

Platelet-Agglutinating Protein p37 from a Patient with Thrombotic Thrombocytopenic Purpura Has Characteristics Similar to Prethrombin 2

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Thrombotic thrombocytopenic purpura (TTP) is characterized by widespread platelet thrombi in arterioles and capillaries. Unusually large or multimeric von Willebrand factor, as well as one or more platelet-agglutinating factors, have been implicated in the pathogenesis of TTP. But, the actual mechanisms of platelet agglutination have not been satisfactorily explained. Recent studies suggested the 37-kDa platelet-agglutinating protein (PAP) p37 to be partially responsible for the formation of platelet thrombi in patients with TTP. We studied mobility in SDS-PAGE, the sequence of N-terminal amino acid residues, DNA and antigenic characteristics of PAP p37, which might be related to the pathogenesis of TTP. PAP p37 was purified from the plasma of a 31-year-old male Korean patient with acute TTP. The findings are as follows: (1) We compared PAP p37 with thrombin through the use of SDS-PAGE, either with or without β -mercaptoethanol. PAP p37 did not appear to be cleaved between the A- and B-chains of prethrombin 2. However, thrombin did cleave between those of prethrombin 2, but linked with disulfide bridge. (2) N-terminal 21 amino acid sequence of PAP p37 was T-F-G-S-G-E-A-D-X-G-L-R-P-L-F-E-K-K-S-L-E. It appeared to be identical to that of 285-305 amino acid residues of human prothrombin (prethrombin 2). (3) No prothrombin gene DNA mutation was revealed. (4) The antigenicity of PAP p37 was similar to thrombin, which was a result of the competitive binding against the anti-thrombin antibody. With these results, we conclude that PAP p37 has similar characteristics to prethrombin 2.

Keywords: Platelet-agglutinating protein (PAP), p37, Prethrombin2, TTP

Introduction

Thrombotic thrombocytopenic purpura (TTP) was first described by Moschowitz in 1924 (Moschcowitz, 1924). It was characterized by the fluctuating-neurologic symptom, thrombocytopenia, microangiopathic hemolytic anemia, fever, and renal dysfunction that was rapidly fatal because of the involved organ dysfunction. Typical pathologic features of this disorder are widespread hyaline thrombi within the vessel wall. In the involved organ, a hyaline material that principally consists of platelet thrombi occludes terminal arterioles and capillaries. Microvascular occlusions are seen in most organs, including the lungs and eyes. The most-frequently-involved organs are the brain, heart, spleen, kidneys, pancreas, and adrenals. These hyaline thrombi consist of platelet-agglutination with little fibrin, which differs from thrombi that consists of platelet-aggregation with rich fibrin that is shown in common pathways of coagulation, due to endothelial cell injury (Moschcowitz, 1924).

The pathogenesis of TTP is still unknown. Recent suggestions are that persisting-endothelial-derived multimeric von Willebrand factor (vWF), or unusually large vWF, which cannot be cleaved normally (Wagner *et al.*, 1995; Tsai and Lian, 1998; Veyradier *et al.*, 2001), and 37 kDa platelet-agglutinating protein (PAP), p37, are associated with these pathologic mechanisms (Lian *et al.*, 1979, 1991; Siddiqui and Lian, 1985, 1992, 1993; Jin *et al.*, 1995; Chen S and Lian 1998). Other investigators reported that there were reduced prostacyclin I₂ (PG I₂) production (Wu KK, 1992), or

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defections of the plasma-binding ability of PG I₂ (Wu KK *et al.*, 1985) in TTP. The causes of multimeric or unusually large vWF production in TTP have been reported. These are associated with the deficiency of the vWF-cleaving protease, or the formation of an antibody against the protease (Tsai and Lian, 1998; Tsai *et al.*, 2000). On the other hand, PAP p37 was found in the plasma of TTP patients, which are known to have potent platelet-agglutination activity that was first described by Siddiqui and Lian (Siddiqui and Lian, 1985).

We isolated and purified the platelet-agglutinating protein, PAP p37, from a 31-yr-old Korean male patient with acute TTP, and studied the characteristics of the PAP p37 (Kim *et al.*, 1992; Jin *et al.*, 1995). This was similar to most of the chemical characteristics of PAP p37 that are described by Siddiqui and Lian (Siddiqui and Lian, 1985, 1992, 1993), except for the weak binding activity to concanavalin A (Con-A). PAP p37 has a molecular weight of 37 kDa, as well as single polypeptide of acid glycoprotein with potent platelet-agglutinating activity.

Only a few of the chemical characteristics of PAP p37 are presently known. Therefore, we studied the characteristics of PAP p37, which might be the pathogenesis of TTP.

Materials and Methods

Purification of PAP p37 We used the plasma that was obtained during plasmapheresis of a 31-yr-old Korean patient with acute TTP (Kim *et al.*, 1992). Clinical manifestation, laboratory data, pathologic features, and a platelet-agglutination test confirmed the diagnosis of TTP. Two liters of the plasma were directly lyophilized and stored at -70°C until use. The purification method for PAP p37 was the same as the previous report (Jin *et al.*, 1995). All of the procedures were performed at 4°C. Briefly, PAP p37 was purified from the resolved lyophilized plasma in a 10 mM Tris-HCl pH 7.4 buffer by ammonium sulfate fractionation, DEAE-Sephacel, and Con-A-Sepharose chromatographies, as previously described (Siddiqui and Lian, 1985). The fraction with maximum-agglutinating activity was eluted by a linear gradient of α -methyl-D-glucoside (0-0.5 M) from a Con A-Sepharose column. The partially-purified PAP p37 was further purified by performing a Superose 12 gel filtration on fast-performance liquid chromatography (FPLC). The PAP p37 fraction after Con-A-Sepharose chromatography was collected, then dialyzed against 20 mM Tris-HCl (pH 7.4) that contained 0.1 M NaCl and concentrated by ultrafiltration with a YM-10 membrane (Amicon Inc., Beverly, USA). The concentrated sample was loaded onto Superose 12 (2.5 × 30 cm) on FPLC that was previously equilibrated with the Tris-saline buffer (pH 7.4), and developed with the same buffer at a flow rate of 0.3 ml per min (Jin *et al.*, 1995).

Measurement of platelet agglutination The platelet-rich human plasma from normal subjects was prepared from freshly-collected blood by 3.8% sodium citrate and subsequently centrifuged at 180 g for 10 min at room temperature, as previously described (Lian *et al.*, 1979). Platelet washing was carried out using the albumin density-gradient method (Walsh *et al.*, 1977). Next, 0.1 ml of the washed-platelet suspension that contained 7×10^8 platelets/ml in a

Tris-saline buffer (pH 7.4) was prewarmed at 37°C for 5 min. The platelet agglutination was initiated by adding 0.4 ml of the samples that were collected during the purification of PAP p37, and measured by recording the light transmission changes at 609 nm using a Chrono-log aggregometer (Haverstown, USA). Light-transmission values were converted to percent agglutination, as previously described (Siddiqui and Lian, 1985).

N-terminal amino acid sequencing of PAP p37 About 1-2 nM of the highly-purified PAP p37 (as judged by SDS-PAGE) in 30 μ l distilled water was bottled onto a fiberglass peptide disk in the sample cartridge of a Porton 2090 E automatic protein sequencer (Beckman Co, Fullerton, USA). Amino acids were separated by an on-line HPLC and quantitated from their peak areas using standard procedures. The N-terminal sequence that contained 21 amino acids was analyzed using a Sequence Homology analysis of NCBI BLAST from <http://www.ncbi.nlm.nih.gov/BLAST1> and Multiple Sequence Alignment of GeneDoc 2.6.0 from <http://www.psc.edu/biomed/genedoc/>.

Enzyme-linked immunosorbent assay (ELISA) Each well of the polystyrene microtiter plates (Plastomed, Warsaw, Poland) that contained 96 wells was coated with either the purified PAP p37 or thrombin in a 0.1 M carbonate/bicarbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6, 0.02% NaN₃). Unoccupied binding sites were blocked by 2% BSA in TBS (100 mM Tris-HCl buffer, pH 7.4, containing 1.5 M NaCl). The plates were sequentially incubated with mouse IgG-horseradish peroxidase conjugate (IgG-HRP; Sigma Co, St. Louis, USA) for 3 h at 37°C each with gentle shaking. After the excess conjugate was removed by washing with TBS, then 4-chloro-1-naphthol (Sigma Co, St. Louis, USA) was added to the reaction mixtures, and the reaction was followed by increases in absorbance at 492 nm and comparison with a standard peroxidase reaction. For competitive ELISA, the wells were coated with 100 μ l (1 μ g/ml) of the carbonate buffer that contained either the purified PAP p37 or thrombin, then incubated with the mouse anti-human thrombin antibody that was premixed with increasing concentrations of the purified PAP p37 before adding the IgG-HRP conjugate.

DNA sequence analysis of the prothrombin gene in the patient with TTP Genomic DNA was isolated from the blood of the same TTP patient, according to the procedure of Degen (Degen *et al.*, 1983; Degen and Davie, 1987). In order to examine the possibility that PAP p37 may be derived from a mutated prothrombin gene, an interesting region (including exons 8 and 9) was amplified by PCR using two oligonucleotides, 5'-ATG GGT GAG GAA TGG CCC AG-3' and 5'-GTC ATC TGT AAA GCC CAG GC-3'. Each cycle included 1 min at 94°C for denaturation, 1 min at 58°C for annealing, and 2 min at 72°C for extension. Following 30 cycles, 3 min for the extension was allowed to complete the PCR reaction. The amplified PCR product was cloned into a T-vector (Amersham, Buckinghamshire, UK) and the insert was sequenced using a CircumVent thermal cycle DNA sequencing kit (New England Biolabs, Hertfordshire, UK) according to the procedures that were supplied by the company.

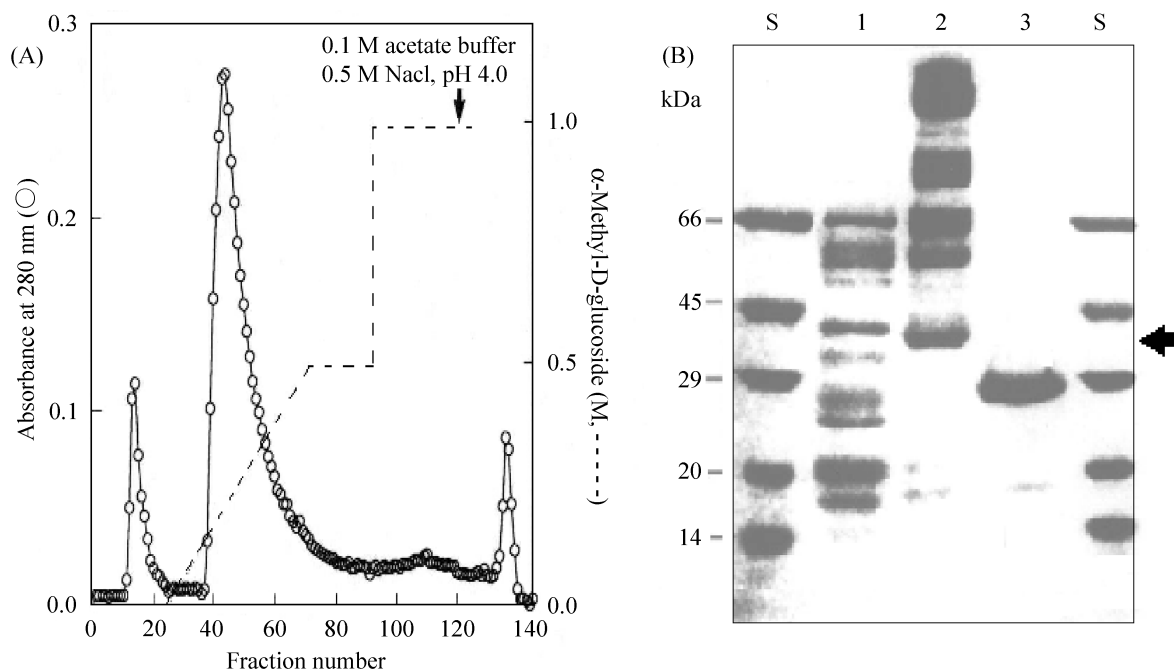


Fig. 1. The elution profile obtained from Con A-Sepharose chromatography (A) and SDS-PAGE analysis of the fractions (B). In panel B, lanes 1, 2, and 3 corresponded to the first (Con A-I), second (Con A-II), and third peak (Con A-III) of the chromatogram, respectively. The second peak with the maximum agglutination activity (Con A-II) was collected for further purification. The S lane shows molecular mass standards. The arrow indicates a 37 kDa PAP.

Other methods SDS-PAGE was performed in a small vertical slab gel unit (Bio-Rad instruments, Hercules, USA) with samples that were heated at 90°C for 1 min, as described by Laemmli (Laemmli, 1970). The protein was determined according to Lowry using BSA as a standard (Lowry *et al.*, 1951).

Results

The characteristics of PAP p37 The characteristics of purified PAP p37 from the plasma of a patient with TTP were the same as the PAP p37 that was described by Siddiqui and Lian (Siddiqui and Lian, 1985), except for the elution differences among the Con A-Sepharose column linear gradients. In our experiment, PAP p37 was tightly bound to Con A (0-0.5 M, Con A-I, -II), especially Con A-II (0.5 M), although less eluted in the presence of a high concentration (1 M, Con A-III) of α -methyl-D-glucoside (Siddiqui and Lian, 1985) (Fig. 1). The proteins from Con A-I and -II had potent platelet-agglutinating activity, but the protein from the third peak (Con A-III) showed no significant platelet-agglutinating activity. This indicates that these two glycoproteins may be slightly different in terms of carbohydrate composition. During further purification, the fourth-purified peak (Fig. 2-A, Super-IV) protein on FPLC appeared to migrate as a single band on SDS-PAGE (Fig. 2-B, lanes 5 and 6, fractions 86 and 91), indicating that it was isolated in a homogeneous state. This Super-IV fraction revealed potent platelet agglutination (Fig. 3-A). This activity was inhibited when the fraction was

preincubated with normal plasma (Fig. 3-B). The purified PAP p37 was determined to be a glycoprotein by Schiff's base-staining (not shown); it was confirmed as an acidic protein with an isoelectric point (pI) of 4.7 by isoelectric focusing (Fig. 4).

N-terminal amino acid sequence of purified PAP p37 is identical to 285-305 amino acid sequences of preprothrombin and prethrombin 2. The N-terminal sequence of PAP p37 was T-F-G-S-G-E-A-D-X-G-L-R-P-L-F-E-K-K-S-L-E, which was determined by the automated-Edman-degradation method. The sequence is identical to that established for 285-305 amino acid residues of human prothrombin and prethrombin 2 (Degen and Davie, 1987) (Fig. 5). The 9th amino acid (X) was ambiguous, but was suggested to be glyco-amino acid or cysteine by comparing a previous prothrombin amino acid sequence (Degen and Davie, 1987). This amino acid is a binding site of the catalytic domain for forming the prothrombin tertiary structure. The Thr285 of prothrombin is the first amino acid sequence of the human prothrombin light chain by the successive proteolytic cleavages for prothrombin activation (Lian *et al.*, 1991). PAP p37 was identical to this amino acid sequence of a part of the prothrombin (B-chain of prethrombin 2), or the α -thrombin light chain of its cleavage product. Human thrombin is composed of two polypeptides, a light chain of 6 kDa and a heavy chain of 31 kDa (Mann *et al.*, 1994).

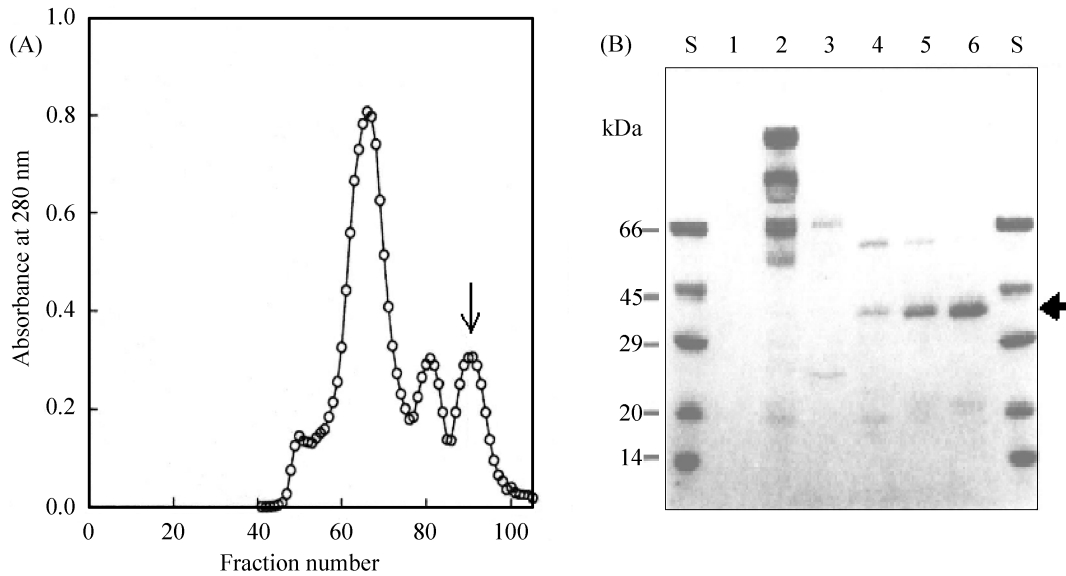


Fig. 2. The Superose 12 gel filtration chromatography of the PAP fraction (\downarrow) from Con A-Sepharose (A). Eluted fractions were analyzed by SDS-PAGE, and the proteins were visualized by silver staining (B). S contains molecular markers. Lanes 1, 2, 3, 4, 5, and 6 are fractions 50, 66, 76, 81, 86 and 91, respectively. The arrowhead (\leftarrow) indicates PAP.

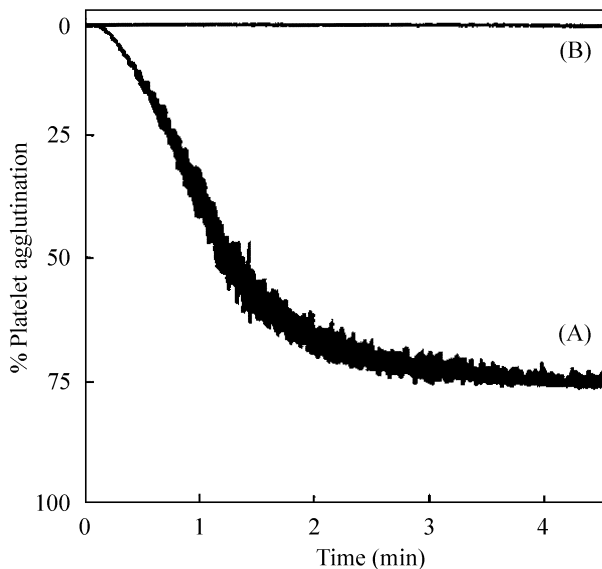


Fig. 3. Agglutination of washed platelets from a normal subject. A shows the agglutination by the purified PAP: 0.4 ml (about 1 μ g) of the purified PAP was added to the 0.1 ml of washed human-platelet suspension in a Tri-saline buffer (pH 7.4) that was prewarmed at 37°C for 5 min. B is the inhibition of PAP-induced platelet-agglutination by normal plasma, respectively: 0.1 ml of the platelet suspension was preincubated with 0.3 ml of either normal plasma and 0.1 ml of the PAP was added to mixture. The percent agglutination of platelets was determined using a Chrono-log aggregometer.

SDS-PAGE with or without β -mercaptoethanol Because PAP p37 was identical with a portion of prothrombin that contained the A- and B-chains (37 kDa), we studied its

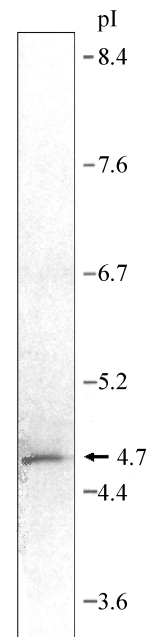


Fig. 4. Isoelectric pH of the purified PAP. The pI value of PAP was determined by isoelectric focusing electrophoresis. The arrow indicates PAP with a pI value of 4.7.

characteristics. We discovered that PAP p37 appeared to be the same size polypeptide band on SDS-PAGE, both in the presence (reducing condition) and absence (unreducing condition) of β -mercaptoethanol; the normal human α -thrombin appeared as two different peptide bands in these conditions (Fig. 6). This was an interesting and important result for analogizing PAP p37. PAP p37 has a similar

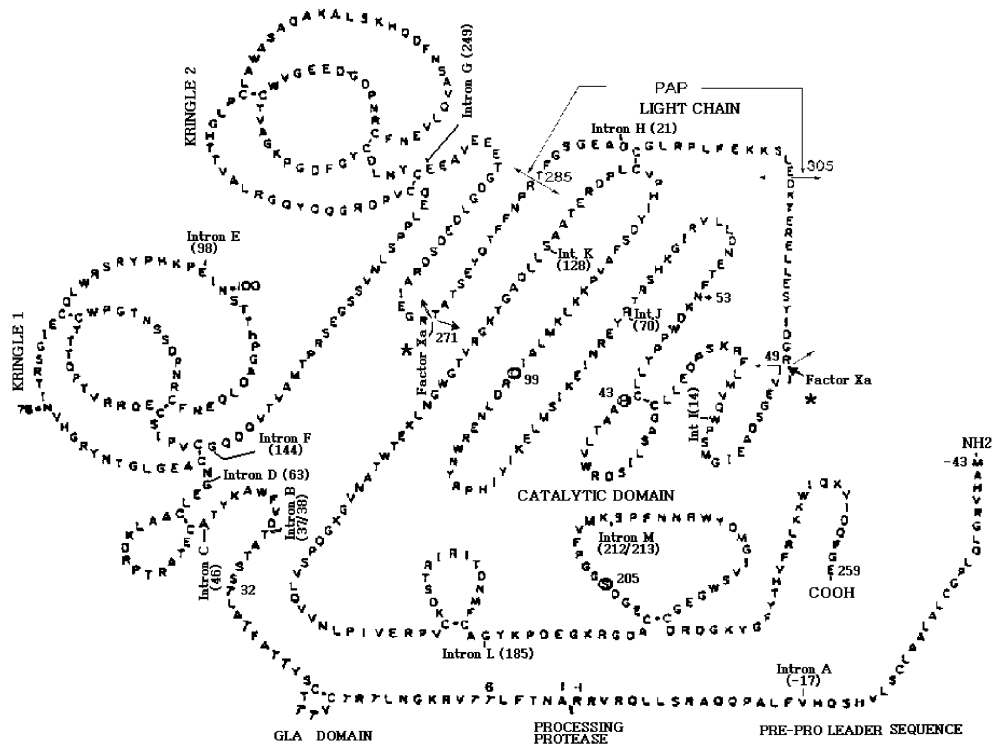


Fig. 5. Amino acid sequence and tentative structure for prepro prothrombin showing the location of the 13 intervening sequences (A-M). The prepro leader sequence is removed during the biosynthesis of the protein by signal peptidase, a protease that hydrolyzes, and R-A peptide bond between -1 and 1. The Gla domain and two kringle structures are located in the amino-terminal region of the protein that is removed after proteolytic activation by factor Xa (*). Thrombin is composed of a light chain (49 amino acids) that is linked to a catalytic domain (259 amino acids). Thrombin undergoes further self-autolysis by removing 13 amino acids (from T-272 to R-284) from the N-terminal of the light chain (T-285 to E-305 of N-terminal amino acid of prothrombin is exactly the same as the sequence of 21 N-terminal amino acid sequence of PAP). The amino acids that participate in catalysis are circled (H-43, D-99, and S-205). Solid diamonds represent the three carbohydrate attachment sites. The positions of the intervening sequences are indicated. The amino acid residues are numbered as follows, beginning with the amino-terminal end: prepro leader sequence, -43 to -1; prothrombin fragment 1, 1-271; light chain of thrombin, 1-49; catalytic chain of thrombin, 1-259. The Asn at position 121 is coded by an AAC codon in the cDNA (Degen *et al.*, 1983), while an ACC at this position in the gene codes for a Thr.

molecular weight (37 kDa) as prothrombin 2 and thrombin. But in thrombin, the A- and B-chains are already cleaved by Xa, which was only linked by the disulfide bond that can be separated in the presence of β -mercaptoethanol into the A- and B-chains. Therefore, two different bands were revealed in SDS-PAGE, with or without β -mercaptoethanol. PAP p37 appeared to be the same-sized band in SDS-PAGE with or without treating β -mercaptoethanol. With these findings, we could conclude that PAP p37 is similar to prothrombin 2. When prothrombin cleaved between fragment 1.2 and the A-chain, then the prothrombin 2 joined A-B chains were produced (Fig. 7).

Enzyme-linked immunosorbent assay (ELISA) The binding activity of PAP p37 to anti-thrombin was similar to thrombin (Fig. 8-A). Two proteins, PAP p37 and thrombin, are suggested to have similar antigenicity from the results of their competitive binding ability to the anti-thrombin antibody (Fig. 8-B).

DNA sequence analysis of the prothrombin gene To identify the gene mutation that causes PAP p37 production, the DNA sequence of the prothrombin gene was analyzed. The exon 8 and 9 regions of the prothrombin gene (including the part of interest) were amplified by a polymerase chain reaction with the genomic DNA from the patients blood cells as a template. The DNA sequence of the amplified PCR product did not reveal any mutation in the regions of the prothrombin gene. Therefore, it was shown that PAP p37 is not a mutant product of the prothrombin gene, but an unsuccessfully-cleaved product of prothrombin through the proteolysis process.

Discussion

PAP p37 has potent platelet-agglutinating activity in the patients with TTP that were first described by Siddiqui and Lian (Siddiqui and Lian, 1985, 1992, 1993). We found and reported the partial chemical characteristics of the Korean

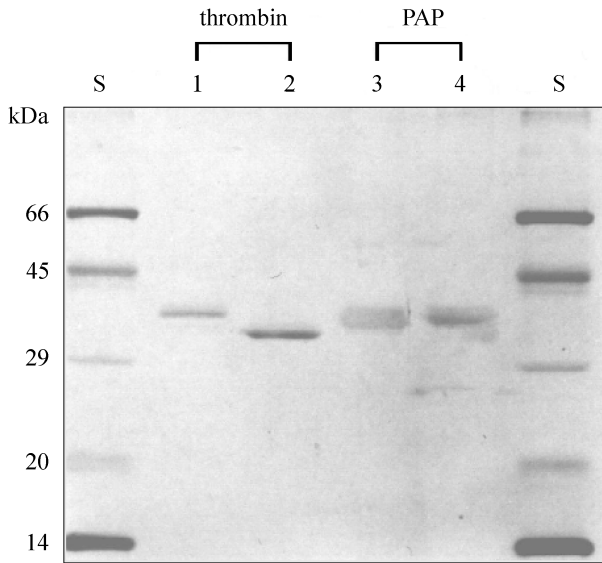


Fig. 6. Comparison of electrophoretic mobility between PAP and thrombin under non-reducing and reducing condition. Human thrombin (lanes 1 and 2) and purified PAP (lanes 3 and 4) were electrophoresed in the absence (lanes 1 and 3) and presence (lanes 2 and 4) of β -mercaptoethanol.

type PAP p37 that was similar to the previous-reported PAP p37, except for the weak Con-A binding activity (Jin *et al.*, 1995). Compared to its important role in the pathogenesis of TTP, however, only some of the protein properties of PAP p37 are known. Studies about its basic structure are presently minimal.

In this study, it was noteworthy that the N-terminal amino acid sequence of PAP p37 is identical to 285-305 amino acid sequences of the prothrombin fragment and prethrombin 2. This may provide a clue to the pathogenesis of TTP. Prothrombin or the coagulation factor II, one of the hepatic-

derived vitamin K-dependent procoagulants (coagulation factors II, VII, IX, and X), are the substrate for the thrombin formation by the activated factor X (Xa) in the common pathway and procoagulant itself, which participates in the platelet-agglutination. The entire amino acid sequence of prothrombin has been determined (Degen *et al.*, 1987). Prothrombin is a single-chain glycoprotein with a molecular weight of 72 kDa. The glutamate residue (Gla domain) and 2 kringle structures are located in the amino-terminal region of the prothrombin that is removed after proteolytic activation by factor Xa. The carboxy-terminal portion is composed of a light chain that is linked to a catalytic domain that contains serine protease of the proteolytic-thrombin function (Fig. 5). After the glutamate residue is carboxylated in the presence of vitamin K, additional binding of the calcium and phospholipid and proteolysis by Xa follows. The first cleavage of prothrombin by Xa occurs by two pathways as follows: (1) Prothrombin fragments 1, 2, or 3 in N-terminal and prethrombin 2, which consists of the A and B chains. (2) The intermediate products of prothrombin cleavage, meizothrombin, which is cleaved between the A and B chains and bridged by a disulfide bond (Kisiel *et al.*, 1974; Walz *et al.*, 1974; Butkowski *et al.*, 1977) (Fig. 7). If the first cleavage site of prothrombin is between the A chain (inhibiting B chain protease action) and the B chain (with serine protease action (meizothrombin)), then it is linked by a disulfide bond, and the proteolytic activation of the B chain is initiated by removing fragments 1, 2 in the N-terminal portion, and then generating active α -thrombin (Walz *et al.*, 1974; Butkowski *et al.*, 1977). However, if prothrombin is initially cleaved by Xa to the prothrombin fragments 1, 2 and prethrombin 2, then the union of the A and B chains persisted and prethrombin 2 (inactive thrombin light chain) accumulated in the plasma. The accumulated-procoagulant, prethrombin 2, binds to glycoprotein IV (GP IV) of the platelet membrane and exaggerates platelet agglutination (Lian *et al.*, 1991; Siddiqui

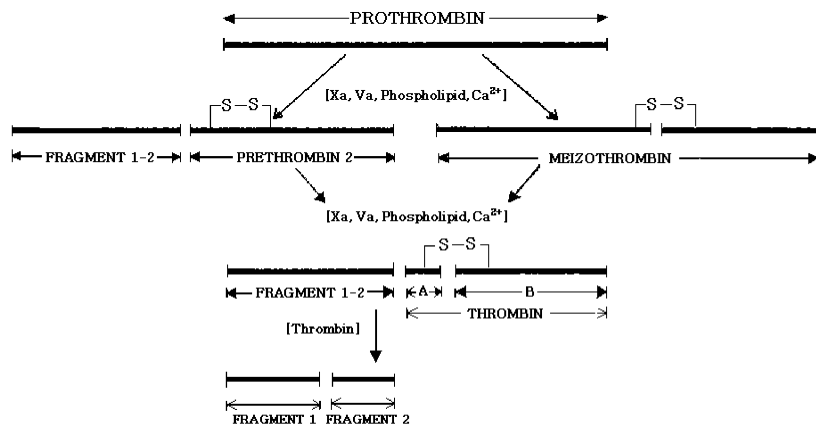


Fig. 7. Products of prothrombin proteolysis by factor Xa and thrombin. Factor Xa-catalyzed proteolysis produces the prothrombin fragment 1.2 and thrombin. Two intermediate reaction products can either be observed (prethrombin 2) or inferred as necessary to account for the rate of production of thrombin from prothrombin (meizothrombin). Thrombin-catalyzed cleavage produces the separate fragments 1 and 2 from the fragment 1.2.

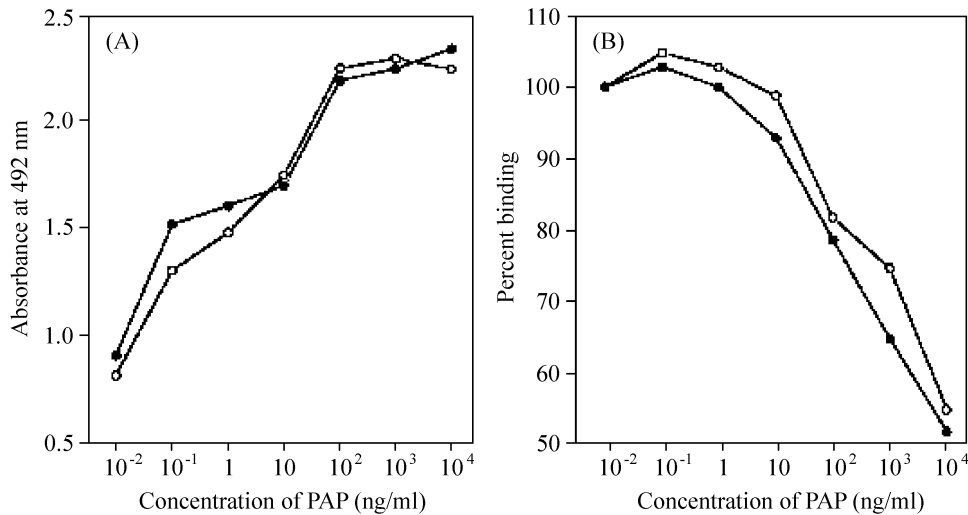


Fig. 8. The antigenic properties' comparison of PAP and thrombin by ELISA. A; The 96 wells that were coated with varying concentrations of either PAP (○) or thrombin (●) were incubated with the anti-thrombin antibody; the bound antibody was detected using the enzyme-conjugated secondary antibody (IgG-HRP) and a chromogenic substrate (4-chloro-naphthol), and quantitated by taking absorbencies at 492 nm. B; The plates were coated with 1 mg/ml of either PAP (○) or thrombin (●). Then, the anti-thrombin antibody that was mixed with increasing concentrations of PAP were added. The plate-bound antibody was similarly detected. The absorbance value in absence of PAP was set to 100% binding. Then, the absorbance value in presence of PAP was converted to percent binding.

and Lian, 1992; Jin *et al.*, 1995). Therefore, vascular occlusion by the platelet agglutination leads to tissue necrosis and disseminated-intravascular coagulation by released tissue factors.

From our study, there was no evidence that prethrombin 2 was the mutant product of the prothrombin gene from the DNA sequence analysis. There is more than one normal cleavage site of prothrombin (Rabiet *et al.*, 1986). The formation of fragments 1,2 and prethrombin 2 in any case exceed the production of meizothrombin, which participates in active thrombin formation; the accumulation of prethrombin 2 seems to make platelet agglutination. This process may be related to the pathogenesis of TTP, but needs further evaluation. The cause of the enhanced prethrombin 2 production in TTP is unclear. Recent studies revealed that the defect or antibody formation against the proteolytic enzyme generated multimeric or usually large vWF in TTP (Wagner *et al.*, 1995; Tsai and Lian, 1998). With the same mechanism, the accumulation of prethrombin 2 can be caused by the unusual proteolysis of prothrombin, which may be related with proteolysis inhibitors, antibody, or quantitative or qualitative defects of proteolytic enzymes (Koo *et al.*, 2000; Bae *et al.*, 2001). With this result, we can postulate that there are multiple proteolytic defects that are due to proteolytic enzyme deficiency and/or presence of proteolytic enzyme inhibitors, not only vWF, but also other procoagulants in TTP. This can be the answer to the infusion of normal plasma for the dilution of inhibitors, and the source of proteolytic enzymes and plasma exchange for the removal of proteolytic enzyme inhibitors that are effective in TTP treatment. Further study will be necessary (George, 2000; Rock, 2000). The

competitive binding of prethrombin 2 to the anti-thrombin antibody with thrombin suggests an antigenic similarity in both proteins. But, prethrombin 2 had no typical thrombin function that would convert fibrinogen to fibrin, because it was not influenced by the thrombin inhibitor, such as hirudin or heparin (data not shown). As previously mentioned, PAP p37 that has potent platelet-agglutinating activity in TTP has similar characteristics to prethrombin 2 that unsuccessfully cleaved the thrombin light chain.

Data presented in this study showed that PAP p37 has similar characteristics to prethrombin 2, inactivated thrombin light chain. This is due to the result of no cleaved disulfide bond between the A and B chains. This product is due to the unsuccessful cleavage of prothrombin. We anticipate that this fact can be helpful in evaluating the pathogenesis of TTP. The possibility of using the recombinant proteolytic enzyme in TTP treatment and abnormal proteolysis (related to the production of multimeric or unusually large vWF and prethrombin 2 that can be a common pathogenesis of TTP) should be studied more.

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