Human Procarboxypeptidase U, or Thrombin-activable Fibrinolysis Inhibitor, Is a Substrate for Transglutaminases

EVIDENCE FOR TRANSGLUTAMINASE-CATALYZED CROSS-LINKING TO FIBRIN*

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Procarboxypeptidase U (EC 3.4.17.20) (pro-CpU), also known as plasma procarboxypeptidase B and thrombin-activable fibrinolysis inhibitor, is a human plasma protein that has been implicated in the regulation of fibrinolysis. In this study, we show that pro-CpU serves as a substrate for transglutaminases. Both factor XIIa and tissue transglutaminase catalyzed the polymerization of pro-CpU and the cross-linking to fibrin as well as the incorporation of 5-dimethylaminonaphthalene-1-sulfon
yl cadaverine (dansylcadaverine), [14C]putrescine, and dansyl-PGQQIV. These findings show that pro-CpU contains both amine acceptor (Gln) and amine donor (Lys) residues. The amine acceptor residues were identified as Gln\(^2\), Gln\(^5\), and Gln\(^292\), suggesting that both the activation peptide and the mature enzyme participate in the cross-linking reaction. These observations imply that transglutaminases may mediate covalent binding of pro-CpU to other proteins and cell surfaces in vivo. In particular, factor XIIa may cross-link pro-CpU to fibrin during the latter part of the coagulation cascade, thereby helping protect the newly formed fibrin clot from premature plasmin degradation. Moreover, the cross-linking may facilitate the activation of pro-CpU, stabilize the enzymatic activity, and protect the active enzyme from further degradation.

Pro-CpU,\(^1\) also known as plasma procarboxypeptidase B (1) and thrombin-activable fibrinolysis inhibitor (2), is a single chain 60-kDa glycoprotein secreted by the liver. The active enzyme is specific for Lys or Arg residues and is therefore referred to as a “basic” carboxypeptidase. Pro-CpU displays high protein sequence identity to other known members of the metallocarboxypeptidase family, including carboxypeptidase B from pancreas (1). The protein was discovered as a plasmino
gen-binding protein, and this property has led investigators to explore a role in fibrinolysis (1–8).

Previous studies have shown that basic carboxypeptidases in general affect the stability of fibrin clots in vitro (9–14). The mechanism most likely involves the removal of exposed COOH-terminal Lys residues on the surface of a “mature” fibrin clot, thus limiting the number of Lys binding sites. COOH-terminal Lys residues are not found on a newly formed clot. However, initial partial plasmin degradation exposes these residues, which then act as binding sites for tissue plasminogen activator, plasminogen, and plasmin (15, 16). Accordingly, the interactions between COOH-terminal Lys residues and Lys binding sites are recognized as potential targets for the regulation of the fibrinolytic system. Early studies examined the ability of pancreatic carboxypeptidase B and carboxypeptidase N to reduce the binding capacity of the fibrin clot (9, 10, 12, 13). More recently, it has been shown that CpU similarly affects the stability of a fibrin clot in vitro (2–7).

Transglutaminases (EC 2.3.2.13) comprise a family of calcium
dependent enzymes, which form N\(^\gamma\)-glutamyl)lysine cross
links between polypeptide chains (17). During the final stages of fibrin deposition, the clot is mechanically stabilized by the factor XIIa-catalyzed formation of N\(^\gamma\)-glutamyl)lysine cross
links between Lys residues (amine donor) and Gln residues (amine acceptor) in adjacent fibrin monomers. In connection with this reaction, other proteins are cross-linked to the clot, including \(\alpha_2\)-antiplasmin (18), von Willebrand factor (19) factor V (20), and thrombospondin (21).

Pro-CpU binds to plasminogen (1), and active CpU binds to \(\alpha_2\)-macroglobulin and pregnancy zone protein (22). Consequently, it is uncertain how CpU is maintained in the clotting milieu. In this study, we present evidence that pro-CpU is a substrate for transglutaminases and is cross-linked to fibrin in vitro. These observations may have important biological significance, and they suggest that pro-CpU is incorporated into the fibrin clot during the latter part of the coagulation cascade. The cross-linked pro-CpU may assist other proteins, including \(\alpha_2\)-antiplasmin in the protection of the newly formed clot from premature plasmin degradation.

EXPERIMENTAL PROCEDURES

Materials—MaxiSorp™ polystyrene tubes (Nunc) were from VWR Scientific Products (West Chester, PA). Putrescine, E-64, phenylmethylsulfonyl fluoride, guinea pig liver (tissue) transglutaminase (EC 2.3.2.13), human thrombin (EC 3.4.21.5), human fibrinogen, bovine serum albumin, bovine carbonic anhydrase, and sweet potato \(\beta\)-amylase were obtained from Sigma. Hg thryoglobulin, horse ferretin, and bovine catalase were from Amersham Pharmacia Biotech. Dansylca
daverine was from Molecular Probes (Eugene, OR). L-1,4-[\(^14\)C]Putrescine (118 mCi/mmol) was from NEN Life Science Products. The dansylated substrate dansyl-PGQQIV (23) was synthesized at Multiple Peptide Systems (San Diego, CA). Sequencing grade porcine trypsin was from Promega, Madison, WI. Recombinant human factor XIIa was a kind gift from Dr. Charles S. Greenberg, Duke University Medical Center. Fresh frozen human plasma was obtained from American Red Cross Blood Services (Charlotte, NC).

Protein Purification—Pro-CpU was purified from human plasma by plasminogen-Sepharose affinity chromatography as described previously (1, 24). Pro-CpU was separated from \(\alpha_2\)-antiplasmin by anion exchange chromatography on a Mono-Q HR 5/5 column (Amersham Pharmacia Biotech) connected to a Amersham Pharmacia Biotech fast

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The abbreviations used are: pro-CpU, procarboxypeptidase U; PAGE, polyacrylamide gel electrophoresis; PTH, phenylthiohydantoin; TAFI, thrombin-activable fibrinolysis inhibitor; dansyl, 5-dimethylami
nonaphthalene-1-sulfon; HPLC, high pressure liquid chromatogra
d; DTT, dithiothreitol.
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Protein liquid chromatography system. The column was equilibrated in 20 mM Tris-Cl, pH 7.4 (Buffer A) and developed using a 0.3% Buffer B/min linear gradient from Buffer A to 20 mM Tris-Cl, pH 7.4, containing 1 M NaCl (buffer B) at a flow rate of 1 ml/min. Plasminogen, α,-antiplasmin, and acetonitrile. Peptides were monitored at 220 nm and collected manually. Radioactive peptides were further analyzed by Edman degradation and plasma desorption time of flight mass spectrometry.

Transglutaminase-catalyzed Cross-linking of Pro-CpU to Fibrin—
MaxiSorp™ polystyrene tubes (Nunc) were coated with fibrin by incubating 200 μl of human fibrinogen at 500 μg/ml and 0.1 National Institutes of Health units of thrombin for 20 min. The tubes were emptied, washed three times with 50 mM Tris-Cl, 100 mM NaCl, pH 7.5 (Buffer A) and blocked overnight with 1% bovine serum albumin in Buffer A at 4 °C. The next day, the tubes were washed with Buffer A, and 0,2 μg of iodinated pro-CpU or 0.225 μg of iodinated α,-antiplasmin were added in triplicates. The cross-linking reactions were initiated by adding tissue transglutaminase or FXIIIs ranging from 0.05 to 10 μg in Buffer A containing 10 mM CaCl2, and 0,5 mM DTT. The volumes were adjusted to 100 μl and the reactions were continued for 4 h at 37 °C. The tubes were washed three times in 1) Buffer A containing 10 mM EDTA, 2) 100 mM glycine-HCl, pH 3, and 3) Buffer A containing 1% SDS. After the last washing step, the tubes were counted for radioactivity.

Amino Acid Sequence Analysis—Automated Edman degradation was carried out in an Applied Biosystems 477A sequencer with on-line analysis of the phenylthiohydantoin (PTH) amino acids using an Applied Biosystems 120A HPLC. The remaining PTH-derivatives were collected and counted for 14C radioactivity. In selected runs, the eluate from the 120A HPLC system was collected at timed intervals and counted for 14C radioactivity to determine the retention of PTH-Gln(14C)putrescine. Some samples were transferred to polyvinylidene difluoride membranes before Edman degradation (27).

Amino Acid Analysis and Determination of Protein Concentrations—
The concentrations of proteins were determined by amino acid analysis (29). The samples were desalted and the absorbances at 280 nm were determined. Aliquots were dried and hydrolyzed for 24 h in 6 M HCl, and the amino acid composition was determined in a Beckman 6300 amino acid analyzer. The determined absorbance coefficient for pro-CpU was A1%, 1 cm = 20,6. The concentration of other proteins were calculated according to the following absorbance coefficients: α,-antiplasmin, A1%, 1 cm = 13,0 (30) and a fibrinogen A1%, 1 cm = 13,9 (31).

RESULTS

Pro-CpU Is a Substrate for Tissue Transglutaminase and Factor XIIa—To test whether pro-CpU serves as a substrate for transglutaminases, we monitored the incorporation of fluorescent donor (dansylcadaverine) or acceptor (dansyl-PGGQ QIV) substrates by tissue transglutaminase or factor XIIa. Pro-CpU and the fluorescent probes were incubated with increasing amount of factor XIIa or tissue transglutaminase. The reactions were continued for 3 h, and the products were analyzed by reduced SDS-PAGE and examined under UV light (Fig. 1). It is apparent that transglutaminases catalyzed the
incorporation of both probes into pro-CpU in a Ca\(^{2+}\)-dependent manner. Other proteins, not expected to be transglutaminase substrates, including human extracellular superoxide dismutase, bovine serum albumin, bovine carboxy anhydride, \(\beta\)-amylase, hog thyroglobulin, horse ferretin, and bovine catalase, failed to incorporate the probes (not shown). However, the known acceptor (\(\alpha_2\)-antiplasmin) (33) and donor (plasminogen) (34, 35) substrates acted as expected (Fig. 1). Taken together, these experiments suggest that pro-CpU incorporated dansylcadaverine and dansyl-PGGQQIV in a specific manner.

Because pro-CpU contains both reactive Gln (amine acceptor) and Lys (amine donor) residues, we tested whether the transglutaminases were able to form inter-pro-CpU N\(^\gamma\)-\(\gamma\)-glutamyllysine cross-links and produce pro-CpU homopolymers. Iodinated pro-CpU was incubated with increasing amounts of tissue transglutaminase or factor XIIIa (not shown), and the reaction products were analyzed by reduced SDS-PAGE (Fig. 2). The result indicated that transglutaminases catalyzed the formation of high molecular weight polypeptides and thus confirmed that pro-CpU is a substrate for transglutaminases. Significantly, the result also suggests that pro-CpU is capable of forming inter N\(^\gamma\)-\(\gamma\)-glutamyllysine cross-links with other proteins.

**Identification of the Reactive Gln Residue(s) in Pro-CpU**—
Pro-CpU (1 mg) and tissue transglutaminase or factor XIIIa (100 \(\mu\)g) were incubated in the presence of \([^{14}\text{C}]\text{putrescine}, as described under "Experimental Procedures." The \([^{14}\text{C}]\text{putrescine labeling was performed under nondenaturing conditions using a low concentration of reducing agent (0.5 mM DTT) to sustain the activity of the transglutaminases. The disulfide integrity was not affected, even by an extended incubation in 0.5 mM DTT, as judged by the inability of pro-CpU to incorporate iodo\([^{125}\text{I}]\text{acetic acid (data not shown). The following the \([^{14}\text{C}]\text{putrescine labeling reaction, pro-CpU was reduced and alkylated and digested by trypsin. The peptides were separated by high performance size exclusion chromatography, and pools containing radioactive peptides were further purified by reverse phase HPLC, as described under "Experimental Procedures." The purified radioactive peptides were characterized by Edman degradation and mass spectrometry (Table 1). Two radioactive tryptic peptides were recovered from both the tissue transglutaminase and the factor XIIIa labeling of pro-CpU: Phe\(^4\)-(Gln\(^2\))-Ser-Gly-(Gln\(^5\))-Val-Leu-Ala-Ala-Leu-Pro-Arg\(^2\) and Ala\(^{283}\)-Tyr-Ile-Ser-Met-His-Ser-Tyr-Ser-(Gln\(^{292}\))-His-Ile-Val-Phe-Pro-Tyr-Ser-Tyr-Thr-Arg\(^{302}\). The yield of the two peptides (~4 nmol) suggested that they represent the major amine acceptors. During Edman degradation of the two peptides PTH-Gln\(^6\), PTH-Gln\(^5\), and PTH-Gln\(^{292}\) were not detected, suggesting that these residues were modified. This conclusion was substantiated by three observations: (i) the PTH-derivatives released during Edman degradation in the positions corresponding to Gln\(^2\), Gln\(^5\), and Gln\(^{292}\) contained \(^{14}\text{C} radioactivity; (ii) the retention time of the PTH-derivative eluting in these positions was consistent with the retention time of PTH-Gln\(^{4}\)-\([^{14}\text{C}]\text{putrescine (Table I); and (iii) the masses of peptide 1 and peptide 2 are consistent with the \([^{14}\text{C}]\text{putrescine modification described above (Table I).}

Because some of the predicted tryptic peptides were rather large, we performed a second digest using pepsin to generate smaller, more manageable peptides. Pro-CpU and tissue transglutaminase or factor XIIIa were incubated in the presence of \([^{14}\text{C}]\text{putrescine, as described under "Experimental Procedures," and digested with pepsin. The analysis of these digests resulted in the purification of two major radioactive peptides containing Gln\(^7\) and Gln\(^{292}\), as identified in the tryptic digests. In addition, small amounts of \([^{14}\text{C}]\text{putrescine modified peptides, including Gln\(^{16}\) and Gln\(^{81}\)-Gln\(^{82}\)-Gln\(^{83}\) were purified from the tissue transglutaminase-catalyzed reaction. The Gln\(^{81}\)-Gln\(^{82}\)-Gln\(^{83}\)-containing peptide was obtained in several cleavage variants due to the low specificity of pepsin, and some of these fragments contained modified Gln\(^{81}\), whereas in others Gln\(^{82}\) or Gln\(^{83}\) was modified.

The analysis of several separate \([^{14}\text{C}]\text{putrescine labeling experiments suggested that Gln\(^7\), Gln\(^5\), and Gln\(^{292}\) are the...
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The fibrin incorporation of pro-CpU was comparable to that of α2-antiplasmin, suggesting that the incorporation of pro-CpU is biologically relevant.

**DISCUSSION**

In this study, we present evidence that pro-CpU is a substrate for transglutaminases and is cross-linked to fibrin during the latter part of the coagulation cascade. Incorporation of dansylecadaverine [14C]putrescine and dansyle-PGGQQIV demonstrates the presence of both amine acceptor and amine donor sites. This observation was substantiated by the formation of transglutaminase induced pro-CpU polymers. We determined the location of the amine acceptor sites to ascertain whether both the zymogen and the mature enzyme participated in the cross-linking reaction.

**FIG. 3. Pro-CpU is cross-linked to fibrin by transglutaminases.** Polystyrene tubes were coated with fibrin and incubated with iodinated pro-CpU or iodinated α2-antiplasmin in the presence of increasing amount of tissue transglutaminase (TG) as indicated. The tubes were washed and counted for γ-radioactivity, and the results of three identical experiments were averaged. Both α2-antiplasmin (●) and pro-CpU (×) were cross-linked to fibrin by tissue transglutaminase in a concentration-dependent manner. Similar results were obtained using FXIIIa. In addition, the incorporation of α2-antiplasmin (○) and pro-CpU (■) were abolished by the addition of 10 mM EDTA. These experiments show that pro-CpU is cross-linked to fibrin by transglutaminases. Moreover, the binding efficiency of the two proteins appeared to be similar, suggesting that the incorporation of pro-CpU is physiologically relevant.

**FIG. 4. Schematic showing the location of amine acceptor sites.** The major sites, including Gln2, Gln5, and Gln292, are utilized by factor XIIIa, and tissue transglutaminase are positioned near the NH₂ terminus and toward the COOH terminus. In addition, tissue transglutaminase incorporated [14C]putrescine in Gln 16 and in a cluster of Gln residues, including Gln81, Gln82, and Gln83. Accordingly, because amine acceptor sites are found in both the activation peptide and in the mature enzyme, it is likely that pro-CpU remains associated with the fibrin clot following removal of the activation peptide.

Three major amine acceptor sites, Gln6, Gln5, and Gln292, were preferred by both tissue transglutaminase and factor XIIIa. Some additional minor [14C]putrescine incorporation was observed in Gln16 and Gln81-Gln82-Gln83 after a detailed analysis of the tissue transglutaminase reaction products (see Fig. 4). This incorporation accounted for only a minor fraction of the total incorporated radioactivity and might reflect differences in the substrate requirement of tissue transglutaminase or factor XIIIa. However, the major reactive Gln residues preferred by both transglutaminases were Gln2, Gln5, and Gln292. Two of the major reactive Gln residues are found near the NH₂-terminal of pro-CpU. The presence of closely spaced reactive Gln residues is quite common and has been shown in other proteins, including plasminogen activator inhibitor-2 (36), β A3-crystallin (37), the γ-chain of fibrinogen (38), and vitronectin (39). It is not possible to derive a consensus sequence for the amine acceptor sites in proteins, but it is common that reactive Gln residues are positioned near the NH₂ or COOH terminus (40).

The transglutaminase-catalyzed cross-linking may influence the biology of pro-CpU in several important ways. Specifically, the reactive Gln residues are located both in the activation peptide and in the mature enzyme, suggesting that CpU remains associated with the matrix after activation. The enzymatic activity of CpU has been reported to be transient in vitro (4, 41) because of a change in the thermodynamic stability after activation (42). It is likely that the Nε(γ-glutamyl)lysine cross-links and other noncovalent interactions promoted by the cross-linking increase the conformational stability and stabilize the enzymatic activity of CpU. Proteolytic inactivation of CpU is similarly less likely as a result of both increased thermodynamic stability and steric hindrance. These factors may also influence the activation kinetics of pro-CpU.

Fibrin functions as a matrix during fibrinolysis and helps confine the activities of the involved proteins to prevent potential damaging effects elsewhere. This is exemplified by the factor XIIia-catalyzed cross-linking of α2-antiplasmin to fibrin during the final stages of the blood coagulation (33, 43, 44). Similarly, pro-CpU activated outside the immediate vicinity of the clotting/fibrinolysis environment is likely to be bound to α2-macroglobulin or pregnancy zone protein and removed (22). The fibrin incorporation of α2-antiplasmin is presumably mediated by plasminogen, which has affinity for both fibrin and α2-antiplasmin (45), thus bringing α2-antiplasmin in close proximity to the fibrin. Significantly, plasminogen also has affinity for pro-CpU (1), and it is likely that this interaction similarly promotes a factor XIIia mediated incorporation of pro-CpU in the newly formed fibrin clot.

The observations described in this study suggest that transglutaminases may mediate covalent binding of pro-CpU to fibrin in vitro.
other proteins and cell surfaces in vivo. In particular, the data suggest that pro-CpU, CpU, or both are incorporated into fibrin during a coagulation event and function in concert with other proteins (e.g., α2-antiplasmin) to prevent premature dissolution of the fibrin clot. This interaction may also have important implications for the pro-CpU activation kinetics and may stabilize CpU after activation.

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