Propolypeptide of von Willebrand Factor Serves As a Substrate for Factor XIIIa and Is Cross-linked to Laminin*

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The propolypeptide of von Willebrand factor (pp-vWF) was found to serve as a substrate for Factor XIIIa (FXIIIa). FXIIIa catalyzed polymerization of pp-vWF as well as the incorporation of monodansylcadaverine and [14C]putrescine into pp-vWF in a Ca2+- and time-dependent manner. The amount of putrescine incorporated into pp-vWF was increased as a function of putrescine concentration and reached a maximum of 3 mol/mol of pp-vWF, indicating that there are at least 3 glutamine residues in the pp-vWF molecule responsible for the reaction. 125I-pp-vWF was incubated with FXIIIa in the presence of various adhesive glycoproteins. Laminin, among the proteins tested, specifically formed a large molecular weight complex with 125I-pp-vWF in a Ca2+- and time-dependent manner. It was also found that FXIIIa catalyzed polymerization of laminin as well as the incorporation of monodansylcadaverine into laminin. The high molecular weight complexes of 125I-pp-vWF (or 125I-laminin) formed in the presence of laminin (or pp-vWF) were immunoprecipitated with anti-laminin (or anti-pp-vWF) antibodies. Taking all the data together it can be concluded that both pp-vWF and laminin have glutamine and lysine residues responsive to FXIIIa and make copolymers by virtue of FXIIIa.

Factor XIII (FXIII) is a blood coagulation factor which is distributed both extracellularly (in plasma) and intracellularly (in megakaryocytes, platelets, and placenta). Upon activation by thrombin, it is converted to the active form, Factor XIIIa (FXIIIa), which catalyzes the formation of e-(γ-glutamyl)-lysyl cross-links between specific protein pairs (2). The best known physiological reaction catalyzed by this enzyme is the stablilization of fibrin clot by cross linking between the α-chains and between γ-chains of fibrin (3, 4) in the final stage of the blood coagulation cascade. Protein substrates for FXIIIa include actin (5) and myosin (6) as well as proteins which exist in plasma and/or in extracellular matrix, such as α2-plasmin inhibitor (7), Factor V (8), lipoprotein (a) (9), fibronectin (10), von Willebrand factor (vWF) (11), vitronectin (12), thrombospondin (13), collagen (14), and osteopontin (15). The formation of covalent cross-links between some of these proteins by FXIIIa is believed to be important during wound healing at the site of blood vessel injury (2).

The propolypeptide of vWF (pp-vWF), which is also called von Willebrand antigen II, is a glycoprotein of M, = 100,000 (16). In endothelial cells, it is synthesized as a part of a large precursor molecule (prepro-vWF), cleaved from the precursor with concomitant production of mature vWF, and either stored in secretory granules called Weibel-Palade bodies or secreted constitutively (17). It is also synthesized in megakaryocytes and stored in α-granules of platelets (18, 19), and a significant amount of this protein is also expressed on the platelet surface (20, 21). Recently, we have found that pp-vWF binds to collagen with an affinity comparable to that of mature vWF (22), and, furthermore, that it inhibits collagen-induced platelet aggregation, in contrast to mature vWF which usually promotes aggregation (23). Upon blood vessel wall injury, platelets are activated by exposed collagen fibers and are aggregated to form a hemostatic plug. We have hypothesized that pp-vWF, released from activated platelets as well as from injured endothelial cells, may accumulate on the exposed collagen layer and may function as a repressor of excess platelet aggregation (23). Laminin is the most abundant noncollagenous glycoprotein in basement membranes and is composed of α1-400-kDa A chain and ~220-kDa B1 and B2 chains (24). Laminin is thought to be an important structural and regulatory molecule; it binds to other components in basement membranes such as type IV collagen, heparan sulfate proteoglycan, and entactin (nidogen), and also binds to cells to regulate various cellular events including attachment, proliferation, migration, and differentiation (25).

Since the physiological significance of pp-vWF as an extracellular matrix protein is still not understood, it is of interest to know whether it is incorporated into the extracellular matrix of the subendothelium and modulates certain cellular responses during wound healing. In the present paper, we show that pp-vWF is a substrate for FXIIIa and is specifically cross-linked to laminin.

EXPERIMENTAL PROCEDURES

Materials—Bovine serum albumin (BSA), monodansylcadaverine (MDC), hirudin, and benzamidine were obtained from Sigma. [1,4-14C]putrescine was obtained from Amersham, mouse laminin was from Biomedical Technologies (Stoughton, MA), monoclonal rabbit polyclonal antibody against mouse laminin was from Iwaki Glass (Tokyo, Japan), type I collagen was from KOKEN Co., Ltd. (Tokyo, Japan), bovine thrombin was from Mochida Pharmaceutical Company (Tokyo, Japan), and immobilized protein A was from Pierce.
Bovine fibronectin was kindly provided from Itoham Foods Inc. (Nishinomiya, Japan). Placental FXIII was kindly provided from Hoechst Japan Co. (Tokyo, Japan) and was separated from human serum albumin using DEAE-cellulose chromatography and p-chloromercuribenzoate-agarose chromatography (26). Bovine pp-vWF was purified from plasma as previously described (27). Monospecific rabbit polyclonal antibody against bovine pp-vWF was prepared as described in Ref. 22. Bovine pp-vWF and bovine fibrinogen were purified from fresh plasma according to the method of Kirby (28) and of Peerschke et al. (29), respectively.

**Activation of FXIII—FXIII (100 mg/ml) was activated by incubation in 10 mM Tris-buffered saline, pH 7.4 (TBS), in the presence of 10 mM dithiotreitol (DTT) for 10 min at 37 °C. Thrombin was then inactivated either with 20 units/ml hirudin or with 20 mM benzamidine. Activated FXIII (FXIIIa) was used immediately.**

**Incorporation of MDC into pp-vWF and Laminin—pp-vWF (1 mg/ml) was incubated at 37 °C with 0.5 mM MDC and FXIIIa (17 mg/ml) in TBS containing 5 mM DTT and 10 mM CaCl₂ or 5 mM EDTA. An aliquot of 10 μl of the reaction mixture was withdrawn at the indicated time points (1–60 min), added to the same volume of 10 mM Tris-HCl, pH 6.8, containing 2% 2-mercaptoethanol, 1.5% sodium dodecyl sulfate (SDS), and 0.01% Brilliant Blue (buffer A), and heated at 100 °C for 2 min prior to electrophoresis. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (30) using 8.75% gels under reducing conditions. Laminin to Other Proteins—Radioiodination of pp-vWF and laminin was carried out as described previously (31). **\[\text{\textsuperscript{125}I}\]pp-vWF or \[\text{\textsuperscript{125}I}\]laminin to Other Proteins—Radioiodination of pp-vWF and laminin was carried out as described previously (31).**

**RESULTS**

To test if pp-vWF serves as a substrate for FXIIIa and cross-linked polymers are formed, it was incubated at a relatively high concentration (0.5 mg/ml) with thrombin-activated FXIIIa at 25 °C and analyzed by SDS-PAGE. pp-vWF was indeed found to form a covalently cross-linked homopolymer which did not enter the gel even in the presence of SDS and the reducing agent, and the original band, observed in the absence of the enzyme, disappeared (data not shown). This indicates that pp-vWF contains both glutamine and lysine residues that participate in the FXIIIa-mediated reaction. The notion that pp-vWF possesses acyl-donating glutamine residues was further verified by experiments using MDC or \[\text{\textsuperscript{14}C}\]putrescine as an acyl acceptor. pp-vWF was incubated with FXIIIa in the presence of 0.5 mM MDC and analyzed by SDS-PAGE under reducing conditions followed by fluorescence visualization (Fig. 1). The band corresponding to pp-vWF was labeled with MDC and became increasingly fluorescent as a function of time, while no MDC incorporation was observed in the presence of EDTA (Fig. 1A). Coomassie staining of the gel showed that formation of homopolymers of pp-vWF was inhibited in the presence of excess amounts of the acyl acceptor (Fig. 1B). The incorporation of another amine, \[\text{\textsuperscript{14}C}\]putrescine, into pp-vWF was studied to quantify the reaction. Putrescine was also incorporated into pp-vWF by FXIIIa in a time- and Ca²⁺-dependent manner (Fig. 2A). Under our experimental conditions, it was calculated that about 1.5 mol of putrescine were incorporated into 1 mol of pp-vWF after an 8-h incubation (Fig. 2A). To see if this is the maximum amount of putrescine incorporated, pp-vWF was incubated for 14 h with increasing concentrations of putrescine (Fig. 2B). The amount of putrescine incorporated increased as a function of putrescine concentration and saturation was achieved above 2 mM. At saturation, 3 mol of putrescine were incorporated per mol of pp-vWF, indicating that in pp-vWF there are at least 3 glutamine residues which can serve as acyl-donating residues in the reaction catalyzed by FXIIIa. In the same assay, guinea pig liver transglutaminase (Sigma) also catalyzed \[\text{\textsuperscript{14}C}\]putrescine incorporation into pp-vWF and gave the same maximum incorporation (3 mol of putrescine/mol of pp-vWF) (data not shown). It is obvious that in pp-vWF there are at least 3 glutamine residues which can serve as acyl-donating residues in the reaction catalyzed by FXIIIa.
that pp-vWF serves as a substrate for both FXIIIa and tissue transglutaminase.

As pp-vWF can bind to collagen and may accumulate on the subendothelium after vessel injury, there is a possibility that pp-vWF cross-links to other proteins by virtue of FXIIIa or transglutaminase and modulates their biological functions in vivo. To search for such proteins, we investigated the cross-linking between pp-vWF and various proteins. 

![Graph A](image1)

**Fig. 2.** A, time course of incorporation of [14C]putrescine into pp-vWF catalyzed by FXIIIa. pp-vWF (0.5 mg/ml) was incubated with FXIIIa (10 

**Fig. 3.** Detection of cross-linking between 125I-pp-vWF and various proteins. 125I-pp-vWF (0.7 

**Fig. 4.** Time course of incorporation of [14C]putrescine into pp-vWF.

For the interface between the separating and the stacking gels, and very high molecular weight complex (VHMW) which was observed at the top of the stacking gel, was quantified by radioluminography using a BAS 2000 system (Table I). Since samples were heated at 100 °C for 5 min under denaturing and reducing conditions before electrophoresis, the materials observed in the HMW and VHMW bands should represent covalently cross-linked products. When 125I-pp-vWF alone was incubated with FXIIIa, about 16% of the radioactivity was detected in the VHMW and HMW bands, indicating that an intermolecular cross-linking reaction took place to some extent even at a very low concentration (0.7 

**Fig. 5.** Time course of incorporation of [14C]putrescine into pp-vWF.

and, during the following 6 h, a large amount of pp-vWF was incorporated into the VHMW band. These results suggest...
Cross-linking between pp-vWF and Laminin by Factor XIIIa

The distribution of radioactivity on the dried gels used for autoradiography represented in Fig. 3 was quantitatively analyzed by radioluminography. Radioactivity associated with bands corresponding to monomeric pp-vWF, HMW which was seen at the interface of the separating and stacking gels, and VHMW which was observed at the top of the stacking gel, was determined. The means of three different experiments are shown. Values are expressed as percentage of total radioactivity in each lane (monomer + HMW + VHMW).

<table>
<thead>
<tr>
<th></th>
<th>None</th>
<th>BSA</th>
<th>Laminin</th>
<th>vWF</th>
<th>Fibronectin</th>
<th>Fibrinogen</th>
<th>Collagen</th>
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<tr>
<td>VHMW</td>
<td>5.2%</td>
<td>7.0%</td>
<td>43.1%</td>
<td>8.6%</td>
<td>4.2%</td>
<td>8.6%</td>
<td>2.7%</td>
</tr>
<tr>
<td>HMW</td>
<td>10.9%</td>
<td>17.5%</td>
<td>18.8%</td>
<td>56.5%</td>
<td>8.5%</td>
<td>12.3%</td>
<td>3.5%</td>
</tr>
<tr>
<td>Monomer</td>
<td>83.9%</td>
<td>75.5%</td>
<td>38.1%</td>
<td>34.9%</td>
<td>87.3%</td>
<td>79.1%</td>
<td>93.8%</td>
</tr>
</tbody>
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* These minor components were not necessarily observed on the autoradiogram shown in Fig. 3 under our experimental conditions.

FIG. 4. Time course of cross-linking between 125I-pp-vWF and laminin catalyzed by FXIIIa. 125I-pp-vWF (0.7 μg/ml) and laminin (100 μg/ml) were incubated with FXIIIa (10 μg/ml) in the presence of 10 mM CaCl₂ at 37 °C. At the indicated time points, the reaction was stopped as described in the legend for Fig. 3, subjected to SDS-PAGE, and analyzed either by autoradiography (inset) or by radioluminography. Distribution of radioactivity among the bands corresponding to monomeric pp-vWF (A), HMW (B), and VHMW (C) (see Table I) at each incubation time point was shown. Inset, an autoradiogram showing the time course of the cross-linking reaction between pp-vWF and laminin. Lane 1, untreated 125I-pp-vWF; lanes 2–6, 125I-pp-vWF incubated with FXIIIa and laminin for 0.5, 1, 2, 4, and 6 h, respectively.

FIG. 5. Cross-linking reaction between 125I-laminin and pp-vWF catalyzed by FXIIIa. 125I-Laminin (1 μg/ml) was incubated at 37 °C for 6 h in the presence of 10 mM CaCl₂ with 10 μg/ml FXIIIa either alone (lane 1) or with pp-vWF at concentrations of 10, 30, and 100 μg/ml (lanes 2–4, respectively). As a negative control, 125I-laminin was incubated with 100 μg/ml pp-vWF and 5 mM EDTA (lane 5). Samples were analyzed by SDS-PAGE using 6% separating gel for SDS-PAGE followed by autoradiography as described in the legend for Fig. 3.

FIG. 6. Incorporation of MDC into laminin catalyzed by FXIIIa. Panel A, laminin (1.5 mg/ml) was incubated with FXIIIa (20 μg/ml) and 0.5 mM MDC in the presence of 5 mM EDTA (lane 1) or 10 mM CaCl₂ (lane 2) at 37 °C for 2 h. Fluorescence was detected after SDS-PAGE as described in the legend to Fig. 1 using 6% gel. Panel B, the same gel as in panel A but stained with Coomassie Brilliant Blue.

These data indicate that laminin also serves as a substrate for FXIIIa and contains both glutamine and lysine residues that participate in the FXIIIa-mediated reaction.

In order to prove copolymer formation between pp-vWF and laminin, the following immunochemical experiments were performed. 125I-pp-vWF was incubated with laminin to produce the large molecular weight complexes. The sample was treated with either anti-laminin antibody, preimmune IgG, or buffer (TBS), followed by further incubation with protein A-immobilized beads. The beads were washed and the radioactivity associated with the beads was counted (Table II). The value obtained when the sample was treated with buffer alone was subtracted to see the specific binding. When the sample was treated with anti-laminin antibody, about 20 times as much radioactivity was observed as with preimmune IgG, suggesting that the large complexes consisted of laminin and...
the methods used to purify laminin were slightly different between these two preparations. Yurchenco et al. (33) reported Ca²⁺-dependent noncovalent self-assembly of laminin. This interaction may create a favorable situation for cross-linked homopolymer formation to take place. The laminin-pp-vWF complex we show here was also formed at laminin concentrations as low as 10 μg/ml (data not shown), at which concentration noncovalent self-assembly of laminin does not occur (33). This indicates that a noncovalently aggregated form of laminin is not required for the formation of cross-linking between laminin and pp-vWF. It is likely that efficient cross-linking between laminin and pp-vWF by FXIIIa is preceded by specific but noncovalent interaction between these proteins. Although the concentration of pp-vWF in plasma is very low (0.2 μg/ml) (22), it increases at a site of vascular injury where pp-vWF is secreted from activated platelets. The possibility is strongly suggested that pp-vWF may be readily cross-linked with laminin in the subendothelial matrix upon endothelial injury and subsequent activation of blood coagulation including platelet aggregation and FXIII activation.

FXIIIa is known to catalyze cross-linking between different proteins such as fibrin and α2-plasmin inhibitor (7), fibronectin and fibrinogen (34), fibronectin and collagen (35), vWF and fibrin (11), or vWF and collagen (36). It is very interesting that proteins which serve as substrates for FXIIIa are often proteins which serve as substrates for FXIIIa are often cross-linked to each other to form copolymers. It seems, however, that only limited pairs of proteins are efficiently cross-linked to each other. Cross-linking of different proteins is important in localizing certain proteins to specific sites, stabilizing the deposited molecules, or modulating some biological functions of either of the cross-linked proteins. α2-Plasmin inhibitor incorporated into a fibrin clot by FXIIIa increases resistivity of the clot against fibrinolysis (57). Cross-linking of fibrin to fibronectin and fibronectin to collagen enhances cell adhesion to these structural proteins (38, 39). Laminin is known to have many biological functions including cell attachment, migration, differentiation, and tube formation in endothelial cells (25, 40). Recently, we have found that bovine pp-vWF promotes melanoma cell attachment in an RGD-independent manner. It is of interest to know whether these activities of laminin and pp-vWF are affected by FXIIIa-catalyzed cross-linking. Recently, we have reported that intact pp-vWF inhibits collagen-induced platelet activation (23). pp-vWF polymerized by FXIIIa may exhibit altered activity in this inhibition. These points as well as identifications of reactive glutamine and lysine residues in pp-vWF are currently under investigation.

REFERENCES

T. Takagi, Y. Sudo, T. Saito, and Y. Saito, unpublished observations.
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