. Immunoblot analysis of BHK cell extracts with anti-prothrombin antibodies. Protein samples were electrophoresed in a 12% polyacrylamide gel in a reduced condition. The blotted filter were then incubated with anti-prothrombin antibodies. Lanes 1 and 2 contain proteins (7.5 μg) from cells grown in media supplemented with 10% PABI-depleted serum added with α_2-macroglobulin without and with leupeptin, respectively; lanes 3 and 4 contain proteins (7.5 μg) from cells grown in media supplemented with 10% PABI-depleted serum added with α_2-macroglobulin and thrombin without and with leupeptin, respectively; lane 5 contains 2.5 μg of α-thrombin. Band a corresponds to B-chain of α-thrombin, while bands b, c and d correspond to nicked derivatives of B-chain (see Fig. 6).

much higher levels (lanes 2, 4 and 6 in Fig. 8) compared to the trace amount of band e, if any, in the presence of leupeptin (lane 3 and 7). Band d was also considered to be an intracellular degradation product of PABI. These results show that bovine α_2-macroglobulin in the media is internalized by BHK cells followed by efficient intracellular degradation in the absence of leupeptin, but significant protection from degradation in the presence of leupeptin resulting in its great accumulation in cells.

As shown in Fig. 9, anti-prothrombin antibodies recognized greatly increased intracellular thrombin in the presence of leupeptin (bands a and d in lane 4), only when both α_2-macroglobulin and thrombin were added to the PABI-depleted calf serum. The increase was not observed when the serum was supplemented only with α_2-macroglobulin. These results indicate that the formation of the complex (PABI) between α_2-macroglobulin and thrombin is a prerequisite for internalization and subsequent intracellular accumulation of PABI in the presence of leupeptin (Fig. 9). The results also suggests that free and active thrombin is essentially absent in the PABI-depleted serum (lane 2 of Fig. 9). Thrombin in serum might have been consumed not only by forming PABI complex and complexes with other proteinase inhibitors such as antithrombin III, but also by degradations. When anti-PABI antibodies were used in place of anti-prothrombin in this experiment, an increased amount of intracellular PABI, and its protection from degradation in the presence of leupeptin were observed as expected (data not shown).

These results show that at least one of the proteinases complexed with α_2-macroglobulin in PABI is thrombin in the serum which is added to the culture media.

Discussion

PABI was originally identified as a large, multicatalytic proteinase that is greatly elevated in its intracellular concentration primarily in the lysosomal fraction in the presence of small proteinase inhibitors, such as leupeptin [1]. In order to determine its biological role, PABI was further characterized in the present study.

We first carried out electron microscopic analysis of PABI to compare with the tertiary structures previously reported for other large proteinases [25]. The electron microscopic image obtained for PABI was unexpectedly very similar to those of tetrameric α_2-macroglobulin complexed with proteinases, such as chymotrypsin and trypsin (Fig. 5) [21–23]. The result strongly suggested that PABI was a complex of α_2-macroglobulin and a proteinase(s), which was internalized by the cultured cells, presumably by the receptor mediated endocytosis [26–28]. The result also provides further evidence that the H shaped electron microscopic image is of the α_2-macroglobulin complexed with a proteinase(s), but not of the native form of α_2-macroglobulin as previously postulated [29].

Tests for immunological cross-reactivity (Figs. 1 and 2) show that anti-PABI cross-reacting material (APCRM) is also present in sera, and that APCRM forms fused precipitine lines only with bovine α_2-macroglobulin. Furthermore, other properties of the purified APCRM, such as tryptic peptide map also agree well with those of α_2-macroglobulin. These results clearly indicate that the major component of PABI is serum α_2-macroglobulin and not other proteins including its homologous proteins, such as α_1-macroglobulin or pregnancy zone protein [12,30].

The proteinases complexed with α_2-macroglobulin in PABI include those which have trypsin-like or chymotrypsin-like specificity, but neither cysteine proteinase, elastase nor plasmin-like proteinases as previously observed [1]. This suggests that intracellular proteinases (particularly lysosomal proteinases) produced by the cells are apparently not complexed with α_2-macroglobulin in PABI. These observations are consistent with the well established fact that the cell receptor

recognition site of α_2-macroglobulin molecule becomes available only after α_2-macroglobulin undergoes a substantial conformational changes upon forming a complex with a proteinase(s) ($E^F\alpha_2M$) [21,27,28,31].

Because synthetic substrates, such as CBZ-Arg-MCA, Z-Phe-Arg-MCA and BOC-Val-Leu-Lys-MCA were very poor substrates for PABI [1], the proteinase in PABI was considered not to be trypsin. The major trypsin-like substrate specificity of PABI, however, was rather similar to that of thrombin [1]. Therefore, we speculated that thrombin is a possible proteinase component of PABI. In immunoblot analysis, both anti-prothrombin antibody and anti-PABI antibody recognized α-thrombin and its derivatives by staining identical bands in Western blot analysis (Fig. 6). This indicates that thrombin present in the supplemented serum is one of the major proteinases complexed with α_2-macroglobulin in PABI. Results of a series of PABI uptake experiments (Figs. 7–9) clearly show that leupeptin, transported to the lysosomes of the cells [1], protected PABI molecules from proteolytic degradation, resulting in high accumulations of PABI in the cells. Furthermore, the results of PABI-depletion and protein supplementation experiments (Fig. 9) show that thrombin complexed with α_2-macroglobulin can be taken up by cells, agreeing well with the previous observations [26–28]. A proteinase(s) with chymotrypsin-like substrate specificity is also contained in some PABI molecules [1]. The intrinsic chymotrypsin-like activity of thrombin may be responsible for this activity [35–38]. At the present, however, no definitive identity of the proteinase(s) is available. Major proteinases which are complexed with α_2-macroglobulin in PABI are apparently restricted to a few proteinases, such as thrombin, probably due to their availability as active proteinases in serum. This may further suggest that the intracellular accumulation of PABI observed in the present experiments is a phenomenon only relevant to the cultured cells which are exposed to the media containing α_2-macroglobulin complexed with activated proteinases such as thrombin.

We have also observed that cultured rat capillary endothelial cells can elevate their PABI concentration to a very high level in the presence of leupeptin (unpublished data). These results may further suggest that α_2-macroglobulin apparently functions as an internalization vehicle for thrombin and some other specific proteinases in the plasma not only for the clearance of those proteinases from the plasma, but also for bringing those proteinases to their potential, alternative function site in cells. Recently, α_2-macroglobulin bindings were reported for interleukin-1β [39], basic fibroblast growth factor [40] as well as for transforming growth factors [41,42]. These observations further suggest an important function of α_2-macroglobulin as a general carrier protein for heterologous, but specific proteins in the plasma.

Osada et al. [43,44] reported the use of α_2-macroglobulin-proteinase complex as a carrier vehicle to transport α-galactosidase into the cells. This approach is of a great importance in considering a possible enzyme replacement therapy for lysosomal enzyme deficiencies, such as Fabry's disease [43]. Small proteinase inhibitors may serve to augment the effect of such therapies by enriching the α_2-macroglobulin complex in the cells.

Tanaka et al. [45] previously reported isolation of an acidic thiol proteinase induced in rat liver by the intraperitoneally administered leupeptin. Interestingly, when leupeptin was injected into peritoneal cavity of rats in our preliminary study, a significant increase in the intensity of an immunostained band of about 90 kDa, which corresponds to PABI, was observed in the liver as well as in the kidney (unpublished data). This observation suggests a significant accumulation of PABI in vivo may also take place when leupeptin is given to the animals. The proteinase(s) component of the PABI in vivo is to be determined. The observations made in the present study are also important for better understanding of the in vivo mechanism of action of small peptide proteinase inhibitors which are given to patients for muscle dystrophy treatment or in other pathological conditions [46–48].

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