Collagen-binding Domain within Bovine Propolypeptide of von Willebrand Factor*

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Two reduced/alkylated fragments of bovine propolypeptide of von Willebrand factor (pp-vWF) that inhibit pp-vWF binding to collagen were isolated. One is a tryptic fragment of molecular mass of about 30 kDa and inhibits the binding at a molar concentration about 20 times higher than the intact pp-vWF. Amino acid sequence of this fragment was determined almost completely, and it was revealed that this fragment corresponded to the carboxyl-terminal region of pp-vWF molecule beginning with Phe. The other active fragment was obtained by lysyl endopeptidase digestion. This migrated as a 21.5/21-kDa doublet in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, but deglycosylation of this doublet resulted in production of single species of 19 kDa. The difference between the doublet constituents, therefore, was of carbohydrate composition. The extent of inhibition of collagen-binding by this 21.5/21-kDa fragment was comparable to that of the 30-kDa fragment, and furthermore, location of this fragment in the molecule was established to be between Phe and Lys. These were the only fragments among those obtained by proteolytic digestions that had significant competitive effect on the binding of intact pp-vWF to collagen. These results strongly suggest that at least one collagen-binding site should be present in the carboxyl-terminal region of bovine pp-vWF extending from residue 570 to 682.

Elucidation of structure-function relationship of proteins is one of the most attractive aims for those who are working in the field of protein chemistry. Adhesive proteins are suitable materials for that kind of investigation, since in most of them are high molecular weight glycoproteins and consist of several domains which are structurally independent. In fact, functional domains in some adhesive proteins have already been determined. Fibronectin, a typical adhesive protein exists in both tissues and plasma (1), consists of repeated domains, and its functional regions responsible for binding to heparin (2), gelatin (3), and fibrin (4) have been proposed. Furthermore, its binding site to the cell surface receptor, integrin, has been identified on amino acid residue level as an arginine-glycine-aspartic acid (R-G-D) sequence (5, 6), von Willebrand factor (vWF)1 is also capable of binding to various molecules such as heparin (7), collagen (8, 9), factor VIII (10), and platelet membrane glycoprotein Ib (11). Moreover, the importance of its interaction with glycoprotein Ib-IIIa complex on activated platelets in the adhesion process has come to be accepted (12–15). Though much information was obtained as to the binding sites of vWF to these molecules through active investigations using protease digestions or monoclonal antibodies (16–22), such an attempt has not been made on the binding site of propolypeptide of von Willebrand factor (pp-vWF) to collagen. pp-vWF, which is also called von Willebrand antigen II (23), is a glycoprotein of relative molecular weight of 100,000. It is present in endothelial cells, platelets, and plasma and is released from platelets upon stimulation (24, 25). Though its physiological role had not been determined, we have shown that pp-vWF binds to collagen with an affinity comparable to that of mature vWF and, furthermore, that it inhibits collagen-induced platelet aggregation in contrast to mature vWF (26, 27). In this report, we tried to determine the collagen-binding site(s) in the pp-vWF molecule, and we obtained evidence that the carboxyl-terminal portion of bovine pp-vWF plays an important role in interaction with collagen.

EXPERIMENTAL PROCEDURES

Inhibition Binding Assay—These experiments were designed to evaluate the effect of proteolytic fragments of pp-vWF on the binding of intact 125I-labeled pp-vWF to collagen. The binding assay was carried out according to the method described previously with slight modifications. Bovine fibrin type I collagen (gift from Dr. Kronenthal in Ethicon Inc., Sommerville, NJ) was used at a concentration of 6.7 μg/ml in an incubation volume of 120 μl. 125I-pp-vWF was added at a concentration of 0.44 μg/ml and competing ligand at a concentration as described in the figure legends. Binding was measured after 20 min of incubation at ambient temperature as described previously (27).

Reduction and S-Acetamidemethylation of pp-vWF—pp-vWF was purified from bovine platelets according to the method described previously (27), and was S-acetamidemethylated with iodoacetamide either before or after the treatment with proteases. One milligram of intact pp-vWF or its proteolytic digest was dissolved in a solution containing 5 M urea, 0.5 M Tris, and 5 mM EDTA, pH 8.6. The solution was degassed, and 0.12 mmol (3) of β-mercaptoethanol was added. After incubation at 40 °C for 2 h, the addition of 0.12 mmol of iodoacetamide was followed by further incubation for 2 h at 40 °C. The S-acetamidemethylated protein was separated from other low molecular weight reagents by gel filtration on a column of Sephadex

1 The abbreviations used are: vWF, von Willebrand factor; pp-vWF, propolypeptide of von Willebrand factor; TPCA, 1-t-octylamido-1-phenylethyl chloromethyl ketone; HPLC, high performance liquid chromatography; S-AcM, S-acetamidemethylated; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[N,N-di[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonic acid.

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Generation and Characterization of Proteolytic Fragments of pp-vWF—Purified pp-vWF, at a concentration of 3 mg/ml in 50 mM phosphate buffer containing 10 mM EDTA, pH 7.2, was digested with TPCK-trypsin at a molar ratio of 100:1. After 20 h incubation at 37°C, the reaction was terminated by addition of 2 mM phenylmethylsulfonyl fluoride, 3 μg/ml leupeptin, and 10 μg/ml soybean trypsin inhibitor. The digest was S-acetamidemethylated and separated into several fractions by reverse phase high performance liquid chromatography (HPLC) on a TSK-gel TMS-250 (C-1) column (0.46 x 7.5 cm; Tosoh Ltd., Tokyo, Japan). Each fraction was lyophilized, dissolved in 10 mM Tris-buffered saline, pH 7.4, and assayed for inhibition of collagen binding. The 30-kDa fragment was further digested with TPCK-trypsin under a denaturing condition in the presence of 4 M urea at an enzyme/substrate ratio of 1:50 for 20 h at 37°C. Peptides generated by this complete digestion were separated by reverse phase HPLC on a 2Bondapak C-18 column (0.78 x 30 cm; Waters, Milford, MA) and subjected to amino acid sequence analysis.

Digestion of pp-vWF with lysyl endopeptidase was carried out as follows. S-acetamidemethylated pp-vWF (S-AcM) (4 mg) was dissolved in 2 ml of 10 mM Tris, 4 M urea, pH 9.0, and 7.5 μg of lysyl endopeptidase (Wako Pure Chemical Ltd., Osaka, Japan) were added (E/S ratio = 1:200). After incubation at 30°C for 7 h, the mixture was directly subjected to reverse phase HPLC on a Biofine RPC-SC18 column (0.46 x 25 cm; Japan Spectroscopic Co., Ltd., Tokyo, Japan) and a linear gradient of acetonitrile was used.

Deglycosylation of 21.5/21-kDa fragments was performed as follows. About 5 μg of fragments were dissolved in 0.1 M phosphate buffer containing 20 mM EDTA, 0.6% CHAPS, and 0.1% SDS, pH 6.5, and after 0.05 unit of endo-β-N-acetylgalcosaminidase (Boehringer Mannheim) was added, the mixture was incubated at 37°C for 15 h and analyzed by SDS-PAGE. Amino acid sequence analysis of purified fragments was performed on an automated gas-phase Sequencer (Applied Biosystems, model 477A) followed by identification of the phenylthiohydantoin-derivatives by on-line HPLC (model 120A).

Protein concentration was determined with BCA protein assay kit (Pierce Chemical Co.) using bovine serum albumin as a standard.

RESULTS

Effect of S-Acetamidemethylation on PP-vWF Binding to Collagen—As pp-vWF contains many intramolecular disulfide bridges, reduction/alkylation of cysteine residues is necessary to separate its fragments. However, reduction of S-S bonds of protein destroys its native structure and often leads to disappearance of biological activity. In order to examine the effect of this chemical modification, activity of S-AcM pp-vWF to compete with native pp-vWF on the binding to collagen was investigated. Surprisingly enough, pp-vWF retained fairly well the collagen-binding activity upon S-acetamidemethylation (Fig. 1). The dose-response curve of the inhibition by S-AcM pp-vWF indicated that it retained one-fifth to one-third of the activity in comparison with the native protein. The electrophoretic mobility of S-AcM pp-vWF under reducing and nonreducing conditions was identical (100 kDa), indicating that complete S-acetamidemethylation was achieved (data not shown).

Characterization of Tryptic Fragment of pp-vWF—Limited digestion of pp-vWF by trypsin produced several fragments of apparent molecular mass ranging from 0.7 to 30 kDa under reducing conditions. After reduction and S-acetamidemethylation, these fragments were separated by reverse phase HPLC on a C-1 column (Fig. 2). The pooled fractions (A-G) were tested for their activity to interfere with binding of pp-vWF to collagen. As shown in Fig. 3, only fraction F significantly inhibited the binding of 125I-labeled pp-vWF to collagen. This fraction contained mainly a fragment which had molecular mass of 30 kDa in addition to small minor contaminants (Fig. 3). The 30-kDa fragment was further purified on a C-18 column and its inhibiting activity was investigated more precisely. On a molar basis, the 30-kDa fragment retained 1/10 to 1/5 inhibiting activity of S-AcM pp-vWF (Fig. 4). The IC50 value of the 30-kDa fragment was about five times higher than that of S-AcM pp-vWF. This may reflect lack of a certain region(s) in the 30-kDa fragment, which is required for complete collagen-binding activity.

The 30-kDa fragment was further digested by trypsin. The reaction was conducted under a denaturing condition so that complete digestion was achieved. This treatment with trypsin generated more than eight peaks on the chromatogram of reverse phase HPLC (Fig. 5). None of these fractions, however, showed any significant inhibitory effect on collagen binding at the concentration tested. Whether this lack of inhibition is due to cleavage of a collagen-binding site by the second digestion or insufficient amount of peptide added in the assay system is not clear. The amino-terminal sequences of the 30-kDa fragment and its tryptic peptide (named TP1 to TP8) were determined (Fig. 6). Some fractions (TP2, TP3, and TP8) contained minor contaminating peptides as judged by small peaks in a chromatogram of phenylthiohydantoin-derivative identification. Sequence of TP4 could
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FIG. 3. Effect of tryptic fragments of pp-vWF on the binding of intact pp-vWF to collagen. Fractions from the reverse phase HPLC were analyzed on SDS-PAGE using 10–15% gradient gel and stained with Coomassie Brilliant Blue. The mobilities of known molecular mass markers are indicated. Under these lanes (A–G) are indicated the extent of inhibition of 125I-pp-vWF binding to collagen. The concentration of each fraction in the reaction mixture was about 5.2 μM, assuming the recovery of the fragment from the starting material was 100%. W, whole digest.

FIG. 4. Dose-dependent inhibition of pp-vWF binding to collagen by the 30-kDa fragment. S-AcM pp-vWF or the 30-kDa fragment was dissolved in 10 mM Tris-buffered saline, pH 7.4, and added to the reaction mixture to produce the concentration as indicated. The experimental conditions were the same as those described in the legend for Fig. 1.

not be identified because of the impurity of this fraction. Considering very high sequence homology between bovine and human pp-vWF which was observed in their amino-terminal 25 residues (27), these sequences were aligned according to that of human pp-vWF predicted from cDNA sequence (29, 30). All fragments identified had their corresponding region in human pp-vWF (Fig. 7). Though the combination of these tryptic peptides could not cover the entire sequence of 30-kDa fragment (the sequence between TP8 and TP2*, and that between TP2* and the carboxy-terminal residue were not identified), it is certain that the 30-kDa fragment is a 185 (or at least 179)-residue polypeptide containing from Phe305 to Arg461 (or Lys463) in the bovine pp-vWF molecule. The discrepancy between molecular mass determined by SDS-PAGE (30 kDa) and the calculated value (20,255 for Phe305–Arg461 or 19,485 for Phe305–Lys463) can be explained by the presence of carbohydrate chains since at least three potential N-glycosylation sites are present in this region.

Active Fragment Generated by Lysyl Endopeptidase—In order to get more information about the collagenn-binding site in pp-vWF molecule, another fragmentation technique was employed. Lysyl endopeptidase (Achromobacter protease I, EC 3.4.14.50) is a serine protease that specifically cleaves peptide bond at carboxyl-terminal of lysine residues (31). Because of its very high specificity, this enzyme is often used as a fragmentation tool in analysis of protein primary structure. As lysine is a relatively rare amino acid in this protein, complete digestion by lysyl endopeptidase is expected to result in production of larger fragments than those obtained by other proteases. S-AcM pp-vWF was subjected to complete digestion by lysyl endopeptidase followed by separation with
reverse phase HPLC on a C-18 column and fractions were tested for their ability to inhibit the {sup}125I-{pp-vWF} binding to collagen. As shown in Fig. 8, significant inhibitory activity was observed only in fraction III, which contained a fragment that appeared as a 21.5/21-kDa doublet band when analyzed by SDS-PAGE (Fig. 9). Fractions II, IV, V, and VI corresponded to fragments of 17, 6.5, 8, and 6.2 kDa, respectively. Recovery of the 14-kDa fragment from the reverse phase HPLC was very low, and a trace amount of the 14-kDa band existed in fraction VI together with a 6.2-kDa band, though it is not seen in this figure. Fraction I did not show any obvious band on SDS-PAGE, indicating that it contained a short peptide fragment that could not be stained under these conditions. The 21.5/21-kDa fragment inhibited pp-vWF binding to collagen in a dose-dependent manner, and the inhibition reached as much as 90% when the fragment was added in the concentration of 11 μM (Fig. 10). The dose dependency was comparable to that of a trypsin-generated 30-kDa fragment. Other isolated fragments, 17 kDa (fraction II), 6.5 kDa (fraction IV), and 8 kDa (fraction V) were much less effective.

The apparent existence of two components in fraction III (21.5 and 21 kDa) was due to the microheterogeneity or partial decomposition of carbohydrate moieties attached to the same polypeptide since endo-β-N-acetylglucosaminidase treatment of the 21.5/21-kDa fragments produced only one band with reduced molecular mass of 19-kDa (Fig. 11). Furthermore, sequence analysis of this fraction containing 21.5/21-kDa fragments revealed that this fraction contained a single class amino-terminal sequence. The determined amino-terminal sequence was as follows: FEACHSAVSPYLRNCRYDV. The identical sequence was found in a part of the 30-kDa fragment (Fig. 7, sequence beginning with Phe{sup}1439). Based upon both the specificity of the protease used and the determined sequence of the 30-kDa fragment shown in Fig. 7, we concluded that the active 21.5/21-kDa fragment corresponded to the region Phe{sup}1430} to Lys{sup}1442} in bovine pp-vWF molecule.

**DISCUSSION**

The studies reported here provide evidence that the carboxy-terminal region of the pp-vWF molecule mediates an interaction with collagen using different fragmentation techniques. Both the trypsin-generated 30-kDa fragment and lysyl endopeptidase-generated 21.5/21-kDa fragment were capable of inhibiting the binding of intact pp-vWF to collagen and were shown to originate from the same carboxy-terminal region. Though direct evidence that shows these fragments do bind to collagen has not been obtained, the competing effect strongly suggests that this region (Phe{sup}1430} to Lys{sup}1442}) is involved in the interaction between pp-vWF and collagen. Evidence for the idea that pp-vWF interacts with collagen through this region is also provided by the study using monoclonal antibodies. We have obtained a monoclonal antibody against pp-vWF that strongly blocks its binding to collagen and have found using immunoblotting that the epitope for this monoclonal antibody is present in the 21.5/21-kDa fragment. As S-AcM pp-vWF retains considerable collagen-binding activity.
ing activity, it seems likely that the interaction with collagen does not necessarily require its three-dimensional structure, so part of the binding site(s) may not be composed of some different regions in primary structure which are arranged close to each other in their three-dimensional structure. However, both 30-kDa and 21.5/21-kDa fragment showed significantly decreased inhibitory activity when compared to the S-AcM pp-vWF, suggesting that certain additional element(s) destroyed by the fragmentation is necessary to exhibit the full activity. As the possibility of existence of multiple collagen-binding sites per pp-vWF molecule still remains, different fragmentation tool may generate another fragment that enhances the effect of 30-kDa or 21.5/21-kDa fragment.

One of the structural features of the carboxyl-terminal collagen-binding region is that it contains many cysteine residues, probably forming disulfide bridges among one another (29, 30). Thus this region may construct a rigid protease-resistant domain in native conformation. As cysteine is rather uniformly distributed throughout this region and there is no sequence that has significantly high hydrophobicity scores, it is hard to predict which part in this domain faces outward to be utilized as the collagen-binding site. Several residues within this region could not be identified by SequenEdman degradation. Among these, positions 644, 658, and 676 are very likely to be glycosylated asparagine residues, considering the appearance of Thr or Ser residue in position 646, 660, and 678, respectively. Though the "R-G-D" sequence is present at residues 676-678 in human pp-vWF sequence predicted from cDNA clone (29, 30), this cell attachment sequence is not conserved in bovine protein, suggesting that this sequence may not be involved in general physiological function of pp-vWF. Another unidentified residue in position 652, which is Tyr in human sequence, is possibly a sulfated tyrosine residue, since amino acid sequences surrounding this residue satisfy most of the criteria for tyrosine sulfation (11). Whether these post-translationally modified residues present in the active 21.5/21-kDa fragment are indispensable for the collagen-binding activity remains to be elucidated.

Since pp-vWF is composed of two tandem repeat of homologous "D" domains that also exist in mature vWF molecule (30), a sequence that is similar to the collagen-binding site in pp-vWF may be found in mature vWF. In preliminary experiments, however, purified vWF did not interfere with pp-vWF binding to collagen. In addition, there are several reports in which it is demonstrated that certain region present in midportion of vWF subunit called "A" domain is responsible for interaction with collagen (17-21). So it seems likely that the collagen-binding site(s) of vWF and pp-vWF are unique and distinct from each other, and they recognize different sites on collagen molecule.

In conclusion, at least one binding site for type I collagen is present in the sequence comprising residues 570-682 of bovine pp-vWF. Considering the fact that pp-vWF inhibits collagen-platelet interaction probably by utilizing its collagen-binding sequence, determination of a definite collagen-binding sequence may give certain general information as to the molecular mechanism involved in the collagen-platelet interaction. The determination of the minimal segment required for collagen-binding activity needs further study.

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