A novel plasminogen-binding protein has been isolated from human plasma utilizing plasminogen-Sepharose affinity chromatography. This protein copurified with α2-antiplasmin when the plasminogen affinity column was eluted with high concentrations of ε-amino caproic acid (>20 mM). Analysis by sodium dodecyl sulfate suggests this protein has an apparent Mr of 60,000. The amino-terminal amino acid sequence showed no similarity to other protein sequences. Based on the amino-terminal amino acid sequence, oligonucleotide probes were designed for polymerase chain reaction primers, and an ~1,800 base pair cDNA was isolated that encodes this Mr, 60,000 protein. The deduced amino acid sequence reveals a primary translation product of 423 amino acids that is very similar to carboxypeptidase A and B and consists of a 22-amino acid signal peptide, a 92-amino acid activation peptide, and a 309-amino acid catalytic domain. This protein shows 44 and 40% similarity to rat procarboxypeptidase B and human mast cell procarboxypeptidase A, respectively. The residues critical for catalysis and zinc and substrate binding of carboxypeptidase A and B are conserved in the Mr, 60,000 plasminogen-binding protein. The presence of aspartic acid at position 257 of the catalytic domain suggests that this protein is a basic carboxypeptidase. When activated by trypsin, it hydrolyzes carboxypeptidase B substrates, hippuryl-Arg and hippuryl-Lys, but not carboxypeptidase A substrates, and it is inhibited by the specific carboxypeptidase B inhibitor (DL-5-guanidinoethyl)mercapto succinic acid. We propose that the Mr, 60,000 plasminogen-binding protein isolated here is a novel human plasma carboxypeptidase B and that it be designated pCPB.

The importance of lysine-binding sites contained in the kringle domains of plasminogen in the regulation of fibrinolysis is well documented and underscored by the antifibrinolytic effect of amino acids such as ε-amino caproic acid (ε-ACA). These sites not only mediate the interaction of plasminogen with fibrin but also with several other plasma proteins that include α2-antiplasmin, histidine-rich glycopolypeptide, and the recently identified tetranection (9–11). All of these latter proteins have been isolated by affinity chromatography on plasminogen-Sepharose (9–11). We employed plasminogen affinity chromatography in an effort to identify additional plasma proteins that interact with plasminogen and report here the isolation of a novel plasminogen-binding protein. Molecular cloning of the gene encoding this protein shows that it is very similar to tissue procarboxypeptidase B.

**EXPERIMENTAL PROCEDURES**

**Materials**

Lysine and protein A-Sepharose were from Pharmacia LKB Biotechnology Inc.; Affi-Gel 10 was from Bio-Rad. Dissopropyl fluorophosphosphate (DFP), ε-amino caproic acid, carboxypeptidase B, o-phenanthroline, and hippuric acid derivatives of L-arginine, L-lysine, L-phenylalanine, L-glycine-lysine, and phenylactic acid were from Sigma. (dl-S-Guanidinoethyl)mercapto succinic acid (GEMSA) was from Fluka. N-Glycosidase F was from Boehringer Mannheim.

**Methods**

Plasminogen Affinity Column—Plasminogen was purified as described by Deutsch and Mertz (28), dialyzed into PBS containing 1 mM DFP, and stored lyophilized at −80 °C. Approximately 500 mg of plasminogen was coupled to 70 ml of Affi-Gel 10 according to the manufacturer’s specifications. The column was stored at 4 °C in PBS containing 1 mM N3 and 1 mM DFP, and 100 μg/ml of aprotinin.

Purification of Plasma Carboxypeptidase B—Twenty units of outdated human plasma was depleted of plasminogen by chromatography on lysine-Sepharose and subsequently fractionated with ammonium sulfate, as previously described (9). Briefly, the plasma was successively fractionated with 0.8 and 2.7 M (NH₄)₂SO₄, and the resultant 2.7 M (NH₄)₂SO₄ pellet was exhaustively dialyzed into PBS and chromatographed on a plasminogen Affi-Gel 10 column equilibrated in PBS containing 1 mM each N3, DFP, and 100 μg/ml aprotinin (buffer A). The column was washed with 10 column volumes of buffer A containing 0.5 M NaCl, re-equilibrated into buffer A, and eluted with 200 mM ε-ACA in PBS. Fractions containing plasma carboxypeptidase B were pooled, and containing IgG was removed by chromatography on protein A-Sepharose. Plasma carboxypeptidase B (pCPB) was subsequently rechromatographed on the plasminogen affinity column as described above, except the column was developed with a 0–50 mM ε-ACA gradient in PBS containing 1 mM N3 and DFP; pCPB was initially identified in column fractions by amino-terminal amino acid sequence after electrolotting to polyvinylidene difluoride (PVDF) (12). Protein sequencing was carried out on

1 The abbreviations used are: ε-ACA, ε-amino caproic acid; DFP, diisopropyl fluorophosphosphate; GEMSA, (dl-S-guanidinoethyl)mercapto succinic acid; PBS, phosphate-buffered saline; CPA and CPB, carboxypeptidase A and B; pCPB, plasma carboxypeptidase B; tCPA and tCPB, tissue carboxypeptidase A and B; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBP, plasminogen-binding protein; PCR, polymerase chain reaction; bp, base pairs.

2 To whom correspondence should be addressed: Dept. of Cardiovascular Research, Genentech, Inc., 460 Pt. San Bruno Blvd., South San Francisco, CA 94080.
Applied Biosystems 470A and 473A vapor-phase sequenators as previously described (13, 14). For removal of potential Asn-linked carbohydrate, pCPB in 0.2 m phosphate, pH 8.6, was incubated with 1% SDS for 5 min at 95 °C. Subsequently, EDTA and Nonidet P-40 were added to 5 mM and 5%, respectively, and 4 units of N-glycosidase F was added. After 24 h at 37 °C, the reaction was extracted with chloroform and methanol, and proteins were resolved by SDS-PAGE.

Cloning of the cDNA Encoding Plasminogen-binding Protein (PBP) by Polymerase Chain Reaction (PCR)—Cloning of pCPB was accomplished using 37 residues of the amino-terminal amino acid sequence obtained from plasma-derived pCPB. We designed oligonucleotides for use as PCR primers based on this sequence. Three groups of highly degenerate PCR primers were designed. All primers were 26 bases in length. Primer pools pbp. 8 and pbp.9 were 16,384- and 49,152-fold degenerate, respectively, and represented all possible coding sequences for the first and last 9 amino acids, respectively, of the 97 amino acid sequence. Primer pbp.10 was 54,576-fold degenerate and was internal to pbp.8 and pbp.9. This primer represented all possible coding sequences for amino acids 9–17 of the known sequence. The expected PCR product from primers pbp.8 and pbp.9 was 105 bp, whereas the expected product from primers pbp.8 and pbp.10 was 98 bp in length.

DNA from human liver and kidney was reverse-transcribed with reverse transcriptase, and both single- and double-stranded cDNA were amplified with our degenerate primers. We used a biphasic PCR protocol in which the first 10 rounds of amplification were done at 50 degrees annealing, and the subsequent 20 rounds were done at reduced (40 degrees) stringency. In addition, we took the product of the first 10 rounds as the outermost primer (pbp.8 and pbp.9) and subjected it to a second round of biphasic PCR using the more internal primers (pbp.8 and pbp.10). This second round of PCR produced the expected 98 bp band from liver, but not kidney cDNA (Fig. 2). Sequencing of the 98 bp band PCR product indicated that it encoded the correct amino acid sequence. A 46-bp probe from the interior of this 98-bp sequence was synthesized, labeled, and used to probe a human liver cDNA library for use as PCR primers based on this sequence. Three groups of highly degenerate PCR primers were designed. All primers were 26 bases in length. Primer pools pbp.8 and pbp.9 were 16,384- and 49,152-fold degenerate, respectively, and represented all possible coding sequences for the first and last 9 amino acids, respectively, of the 97 amino acid sequence. Primer pbp.10 was 54,576-fold degenerate and was internal to pbp.8 and pbp.9. This primer represented all possible coding sequences for amino acids 9–17 of the known sequence. The expected PCR product from primers pbp.8 and pbp.9 was 105 bp, whereas the expected product from primers pbp.8 and pbp.10 was 98 bp in length.

DNA from human liver and kidney was reverse-transcribed with reverse transcriptase, and both single- and double-stranded cDNA were amplified with our degenerate primers. We used a biphasic PCR protocol in which the first 10 rounds of amplification were done at 50 degrees annealing, and the subsequent 20 rounds were done at reduced (40 degrees) stringency. In addition, we took the product of the first 10 rounds as the outermost primer (pbp.8 and pbp.9) and subjected it to a second round of biphasic PCR using the more internal primers (pbp.8 and pbp.10). This second round of PCR produced the expected 98 bp band from liver, but not kidney cDNA (Fig. 2). Sequencing of the 98 bp band PCR product indicated that it encoded the correct amino acid sequence. A 46-bp probe from the interior of this 98-bp sequence was synthesized, labeled, and used to probe a human liver cDNA library to obtain full-length clones of this cDNA. 110 positives were obtained from 1.3 million clones. Five of these, 1a, 1b, 2a, 2b, and 2c, were chosen for further characterization. Inserts in these clones ranged up to ~1.8 kilobases in length. Northern blot analysis of liver mRNA indicated a pCPB transcript size of 2 kilobases.

Activation of pCPB and Enzyme Assay—Purified pCPB in 50 mM Tris, pH 7.65, containing 150 mM NaCl was incubated with trypsin at a 1:10 protease/pCPB (w/w) ratio for various times at 37 °C. An aliquot was removed for analysis by SDS-PAGE, and the reaction was terminated by the addition of soybean trypsin inhibitor. pCPB activity was subsequently determined using the carboxypeptidase B substrates hippuryl-L-Arg or hippuryl-L-Lys as described (15). One unit hydrolyzes 1 nmol of substrate/min. Trypsin cleavage sites were determined by incubating 20–40 μg of pCPB with 2–4 μg of trypsin for 60 min. The reaction was terminated by the addition of SDS to 2% and heating the sample to 95 °C for 5 min. Proteins were resolved on 4–20% SDS-polyacrylamide gels, electroblotted to PVDF, and subjected to amino-terminal amino acid sequencing, as described above.

RESULTS

Purification of pCPB—While purifying α2-antiplasmin as described previously (9), we observed that elution of a plasminogen-Sepharose column with 200 mM ϵ-ACA not only yielded α2-antiplasmin but also an additional plasma protein of M, 60,000. Shown in Fig. 1, inset, is the analysis of the ϵ-ACA eluted proteins by SDS-PAGE. When resolved under nonreducing conditions, the major proteins observed had M, of 200,000, 67,000, and 60,000. These proteins were identified by amino-terminal amino acid sequencing following electroblotting to PVDF. The proteins with apparent M, of 200,000 and 67,000 were identified as IgG and α2-antiplasmin, respectively (data not shown). On occasion, we also observed the elution of tetraclexin. The amino-terminal amino acid sequence obtained from the M, 60,000 protein (FQSGQVLAALRRTSRQVQLXLLTYEIVLXQPVTP ADXIVK) showed no similarity to any protein sequence in the computer data bases. After removal of the IgG using protein A-Sepharose, the M, 60,000 protein was purified to homogeneity by chromatography on plasminogen-Sepharose that was eluted with a 0–50 mM ϵ-ACA gradient (Inset). Approximately 5 μg of the ϵ-ACA eluted proteins from the plasminogen-Sepharose column eluted with 200 mM ϵ-ACA (A) or with a 0–50 mM ϵ-ACA gradient (B, lane 1, fraction 33) were resolved by SDS-PAGE, and proteins were detected by staining with Coomassie Blue. α2AP, antiplasmin; PBP, M, 69,000 plasminogen-binding protein. pCPB treated with N-glycosidase F is shown in lane B2.

Cloning of pCPB—Based on the amino-terminal amino acid sequence of the purified M, 60,000 plasminogen-binding protein, we performed PCR reactions on reverse-transcribed mRNA from human liver and kidney. The PCR primers were highly degenerative pools, representing all possible DNA sequences that could encode the two ends of our amino-terminal sequence. The PCR product from the authentic M, 60,000 plasminogen-binding protein mRNA was predicted to be 98 bp in length. As shown in Fig. 2, a large number of PCR products were obtained from these mRNAs. However, a prominent product of ~98 base pairs was obtained from liver, but not kidney mRNA. This 98-bp product was cloned into pUC21 and sequenced. The DNA sequence was capable of encoding the known 35 amino-terminal amino acids of the plasminogen-binding protein (data not shown). Since this PCR product was obtained using highly degenerate primers, the DNA sequence at the ends that contain these primers could not be assumed to be identical with the authentic mRNA sequence for the M, 60,000 plasminogen-binding protein. To obtain full-length clones, therefore, we synthesized a single
that encodes the amino terminus peptides, including a generally hydrophobic character and a acids long that is not contained in plasma-derived PBP. This predicts a primary translation product 423 amino acids in sequence for the plasminogen-binding protein revealed that it contains either Ile or Leu at position 255 of the catalytic domain, compared with Asp for tCPB. Presumably, the negatively charged Asp of tCPB attracts basic amino acids, whereas Ile or Leu of tCPA would attract hydrophobic amino acids. In addition, Coll et al. (27) have recently suggested that replacement of Ile-243 and Gly-207 in tCPA by glycine and serine, respectively, also contributes to creating a polar environment in the active-site pocket of tCPB. At the analogous sites in the plasminogen-binding protein, Asp, Gly, and Ser are present, suggesting that it is specific for basic carboxyl-terminal amino acids (Fig. 4). This hypothesis is supported by the observation that activated plasminogen-binding protein hydrolyzes tCPB substrates, but not tCPA substrates, and is inhibited by the specific tCPB inhibitor GEMSA (Table I). Because of these observations, we propose that this protein be designated plasma carboxypeptidase B (pCPB).

Characterization of pCPB—When resolved by SDS-PAGE, purified plasma-derived pCPB migrates with an M, of 60,000, whereas N-glycosidase F-treated pCPB migrates with an M, of 45,000 (Fig. 1). When analyzed by isoelectric focusing-PAGE, pCPB resolved to a pl of approximately 5.0. Human pancreatic procarboxypeptidase B1 and B2 migrate on SDS-PAGE with an M, of 47,000 and have isoelectric points of 7.1 and 6.6, respectively (5). These results indicate pCPB is a glycoprotein.

Unlike tissue procarboxypeptidase, pCPB is unstable to trypsin activation. Trypsin treatment of pCPB resulted in only a modest activation followed by inactivation. During a 60-min incubation with trypsin, the specific activity of pCPB initially rose to 10 units/mg by 20 min and then decreased to 5 units/mg by 60 min (Fig. 5). Activated porcine tCPB had a specific activity of 140 units/ml, suggesting that activated pCPB is relatively inactive on these substrates. Determination of the trypsin cleavage sites shows that trypsin concomitantly activates and inactivates pCPB. Analysis of pCPB trypsin cleavage products by SDS-PAGE and their subsequent identification by amino-terminal amino acid sequencing shows that trypsin cleaves at Arg-92 and Arg-330 (Figs. 3 and 5). Cleavage at Arg-92 releases the activation peptide (M, = 35,000). However, simultaneous cleavage at Arg-330 yields an M, = 25,000 inactive fragment (Fig. 5). This latter cleavage results in the loss of a 71-amino acid (M, = 7,000) carboxy-terminal fragment from the catalytic domain, which contains residues implicated in substrate binding. We found that thrombin and plasmin proteolyzed pCPB in a similar manner, whereas urokinase, tissue plasminogen activator, activated protein C, and factors IX, Xa, and VIIa had little or no effect (data not shown). Activated pCPB hydrolyzes hippuryl-L-arginine, hippuryl-L-lysine but not hippuryl-L-phenylalanine, hippurylphenylactic acid, or hippuryl-Gly-Phe (Table I). Ac-
A Novel Human Plasma Carboxypeptidase B

Fig. 3. Sequence and structure of plasma carboxypeptidase B. A, the arrows indicate the cleavage site of the hydrophilic signal, as well as trypsin cleavage sites. The amino-terminal sequence obtained from the direct amino-terminal protein sequence is underlined, as are the stop codon and polyadenylation site. Residues implicated in zinc (circles) and substrate (squares) binding previously determined for tCPA and tCPB are indicated. Asn-linked glycosylation sites are designated by asterisks.

B, M, values shown are calculated from 10% recovery, this would suggest a plasma concentration of 2-5 μg/ml.

Plasma CPB shows 44% identity with rat tissue pro-CBP and 40% identity with human mast cell pro-CPA. When comparing the catalytic domains, pCPB shows ~50% identity with the catalytic domains of rat, bovine, and human tCPA and tCPB. The apparent glycosylation of the activation peptide distinguishes pCPB from tissue carboxypeptidases, which are not glycosylated. The glycosylation of pCPB may act to stabilize and increase the half-life of circulating pCPB. The residues implicated in catalysis and substrate and zinc binding of carboxypeptidases A and B are conserved in pCPB. The studies, indicate that pCPB is a basic carboxypeptidase and, therefore, specific for carboxyl-terminal arginine and lysine residues.

Unlike pancreatic tCPB, pCPB is not stable to trypsin digestion. The plasma carboxypeptidase B isolated here is synthesized and secreted by the liver into the plasma. Based on the frequency of positive clones obtained from a cDNA library screen and the intensity of a pCPB hybridizing band in a Northern blot, we estimate pCPB represents 0.008-0.05% of the total liver mRNA. From 5 liters of plasma, 200-500 μg of pure pCPB was recovered; assuming 10% recovery, this would suggest a plasma concentration of 2-5 μg/ml.

Plasma CPB shows 44% identity with rat tissue pro-CBP and 40% identity with human mast cell pro-CPA. When comparing the catalytic domains, pCPB shows ~50% identity with the catalytic domains of rat, bovine, and human tCPA and tCPB. The apparent glycosylation of the activation peptide distinguishes pCPB from tissue carboxypeptidases, which are not glycosylated. The glycosylation of pCPB may act to stabilize and increase the half-life of circulating pCPB. The residues implicated in catalysis and substrate and zinc binding of carboxypeptidases A and B are conserved in pCPB. The presence of Asp-257, Gly-245, and Ser-208 of the 308-amino acid catalytic domain, along with substrate and inhibitor binding sites, indicate that pCPB is a basic carboxypeptidase and, therefore, specific for carboxyl-terminal arginine and lysine residues.

Unlike pancreatic tCPB, pCPB is not stable to trypsin digestion.

tivated pCPB is inhibited by the Zn\textsuperscript{2+} chelating agent o-phenanthroline and the specific CPB inhibitor GEMSA.\textsuperscript{3}

DISCUSSION

Even though tissue carboxypeptidase B was first purified in 1958 by Folk and Gladner (1) and has been extensively characterized since then (2), it has never been rigorously identified in plasma and, therefore, is thought to function primarily as a digestive enzyme secreted only from the pancreas or small intestine (6, 8). The plasma carboxypeptidase B isolated here is synthesized and secreted by the liver into the plasma. Based on the frequency of positive clones obtained from a cDNA library screen and the intensity of a pCPB hybridizing band in a Northern blot, we estimate pCPB represents 0.008-0.05% of the total liver mRNA. From 5 liters of plasma, 200-500 μg of pure pCPB was recovered; assuming 3 D. L. Eaton and B. E. Malloy, unpublished observations.
activation. Our initial studies reported here suggest that trypsin simultaneously cleaves pCPB at (at least) two sites, ultimately causing inactivation. Tissue CPB is relatively stable to further cleavage by trypsin following activation. To date, we have not observed activation of pCPB without concomitant inactivation utilizing a variety of plasma proteases. The reasons for this susceptibility to trypsin inactivation are unclear at this time. Interestingly, both murine tCPB and human tCPA contain either an arginine or lysine at the analogous site, yet both of these proteases are stable to trypsin (3, 5, 20). Furthermore, activation of porcine procarboxypeptidase B by trypsin yields a stable M₃₆,₀₀₀ protease (29). It is tempting to speculate that pCPB activation is tightly regulated and perhaps occurs only at its site of action. The loss of the glycosylated activation peptide after activation may also necessitate this. Significantly, tCPB has a half-life of only minutes (21). Alternatively, a binding protein may be required that would protect pCPB from proteolytic inactivation.

The importance of lysine-binding sites in the regulation of plasminogen activation, as well as its interaction with other proteins, is well documented. The interactions between plasminogen and histidine-rich glycoprotein, tetranectin, α₂-antiplasmin, fibrin, or plasminogen cell surface receptors are all mediated by lysine-binding sites of the kringle domains of plasminogen (9-11, 23, 24). It is estimated that 30% of the circulating plasminogen may be bound to α₂-antiplasmin, whereas 50% may be bound to histidine-rich glycoprotein (10). The importance of carboxyl-terminal lysine residues in some of these interactions has recently been demonstrated utilizing tCPB. The initial rapid interaction between α₂-
TABLE I

Hydrolysis of substrates by pCPB

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippuryl-Arg</td>
<td>11.4</td>
</tr>
<tr>
<td>Hippuryl-Lys</td>
<td>8.0</td>
</tr>
<tr>
<td>Hippuryl-Phe</td>
<td>0</td>
</tr>
<tr>
<td>Hippuryl-Gly-Lys</td>
<td>0</td>
</tr>
<tr>
<td>Hippurylphenyllactic acid</td>
<td>0</td>
</tr>
</tbody>
</table>

![Fig. 5. Trypsin activation of pCPB. Plasma CPB was incubated with trypsin for the times shown and terminated by the addition of soybean trypsin inhibitor. Activity was determined using hippuryl-Arg, and an aliquot of each incubation was analyzed by SDS-PAGE (inset), as described under “Experimental Procedures.” In a parallel experiment, pCPB was incubated with one-tenth the amount of trypsin for 30 min at 37 °C. Activated pCPB was resolved on SDS-PAGE, electroblotted, and sequenced, as described under “Methods.”](image)

**REFERENCES**


7. Deleted in proof


---

*4* D. L. Eaton, unpublished observation.