Identification and Purification of a Novel Serine Proteinase Inhibitor*

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We report the identification, purification, and partial amino acid sequence of a novel serine proteinase inhibitor which is present in extracts from human placentas and in the cytosolic fraction of the leukemic cell line K562. Extracts from these tissues exhibited time-dependent inhibition of the serine proteinase thrombin. This activity was not accelerated by heparin and corresponded to a protein which formed a 67-kDa complex with 125I-thrombin. The complex was stable on reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A cleaved and functionally inactive form of the protein was purified from placental tissue by chromatography on DEAE-Sepharose, followed by affinity chromatography on thrombin-Sepharose. Antibodies raised against the placental protein recognized the inhibitor from K562 cells and placental extract. Western blotting experiments using the antibody showed that the uncleaved inhibitor has a molecular mass of 38 kDa.

Amino acid sequencing was performed on the purified protein. Sequences of peptides resulting from digestion with cyanogen bromide followed by Endoproteinase Lys-c confirmed that this is a novel inhibitor with significant homology to the serpin family.

Since the serpin family of proteinase inhibitors was defined, an increasing number of proteins have been described which belong to the group (1). The majority of these are extracellular proteins which regulate the proteolytic cascades of the coagulation, fibrinolytic or complement systems (2).

There are few examples of serpins which are resident intracellularly. Plasminogen activator inhibitor-2 is produced by monocytes and placentas and is only secreted under some conditions (3). An intracellular function for the protein has not been determined. Another intracellular serpin, human monocyte/neutrophil elastase inhibitor, has recently been described and a similar cytosolic protein has been reported in horse leukocytes (4–6). Their presence in monocytes and neutrophils suggests a role in the regulation of leukocyte proteases. The cowpox virus gene crm A has been shown to encode an intracellular serpin which inhibits human interleukin-1β-converting enzyme, thereby modifying the immune response to cowpox virus infection (7).

We report the identification and purification of a novel intracellular serine proteinase inhibitor. The protein is present in the cytosolic fraction of K562 cells and also in human placental tissue.

EXPERIMENTAL PROCEDURES

Materials

Chemicals and reagents were obtained from the following sources: sodium chloride, ammonium hydrogen carbonate, glycine, and sodium hydrogen carbonate from Ajax Chemicals, Sydney, Australia; Tris, gelatine, acetic acid, methanol, urea, acetonitrile, sodium phosphate, and ammonium sulfate from BDH Chemicals; Triton X-100, Coomassie Blue R-250, phenylmethylsulfonyl fluoride (PMSF), benzamidine, EDTA, diisopropyl-fluorophosphosphate (DFP), cyano gen bromide (CNBr), iodoacetamide, dithiothreitol (DTT) and hepta-fluorobutyric acid (HFBA) from Sigma; trifluoroacetic acid from Auspep Pty. Ltd., Melbourne, Australia; sodium 125I from Du Pont-New England Nuclear; 4-chloro-1-naphthol, Affi-Gel-10, Affi-Gel-15, and SDS-PAGE molecular weight standards from Bio-Rad; DEAE-Sepharose and Protein A-Sepharose from Pharmacia; chromogenic substrate THI; from Nycomed AS, Oslo, Norway; sodium heparin from David Bull Laboratories, Melbourne, Australia. Bio-Sil SEC-250 columns were purchased from Bio-Rad.

Thrombin

Thrombin was prepared from prothrombin purified from human plasma as previously described (8). The concentration of thrombin in solution was determined by absorbance at 280 nm using an extinction coefficient of 1.83 ml mg⁻¹ cm⁻¹ (9). Thrombin activity was assayed using the chromogenic substrate THI. Thrombin was diluted in 50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% gelatine, 0.05% Triton X-100, and the chromogenic substrate THI added to a final concentration of 300 μM. The linear increase in absorbance at 405 nm was proportional to thrombin activity.

Thrombin inhibition was measured by incubating dilutions of the sample of interest with an equal volume of a known concentration of thrombin in solution for 1 h at 4 °C. Residual binding sites were blocked according to the manufacturer's instructions and incubated with the dialyzed thrombin for 4 h at 4 °C. Residual binding sites were blocked by the addition of glycine to a final concentration of 300 ng of thrombin.

Thrombin was iodinated to a specific activity of 44.7 MBq/μg using Iodogen (1,3,4,6-tetrachloro-3,6-diphenyl glycouril) (10) without loss of thrombin amidolytic activity.

Thrombin-Sepharose and DFP-Thrombin-Sepharose

Thrombin was dialyzed extensively at 4 °C against 100 mM NaHCO₃, pH 8.0, 150 mM NaCl, prior to coupling to the Affi-Gel-15. The final concentration of thrombin was approximately 1 mg/ml in a volume of 10 ml. An equal volume of Affi-Gel-15 was prepared according to the manufacturer's instructions and incubated with the dialyzed thrombin for 4 h at 4 °C. Residual binding sites were blocked by the addition of glycine to a final concentration 100 mM. The thrombin-Sepharose was washed with a solution of 2 mM NaCl and equilibrated with 20 mM Tris, pH 7.4, 150 mM NaCl, prior to use.

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† The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; DFP, diisopropyl-fluorophosphosphate; DTT, dithiothreitol; HFBA, hepta-fluorobutyric acid; PVDF, polyvinylidene difluoride.

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DPF-thrombin-Sepharose was prepared by treating thrombin-Sepharose with DPF at a final concentration of 20 nm.

**SDS-PAGE and Western Blotting**

SDS-PAGE was performed by the method of Laemmli (11). For Western blots proteins were transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore Corporation). The transfer buffer was 25 mM Tris, pH 8.3, 192 mM glycine, 20% methanol. The proteins were electrophoresed for 1 h at 250 mA and 80 V and the membrane was blocked with 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA containing 5% fat-free milk. The secondary antibody was horseradish peroxidase-conjugated sheep anti-rabbit antibody (Silanes Laboratories, Melbourne, Australia) used at a dilution of 1:1000, and the bands were developed with 4-chloro-1-naphthol according to the manufacturer's instructions.

**Determination of Complex Forming Activity with 125I-Thrombin**

Complex formation with 125I-thrombin was demonstrated using reduced SDS-PAGE and autoradiography. Unless otherwise stated, samples to be examined were incubated with an equal volume of 5 nM 125I-thrombin at 37°C for 30 min, then boiled in SDS-PAGE reducing buffer prior to electrophoresis. The gels were dried and exposed to X-Omat, XAR 5 (Kodak).

**Cell Culture**

K562 cells were grown in RPMI 1640 (ICN Biomedicals Inc.) at 37°C in an incubator containing a 5% CO2, 95% air mixture. The medium was supplemented with 10% fetal calf serum, 20 mM glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin (ICN Biomedicals Inc.). The cells were harvested at 80% confluence, washed twice in 50 mM Tris, pH 7.4, 150 mM NaCl. The final resuspension buffer included 150 μM PMSF and 1 mM EDTA and contained 2 x 10^6 cells/ml. The cells were subjected to three cycles of freezing/thawing, sonicated on ice (100 watts, 45 s x 3) using a Labsonic 1510 (B. Braun, Melsungen, Germany) and centrifuged at 100,000 x g for 2 h. The supernatant (cytoplasm) was boiled in the same buffer containing 1% Triton X-100 and extracted at 4°C for 2 h. The extract was centrifuged briefly at 14,000 x g to remove insoluble material and diluted 1:5 in 50 mM Tris, pH 7.4, 150 mM NaCl to give a final volume equal to the cytosolic extract.

**Preparation of Placentas**

Twelve fresh, human placentas were cut into 3-cm cubes and washed free of clotted blood with water. They were minced with a commercial meat grinder and then washed with 10 mM imidazole, pH 6.5, 150 mM NaCl, then boiled in 20 mM imidazole buffer, pH 6.5, 150 mM NaCl, to remove insoluble material, 20 mM imidazole, pH 6.5, 150 mM NaCl, to which the protease inhibitors, 2 mM benzamidine, 150 mM PMSF, and 1 mM EDTA were added. Bacterial growth was inhibited by the addition of 0.02% sodium azide. The material was homogenized in a food blender with equal volumes of 10 mM imidazole, pH 6.5, containing the same protease inhibitors. The homogenate was centrifuged at 4,200 x g for 30 min and the supernatant collected.

**Chromatography on DEAE-Sepharose**

A DEAE-Sepharose column (8.5 x 20 cm) was equilibrated with 20 mM imidazole buffer, pH 6.5, at 4°C. The placental extract was applied to the column at a flow rate of 850 ml/h and the column washed with three column volumes of the equilibrating buffer. The column was eluted with an 8-liter linear gradient, 0–250 mM NaCl, in 20 mM imidazole buffer, pH 6.5, which also contained 0.02% sodium azide. Fractions (15 ml) were collected and assayed for thrombin inhibition and complex formation with 125I-thrombin.

**Affinity Chromatography on Thrombin-Sepharose**

Fractions which contained peak complex forming activity were pooled, titrated to pH 7.4 using 1 M Tris, and stirred at 37°C with 10 ml of thrombin-Sepharose. Aliquots were removed at intervals, centrifuged for a few seconds at 10,000 x g to remove the thrombin-Sepharose, and the supernatant assayed for residual thrombin inhibition and complex formation with 125I-thrombin.

**Preparation of Rabbit Polyclonal Antibodies**

Antibody to the Placental Proteinase Inhibitor—The proteins eluted from thrombin-Sepharose were subjected to SDS-PAGE. After electrophoresis, the gel was stained with 0.1% Coomassie Blue R-250, 0.5% acetic acid, and 10% methanol. The gel was destained in 10% methanol and the thrombin inhibitor band identified as described under "Results." The gel containing the band of interest was cut into small pieces and homogenized in Freund's adjuvant (Connaught Biological Laboratories, Melbourne, Australia) (complete for the first injection and incomplete for subsequent injections) and injected as a 150 μl volume of 1 ml which was injected intramuscularly at 4–6 weekly intervals. A total of six booster injections were delivered, and each rabbit received approximately 20–30 μg of protein/injection. Rabbits were bleed 6 weeks after the first injection. Satisfactory antibody titer was not attained until 6 months after the initiation of the immunization program.

**Antibody to Thrombin—**Rabbit polyclonal antibodies to thrombin were prepared by immunizing rabbits with thrombin. The immunization protocol was similar to the one used for the thrombin inhibitor.

**Immunodepletion and Immunoprecipitation Studies**

Immunoprecipitation—Samples to be used for immunoprecipitation were incubated with 125I-thrombin (2.5 nm) for 1 h at 37°C. The sample (250 μl) was mixed with 50 μl of Protein A-Sepharose and 5 μl of the relevant anti-serum and incubated overnight at 4°C. The mixture was then centrifuged briefly and the supernant removed. The pellet was washed three times in 20 mM Tris, pH 7.4, 250 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, and finally with 10 mM Tris, pH 7.4. The pellet was boiled in reducing buffer and an aliquot analyzed by reduced SDS-PAGE and autoradiography.

Immunodepletion—Immunodepletion was performed on placental extract which had been chromatographed on DEAE-Sepharose and a fraction selected which contained only the inhibitor of interest. Protein A-Sepharose (100 μl) was mixed with 500 μl of the relevant anti-serum for 30 min at 37°C then washed three times with 20 mM Tris, pH 7.4, NaCl 150 mM. The washed pellet was then incubated with 300 μl of the sample to be immunodepleted for 2 h at 4°C. The mixture was centrifuged briefly and the supernatant assayed for thrombin inhibition and complex forming activity with 125I-thrombin.

**Immunodiffusion Purification of the Thrombin Inhibitor**

The IgG fraction of anti-serum raised against the thrombin inhibitor was purified on a protein A-Sepharose column equilibrated with 20 mM Tris, pH 7.4, 150 mM NaCl. The IgG was eluted with 100 mM glycine, pH 2.5, and fractions immediately neutralized with 1 mM Tris (50 μl/ml). The fractions were pooled and concentrated by precipitation with a 50% saturated ammonium sulfate solution. The solution was centrifuged at 12,000 x g for 30 min and the pellet resuspended in 100 mM sodium hydroxide carbonate, pH 8.0, 100 mM NaCl, and dialyzed against the same buffer. The IgG was coupled to Affi-Gel-10 according to the manufacturer’s instructions, at an approximate ratio of 1 mg/ml of gel. The final volume of the gel was 30 ml.

**Pooled fractions from the DEAE-Sepharose eluate containing peak 67-kDa complex forming activity were titrated to pH 7.4 with 1 M Tris buffer. This material was applied to the immunooaffinity column which was equilibrated with 20 mM Tris, pH 7.4, 150 mM NaCl. After extensive washing with the eluting buffer, the column was eluted with 100 mM glycine, pH 2.5, in 3-ml fractions which were immediately neutralized with 1 M Tris 50 μl/ml. Protein concentration in the fractions was estimated by measuring absorption at 280 nm. Fractions with an absorbance greater than 0.01 were pooled to give a final volume of 36 ml and concentrated to 400 μl by centrifugation on a Centricon-10 (Amicon Corp., Danvers, MA). The concentrated material was further purified by HPLC gel filtration on two Bio-Sil SEC-250 columns (600 x 7.5 mm), connected in series and preceded by a Bio-Sil SEC-250 column (80 x 7.8 mm) guard column. The columns were equilibrated with 20 mM imidazole, pH 6.5, 150 mM NaCl at a flow rate of 0.5 ml/min and 0.5-ml fractions collected. Absorbance at 280 nm was used to monitor the protein elution from the column. Fractions which contained pure thrombin were pooled, concentrated, and dialyzed against 0.05 M Tris, pH 7.4, and 1 mM EDTA. The pooled fractions were then lyophilized and redissolved in 0.05 M Tris, pH 7.4, 1 mM EDTA, and concentrated to 1 mg/ml with a Centricon-100 (Amicon Corp., Danvers, MA) for use in further studies.
inhibitor were identified by SDS-PAGE, pooled, and used to derive amino acid sequence.

**Amino Acid Sequencing**

N-terminal amino acid sequencing was attempted using material eluted from the thrombin-Sepharose affinity column which was subjected to reduced SDS-PAGE and electroblotted to PVDF membrane. After staining with Coomassie Blue R-250, the thrombin inhibitor band was identified, cut out, and placed directly in the sequencer. Approximately 5 μg of thrombin was treated in the same way and used as a positive control to exclude the possibility of N-terminal blockage occurring during electrophoresis and electroblotting.

Internal amino acid sequence was derived from placental thrombin inhibitor which had been purified by immunoaffinity chromatography and HPLC gel filtration chromatography (as described above). The purified protein was concentrated to 100 μl in deionized water by centrifugation using a Centricon 10 concentrator. Trifluoroacetic acid was added to a final concentration of 70%. The protein was digested with CNBr, under nitrogen, in the dark, at room temperature, for 24 h. CNBr was used at a ratio of 100 μg of CNBr to 1 μg of protein.

After digestion the sample was dried in a centrifugal evaporator (Savant), dissolved in 100 μl of water, and redried to ensure complete evaporation of trifluoroacetic acid and CNBr. The protein was digested with endoproteinase Lys-C (Boehringer Mannheim) using the method described by Stone et al. (12). In brief, the protein was dissolved in 50 μl of 400 mM ammonium hydroxide carbonate, pH 8, containing 8 M urea and 4.5 mM DTT then incubated at 50 °C for 15 min. Iodoacetamide was added to a final concentration of 10 mM and the incubation continued for a further 15 min at room temperature. The sample was diluted in 140 μl of water and 0.5 μg of endoproteinase Lys-C added and the reaction allowed to proceed overnight.

Peptides resulting from the digestion were separated by reversed-phase HPLC on an RP900 column (1 mm × 50 mm) (ActiVon) which was equilibrated with 0.1% trifluoroacetic acid. Material retained on the column was eluted with a 0–60% acetonitrile gradient in 0.1% trifluoroacetic acid. Fractions corresponding to peaks of absorbance at 214 nm were collected and rechromatographed on the same column. For the second separation, the column was equilibrated with 0.1% HFBA, and peptides were eluted with a 0–60% acetonitrile gradient containing 0.1% HFBA.

Amino acid sequences were determined using an Applied Biosystems 470A protein sequencer fitted with an on-line model 120A phenyl thiobhydroxint (PTH) analyzer.

**RESULTS**

**Thrombin Inhibition by Placental Extract and K562 Cells**

Placental tissue extract contained significant thrombin inhibitory activity. Fig. 1 demonstrates that the inhibition of thrombin amidolytic activity by placental extract was time-dependent. Assays of placental extract indicate that it contains approximately 0.17 thrombin inhibitory units/mg of protein.

The cytosolic fraction of K562 cells also contained thrombin inhibitory activity. Inhibition followed a similar time course to the placental extract and, when assayed, was shown to contain 0.5 thrombin inhibitory units/mg of protein. The addition of heparin (0.5 or 5 units/ml final concentration) to K562 cell extract did not alter the rate of thrombin inhibition (data not shown).

**Identification of Thrombin-binding Proteins by Incubation of Tissue Extracts with 125I-Thrombin**

When crude placental extract was incubated with 125I-thrombin several bands corresponding to complexes with 125I-thrombin were seen (data not shown). Most appeared to correspond to serine proteinase inhibitors from blood present in placental extract. However, a major band which migrated at a molecular mass of 67 kDa could not be accounted for.

Placental extract, prepared as detailed under “Experimental Procedures,” was chromatographed on DEAE-Sepharose. When the fraction with peak thrombin inhibitory activity was incubated with 125I-thrombin a single complex of 67 kDa was seen (Fig. 2, lane 2). A complex of similar molecular weight was present in the extract of K562 cells incubated with 125I-thrombin (lane 5). When the K562 cell extract was centrifuged at 100,000 × g for 2 h, the complex forming activity remained in the supernatant, denoting a cytosolic localization. Complex forming activity with 125I-thrombin was not detected in serum-free medium which had been conditioned by K562 cells, indicating that the inhibitor was not secreted (data not shown). When 125I-thrombin was incubated with the K562 membrane fraction which had been extracted in buffer containing 1% Triton X-100 no complex formation was observed (lane 4). Similarly, 125I-DFP-thrombin failed to form complexes (lanes 1 and 3), suggesting that an intact thrombin active site is essential for the interaction.

The complex formed using placental extract appears to have a slightly lower molecular weight than that obtained using K562 cells. This difference is caused by the presence of a large amount of albumin in placental material which migrates at a molecular mass of 67 kDa.
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Purification from Human Placentas of a Protein Which Forms Complexes with \(^{125}\text{I}\)-Thrombin

Chromatography on DEAE-Sepharose—Placental extract (3.5 liters), with a protein concentration of 24.9 mg/ml and thrombin inhibitory activity of 4.6 units/ml was applied to DEAE-Sepharose with complete retention of the complex forming activity on the column. Elution of the column with a linear NaCl gradient caused complex forming activity to appear at 150 mM NaCl. When thrombin inhibition was assayed there was good correlation between the amount of thrombin inhibition present in fractions (Fig. 3a) and the intensity of \(^{125}\text{I}\)-thrombin complexes (Fig. 3b). The apparent variation in the position of the 67 kDa band is caused by a large amount of albumin in the samples, which migrates at a similar molecular mass. Fractions 120-195 contain a protein which forms an 85-kDa complex with \(^{125}\text{I}\)-thrombin. On the basis of molecular weight this is most likely antithrombin III present in the crude placental extract.

Fractions 210–315 were pooled to give a volume of 1.5 liter, with a protein concentration of 2.1 mg/ml containing 1.4 thrombin inhibitory units/ml. This represents a 14% yield of thrombin inhibitory activity from the DEAE-Sepharose step. Several factors may have operated to produce this outcome: 1) proteinase inhibitors other than the one of interest were present in the starting material, 2) fractions containing the inhibitor of interest were discarded in order to exclude other thrombin complexing proteins, 3) despite the addition of proteinase inhibitors, proteolytic cleavage and inactivation of the inhibitor may have occurred.

Affinity Chromatography on Thrombin-Sepharose—Incubation of the DEAE-Sepharose eluate with thrombin-Sepharose resulted in progressive binding of the thrombin inhibitor and a corresponding loss of thrombin inhibitory activity from the supernatant of the incubation mixture (Fig. 4a). When fractions described in a were incubated for 30 min at 37 °C with 2.5 mM \(^{125}\text{I}\)-thrombin and subjected to reduced SDS-PAGE and autoradiography. Molecular mass markers (kDa) are indicated in the left margin.

FIG. 4. Binding of the thrombin inhibitor to thrombin-Sepharose. a, thrombin-Sepharose was incubated at 37 °C with fractions 210–315 from the DEAE-Sepharose step. At the times shown samples were removed, centrifuged, and the supernatant assayed for the presence of thrombin inhibitory activity. b, the same samples as in a were incubated for 30 min at 37 °C with 2.5 nM \(^{125}\text{I}\)-thrombin and subjected to reduced SDS-PAGE and autoradiography. Molecular mass markers (kDa) are indicated in the left margin.

FIG. 5. SDS-PAGE of the affinity purified thrombin inhibitor. Material eluting from DFP-thrombin-Sepharose (lane 1) or thrombin-Sepharose (lane 2) was neutralized with acetic acid and samples analyzed using reduced SDS-PAGE. The band representing p35 is indicated in the right margin. Molecular mass markers (kDa) are indicated in the left margin.

The proteins eluted from DFP-Sepharose were subjected to SDS-PAGE. Inspection of the Coomassie-stained gel revealed a band at 35 kDa (p35) which was close to the expected molecular mass of the inhibitor (Fig. 5, lane 2). When thrombin-Sepharose was treated with DFP prior to incubation with the placental material, the p35 was absent (Fig. 5, lane 1) indicating that p35 specifically interacted with the thrombin active site. Fig. 5, lane 2, demonstrates other bands which appear to bind specifically to thrombin. The large band migrating at 55 kDa was subsequently shown to be \(\alpha\)-1-antitrypsin by \textit{in situ} tryptic digestion (13) and amino acid sequence (data not shown). The identity of the band samples were incubated with \(^{125}\text{I}\)-thrombin and submitted to SDS-PAGE there was a commensurate loss of complex forming activity (Fig. 4b).
migrating at 30 kDa has not been established.

The band p35 was cut from SDS-PAGE gels and used to develop the rabbit polyclonal antibody employed in the studies which follow.

Characterization of the Polyclonal Antibody to the Protein Forming a 67-kDa Complex with Thrombin

In order to demonstrate that the antibody to p35 recognized the thrombin-binding protein, immunoprecipitation of the 125I-thrombin-inhibitor complex was performed. These studies were performed with either antibody to thrombin (Fig. 6, lanes 1–3) or antibody to p35 (Fig. 6, lanes 4–6). Fraction 255 was selected from the DEAE-Sepharose elution since it contained the inhibitor of interest (Fig. 6, lanes 3 and 6). Fraction 135, containing antithrombin III, was also selected (Fig. 6, lanes 2 and 5). The antibody to p35 selectively immunoprecipitated the radiolabeled 67-kDa complex, whereas the thrombin antibody immunoprecipitated both the 67-kDa complex and the 85-kDa 125I-thrombin-AI3I complex. Immunoprecipitations were also performed on the K562 cytosolic fraction which had also been incubated with 125I-thrombin (Fig. 6, lanes 1 and 4). The antibody to p35 immunoprecipitated the 67-kDa complex indicating the immunological similarity of the complex forming protein from K562 and placenta.

To confirm that p35 is a thrombin inhibitor, immunodepletion studies were performed. These studies were carried out by incubating partially purified placental extract (fraction 255, Fig. 3) with Protein A-Sepharose to which antibody to p35 or control antibody were bound. The placental extract treated in this way was examined for its ability to inhibit thrombin. The results appear in Fig. 7a and demonstrate that the p35 antibody removed virtually all of the thrombin inhibitory activity whereas the control antibody had no effect. Further evidence that p35 is a thrombin-binding protein was derived from the observation that the placental extract failed to form complexes with 125I-thrombin after incubation with anti-p35 (Fig. 7b, lanes 4–6). The sample treated with control antibody demonstrates complex formation (Fig. 7b, lanes 1–3). The appearance of a doublet over the time course probably represents proteolytic cleavage of the 67-kDa complex.

Pooled fractions from DEAE-Sepharose eluate were chromatographed on the anti-p35 immunoaffinity column. The material purified in this way contained a major band on SDS-PAGE of 35 kDa. Further chromatography on an HPLC gel-filtration column resulted in p35 which appeared homogeneous as judged by SDS-PAGE (Fig. 8). This material was used in functional studies to assess its ability to interact with thrombin. The inhibition of the amidolytic activity of thrombin and complex formation with 125I-thrombin were examined. In neither assay could functional activity of the purified p35 be demonstrated (data not shown).

Although there is no doubt that p35 represents a thrombin inhibitory and binding protein, we were concerned that the immunoaffinity purified material failed to demonstrate this activity. A likely explanation is that purified p35 represents a cleaved and inactive form of the inhibitor. In order to examine the proteinase inhibitor in its uncleaved state, we performed a Western blotting study in which the cytosolic fraction of K562 cells, prepared in the presence of proteinase inhibitors, was incubated with thrombin containing trace 125I-thrombin. The samples were submitted to SDS-PAGE and transferred to PVDF membrane. The Western blot was
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Attempts to obtain N-terminal amino acid sequence of the inhibitor purified from placentas revealed N-terminal blockage. Peptides resulting from digestion by CNBr followed by endoproteinase Lys-c were sequenced, and the results are shown in Fig. 10. Searches of protein databases (NBRF Proteins, Swiss-Prot Proteins, and GenPeptide) confirmed that these sequences were novel. Furthermore, significant homology with several members of the serpin family was demonstrated (Fig. 10).

**DISCUSSION**

In this paper we demonstrate that extracts of placental tissue and the leukemic cell line K562 contain heparin-independent inhibitors of the functional activity of thrombin. In addition, a protein which bound 125I-thrombin forming a stable 67-kDa complex was observed on reduced SDS-PAGE. Preliminary evidence that the thrombin-binding protein was a thrombin inhibitor was based on the following observations: (a) formation of the complex was dependent on an intact thrombin active site, and (b) when placental extract was applied to, and eluted from, a DEAE-Sepharose column the ability of fractions to form 67-kDa complexes with 125I-thrombin correlated closely with the major peak of thrombin inhibition.

In order to further characterize the inhibitor and determine its relationship to other proteinase inhibitors, we proceeded to purify the protein from extracts of placentas and K562 cells. Conventional approaches to purification were unsuccessful. The principal problem with placental tissue was the low specific activity in the starting material while with K562 cells it was not feasible to obtain sufficient quantities of cellular extract for purification. To overcome these problems we developed a method of affinity purification using active thrombin as a ligand, immobilized on Sepharose. It was expected that only a small proportion of immobilized thrombin would be available for macromolecular interaction, therefore the purification was performed using an excess of immobilized thrombin over inhibitor. Confirmation that sufficient thrombin was available to interact with the inhibitor was indicated by the loss of inhibitory activity from the placental extract during the incubation. The eluate from this step, when visualized on SDS-PAGE, contained several proteins, three of which (55, 35, and 30 kDa) appeared to bind specifically to thrombin as evidenced by the following observations: (a) the bands were not present in the eluate of DFP-thrombin-Sepharose and (b) the same bands were absent from the eluate of thrombin-Sepharose when placental extract was preincubated with excess free thrombin (data not shown).

Based on the size of the complex (67 kDa) and the molecular mass of reduced thrombin (32 kDa) we predicted that the molecular mass of the thrombin inhibitor would be 35 kDa. The band at 35 kDa in the eluate of thrombin-Sepharose was therefore thought to represent the inhibitor. Evidence that p35 represented a thrombin inhibitory and binding protein was derived from immunoprecipitation and Western blot.

**Amino Acid Sequence of p35**

The band at 35 kDa in the eluate of thrombin-Sepharose was significant and interesting. However, further experiments are necessary to confirm this identity. The amino acid sequence of the p35 from placental tissue was determined by Edman degradation after isolation and purification. The sequence was compared with those of known serpin family members, and the results are shown in Table 1. The sequence of p35 from placental tissue differed significantly from those of known serpin family members. However, the sequence was similar to that of p35 from K562 cells, which was previously reported to be a novel serine proteinase inhibitor.

**Fig. 8.** Immunoadfinity purified p35 from placental tissue. Placental extract was chromatographed on DEAE-Sepharose and fractions containing the inhibitor of interest were applied to the immunoadfinity affinity column. The eluate from this column was concentrated and further purified by HPLC gel filtration. A sample from the peak fraction containing p35 was subjected to SDS-PAGE under reducing conditions. The material from this fraction was used to obtain amino acid sequence.

**Fig. 9.** Western blot of cytosol from K562 cells. The cytosolic fraction of K562 cells was prepared as described under "Experimental Procedures" and incubated with 55 nM thrombin (containing 5 nM 125I-thrombin) at 37 °C. Aliquots were removed at intervals of 5 and 30 min (lanes 2 and 3, respectively), immediately boiled in SDS-PAGE reducing buffer to stop the reaction, and subjected to SDS-PAGE under reducing conditions. A further sample was incubated for 30 min at 37 °C with 55 nM DFP-thrombin containing 5 nM 125I-DFF-thrombin (lane 4). Lane 1 represents cytosol from K562 cells incubated in the absence of thrombin. Lane 5 contains 0.5 μg of immunoadfinity purified p35. The resulting SDS-PAGE gel was electrotomed to PVDF membrane. The membrane was incubated with antibody to p35 followed by horseradish peroxidase-conjugated antirabbit antibody. A further sample was incubated with antibody to p35 followed by horseradish peroxidase-conjugated antirabbit antibody.

**Fig. 10.** Western blot of cytosol from K562 cells. The cytosolic fraction of K562 cells was prepared as described under "Experimental Procedures" and incubated with 55 nM thrombin (containing 5 nM 125I-thrombin) at 37 °C. Aliquots were removed at intervals of 5 and 30 min (lanes 2 and 3, respectively), immediately boiled in SDS-PAGE reducing buffer to stop the reaction, and subjected to SDS-PAGE under reducing conditions. A further sample was incubated for 30 min at 37 °C with 55 nM DFP-thrombin containing 5 nM 125I-DFF-thrombin (lane 4). Lane 1 represents cytosol from K562 cells incubated in the absence of thrombin. Lane 5 contains 0.5 μg of immunoadfinity purified p35. The resulting SDS-PAGE gel was electrotomed to PVDF membrane. The membrane was incubated with antibody to p35 followed by horseradish peroxidase-conjugated antirabbit antibody. A further sample was incubated with antibody to p35 followed by horseradish peroxidase-conjugated antirabbit antibody.
FIG. 10. Homology of p35 peptides with other members of the serpin family. p35 sequences are aligned with sequences of members of the serpin family. Abbreviations: EI, human monocyte/neutrophil elastase inhibitor (4); HLEI, horse leukocyte elastase inhibitor (6); PAI-2, human plasminogen activator inhibitor-2 (18); ATIII, human antithrombin III (19); a1AT, human α-1-antitrypsin (20). The numbers below the sequences refer to α-1-antitrypsin.

studies which employed a polyclonal anti-p35 antibody.

The availability of a specific polyclonal antibody to the thrombin inhibitor allowed the affinity purification of the protein and when combined with HPLC gel filtration this yielded homogeneous p35 as judged by SDS-PAGE. This protein was shown to be functionally inactive. Subsequent Western blot studies indicated that p35 was a proteolytically cleaved form of the inhibitor.

The question remains as to why immunoaffinity purification from placental extract yielded this form of the inhibitor. It is known that serpins undergo a conformational change upon cleavage at the active site (17). The polyclonal antibody developed in the course of our studies was obtained by immunizing rabbits with the cleaved, inactive form of the protein. While the antibody would be expected to recognize both the intact and cleaved molecule, there may still be a significant difference in the relative affinities for the two forms. The use of this antibody for the purification of the inhibitor may have resulted in preferential isolation of the cleaved, inactive form of protein. The antibody successfully immunodepleted the native protein from placental extract indicating that it has some affinity for the native protein. However, these studies were performed with relatively large amounts of antibody bound to the protein A-Sepharose, which may have facilitated a low affinity interaction.

Amino acid sequencing of p35, which had been subjected to SDS-PAGE and transferred to PVDF membrane, revealed N-terminal blockage. While cleavage of the native thrombin inhibitor would give rise to a protein containing a new, unblocked N terminus at or near the reactive site, this fragment would undoubtedly separate from p35 during reduced SDS-PAGE. It would therefore be unavailable for sequencing.

Derivation of internal amino acid sequence was complicated by the resistance of p35 to enzymatic digestion. Furthermore, treatment with CNBr alone generated hydrophobic peptides which could not be separated by reversed-phase HPLC. However, the use of CNBr followed by endoproteinase Lys-c produced peptides which were readily separated by reversed-phase HPLC. The amino acid sequences of these peptides showed clear homology with other members of the serpin family and confirmed the novelty of the thrombin inhibitor protein.

Although p35 binds to, and inhibits, thrombin it is unlikely that this represents a physiological event. p35 appears to be primarily cytosolic, and it therefore may be involved in the regulation of intracellular proteases. Studies are in progress to identify and characterize proteins which interact with the inhibitor.

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