The interaction between human blood-coagulation Factor VIII and von Willebrand Factor

Characterization of a high-affinity binding site on Factor VIII

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The interaction between human Factor VIII and immobilized multimeric von Willebrand Factor (vWF) was characterized. Equilibrium binding studies indicated the presence of multiple classes of Factor VIII-binding sites on vWF. The high-affinity binding ($K_a = 2.1 \times 10^{-10} \text{ m}$) was restricted to only 1–2% of the vWF subunits. Competition studies with monoclonal antibodies with known epitopes demonstrated that the Factor VIII sequence Lys$^{1673}$–Arg$^{1689}$ is involved in the high-affinity interaction with vWF.

INTRODUCTION

The complex between blood-coagulation Factor VIII and von Willebrand Factor (vWF) serves an essential role in the haemostatic process [1]. Factor VIII functions as a cofactor in the activation of Factor X in the intrinsic coagulation pathway [2–4]. The single polypeptide chain of $M_r$ 260000 [5,6] is susceptible to limited proteolysis, and consequently circulates as heterodimers of a C-terminal light chain ($M_r$ 80000) and variable derivatives ($M_r$ 50000–180000) of the N-terminal heavy chain [7–9]. The persistence of Factor VIII in the circulation is strongly dependent on complex-formation with vWF [1,10,11]. vWF circulates in plasma as a heterogeneous population of multimerized dimeric forms [1,12]. These dimers are composed of monomeric subunits of $M_r$ 250000 that are disulphide-linked in their C-terminal regions; disulphide linkage between the free N-termini then results in multimerization [1,13]. A minor proportion of the vWF subunits in the multimers contains an uncleaved N-terminal pro-sequence of $M_r$ 100000 [1,14].

The nature of the interaction between vWF and Factor VIII is poorly documented. On vWF, a major Factor VIII-binding domain has been located within amino acid residues 1–272 of the mature subunit [15]. The Factor VIII light chain has been shown to be involved in vWF binding [16]. In the present study we describe a method for studying the binding of Factor VIII to vWF. Data are presented on the characteristics of the interaction and on the fine mapping of the vWF-binding site on the Factor VIII light chain.

MATERIALS AND METHODS

Purified proteins

Human Factor VIII was purified by a method devised by A. Leyte, M. J. M. de Keyzer-Nellen, M. M. C. L. Groenen-van Dooren, J. de Bruin, H. Pannekoek, J. A. Van Mourik & M. Ph. Verbeet (unpublished work). In summary, Factor VIII concentrate (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service) was dissolved in a buffer containing 0.25 m-CaCl$_2$ to dissociate the Factor VIII–vWF complex. Factor VIII then was isolated by affinity chromatography using a monoclonal antibody against Factor VIII (CLB-CAg 117; see ref. [17]). Purified Factor VIII obtained by this method had a ratio of Factor VIII activity over antigen of 0.9, had a specific activity of 2500–3000 units/mg, and contained less than 0.1% (w/w) vWF. Human vWF was prepared from the same source, by gel filtration of the Factor VIII-depleted concentrate over Bio-Gel A-5m (Bio-Rad Laboratories, Richmond, CA, U.S.A.) in 150 mM-NaCl/50 mM-imidazole/HCl buffer, pH 6.8. The void-volume fractions were pooled and the vWF was concentrated by (NH$_4$)$_2$SO$_4$ precipitation (35% saturation, at 4°C), and dissolved in 150 mM-NaCl/50 mM-Tris/HCl buffer, pH 7.4. The specific vWF antigen content of the final preparation was 100 units/mg. Less than 0.1% of the total protein was residual Factor VIII. The material migrated as a single band ($M_r$ 250000) on SDS/polyacrylamide-gel electrophoresis under reducing conditions. Multimer analysis demonstrated the presence of vWF multimeric forms with $M_r$ values up to at least $5 \times 10^6$ (cf. Fig. 1).

Monoclonal antibodies

Mouse monoclonal antibodies against human Factor VIII were produced and purified as described previously [18]. The epitope of antibody CLB-CAg 69 was mapped on the same region of the Factor VIII light chain (amino acid residues 1649–1778) as reported previously for the antibody CLB-CAg 65 [19]. The control antibody CLB-CAg 9 recognizes an epitope located between residues 712 and 741 on the heavy chain of Factor VIII [19]. These antibodies do not inhibit Factor VIII activity when tested in the spectrophotometric Factor VIII assay (see below).
Synthetic Factor VIII peptide

The synthetic peptide representing the Factor VIII sequence Lys<sup>1672</sup>–Arg<sup>1689</sup>, preceded by Cys-Gly- at the N-terminus, was provided by Dr. M. Lacroix, Centre de Fractonnement Sanguin 'Armand Frappier'.

Factor VIII–vWF binding assay

Micro-titre wells (Immulon; Dynatech G.m.b.H., Plochingen, Germany) were coated with vWF, as follows: various amounts (0–50 munits) of purified human vWF were incubated in 150 mM-NaCl/50 mM-Tris/Cl buffer, pH 7.4 (100 µl/well), for 2 h at 37 °C. The wells were washed once with 250 mM-CaCl<sub>2</sub>/150 mM-NaCl/50 mM-Tris/Cl buffer, pH 7.4, to eliminate any traces of Factor VIII, and twice with the same buffer without CaCl<sub>2</sub>. Subsequently, the wells were incubated with, per well, 100 µl of 2% (w/v) human serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.)/0.1% (w/v) Tween 20 (Merck, Darmstadt, Germany) in 150 mM-NaCl/50 mM-Tris/Cl buffer, pH 7.4, for 1 h at 37 °C or overnight at 4 °C. After this blocking the wells were washed three times with the same buffer containing 0.2% (w/v) human serum albumin. The amounts of vWF immobilized by this protocol were determined by the use of radiolabelled [20] vWF as a tracer, and ranged from 0.7 to 5 munits of vWF per well (about 10% of the input). The vWF-coated wells were incubated with various amounts of Factor VIII (0–370 munits) in 100 µl of 2% (w/v) human serum albumin/0.1% (w/v) Tween 20 in 2.5 mM-CaCl<sub>2</sub>/150 mM-NaCl/50 mM-Tris/Cl buffer, pH 7.4, for 2 h at 37 °C. Non-bound Factor VIII was removed by two immediately successive washes with the same buffer containing 0.2% (w/v) human serum albumin. Dissociation studies (see Fig. 1) demonstrated that this rapid washing procedure (less than 5 s) did not disturb the binding equilibrium. Bound Factor VIII was then quantified spectrophotometrically (see below), with the purified Factor VIII preparation as reference.

Quantification of proteins

Concentrations of total protein were determined by the method of Bradford [21]. vWF antigen was quantified as described previously [22]. Factor VIII light chain was measured by using an enzyme-linked immunosorbent assay based on a previously described method [17]. Factor VIII activity was determined spectrophotometrically, by using a method employing a chromogenic substrate and purified bovine coagulation factors (Coastal Factor VIII; Kabivitrum, Stockholm, Sweden). The assay was performed in micro-titre wells essentially as prescribed by the manufacturer. Factor VIII and vWF concentrations are expressed in units/ml; this represents the concentration of activity or antigen in 1 ml of pooled normal human plasma. One unit of Factor VIII and 1 unit of vWF are approximately equivalent to 0.1 µg and 10 µg of protein respectively [1].

RESULTS AND DISCUSSION

In the present paper we describe a method for studying the interaction between Factor VIII and vWF. The assay system differs from previously described methods also employing immobilized vWF [16,23,24] in that bound Factor VIII is quantified by its biological activity by using a spectrophotometric assay. This approach is based on the notion that the presence of vWF has no effect on the cofactor function of activated Factor VIII [3,25] and allows expression of specific binding of Factor VIII in quantitative terms. Control experiments demonstrated that Factor VIII does not bind to micro-titre wells lacking vWF coating. Factor VIII binding to multimeric vWF was found to be maximal after 1 h of incubation, and could be reversed by the addition of CaCl<sub>2</sub> (final concentration 0.25 mM), a condition known to dissociate the Factor VIII–vWF complex [1]. Reversibility of binding was further studied with dissociation experiments in the absence and in the presence of multimeric vWF in solution (Fig. 1). In the absence of vWF, dissociation occurred with an apparent t<sub>1/2</sub> of about...
Factor VIII–von Willebrand Factor interaction

Factor VIII was incubated in microtitre wells that had been coated with various concentrations of vWF, and coated vWF and bound Factor VIII were quantified, as described in the Materials and methods section. The inset shows the amount of high-affinity Factor VIII-binding sites per well as a function of the amount of vWF coated.

13 min. This was consistent with the first-order rate constant of 5.4 × 10^{-2} min^{-1}, which was derived from a linear plot of the logarithm of the concentration of residual Factor VIII bound versus time (not shown). In the presence of vWF multimers (see Fig. 1 inset) the rate of dissociation increased in a dose-dependent manner. This demonstrates that Factor VIII binding to immobilized vWF was specific and reversible, thus permitting equilibrium binding studies. Fig. 2 shows the Scatchard analysis of binding data obtained at various amounts of vWF coated. These data suggest the presence of more than one class of binding sites. For the high-affinity sites, the dissociation constant was independent of the amount of vWF coated and was calculated assuming that the $M_r$ and plasma concentration of Factor VIII are 260,000 and 0.1 μg/ml respectively [1], to be 2.1 (± 0.3) × 10^{-19} M (mean ± s.d., n = 8). The number of high-affinity binding sites per well increased linearly with the amount of vWF coated (Fig. 2 inset). Per 1 munit (or 10 ng) of vWF, the number of Factor VIII-binding sites was equivalent to 1.5 munit (or 0.15 ng). Given an $M_r$ value of 250,000 for monomeric vWF [1], the apparent Factor VIII/vWF stoichiometry is about 1:70. This value indicates that only 1–2 % of the vWF subunits participates in this interaction. At high Factor VIII concentrations (higher than 1750 munits/ml, 175 munits/well) the binding data suggested the existence of additional binding sites with lower affinity. The extensive washing required in binding studies at higher Factor VIII concentrations introduced experimental variations that hampered precise characterization of the lower-affinity binding.

In order to identify the sites involved in the high-affinity Factor VIII–vWF interaction, competition studies were performed with several monoclonal antibodies against Factor VIII. One antibody against plasma Factor VIII, coded CLB-CAG 69, was found to interfere in the Factor VIII–vWF interaction (Fig. 3a). Similarly, vWF was an effective inhibitor of the binding of Factor VIII to the immobilized antibody (Fig. 3b). This indicates that the epitope of this antibody may be involved in vWF binding. Previous studies have demonstrated that the epitope of the antibody CLB-CAG 69 is located between amino acid residues 1648 and 1779 in Factor VIII [19]. Pilot experiments (results not shown) using immunoblotting of purified Factor VIII have indicated that antibody CLB-CAG 69 recognizes the Factor VIII light chain (residues 1649–2332; see Fig. 4), but not the cleavage product (residues 1690–2332) that is obtained after thrombin digestion. This provided indirect evidence that the sequence at residues 1649–1689, which represents a remarkably acidic region in Factor VIII [5], contains at least part of the epitope of the antibody that interferes with vWF binding. In agreement with this hypothesis, the antibody was found to recognize a synthetic peptide representing the sequence Lys^{1672}_Arg^{1689} (see Fig. 4).

During the course of our investigations, similar findings were reported by Foster et al. [23], who described that the Factor VIII–vWF binding could be blocked by an antibody against plasma Factor VIII recognizing an epitope on a synthetic peptide representing the sequence Val^{1670}_Glu^{1684}, whereas antibodies directed against adjoining sequences did not affect vWF binding. The partial overlap between the Val^{1670}_Glu^{1684} sequence and that of our peptide (see Fig. 4) suggests that the site
involved in vWF binding is located within the sequence Lys1672-Glu1684. Since all antibodies known so far to recognize this specific Factor VIII sequence appear to interfere in vWF binding, it seems likely that this site is directly involved in the Factor VIII–vWF interaction. This would be in agreement with recent observations that the presence of the 41-residue acidic region in the light chain is required for vWF binding [26]. Competition experiments demonstrated that the peptide Ly1672-Arg1684 effectively inhibits the binding of the antibody CLB-CAG 69 to Factor VIII: at a peptide concentration of 1 mM, more than 90% inhibition occurred under the conditions of Fig. 3(b). In contrast, no inhibition of the Factor VIII–vWF interaction was found with peptide concentrations up to 0.5 μM. An explanation for this finding may be that the synthetic peptide does not reflect the complete vWF-binding site. In this regard, it may be noted that this sequence contains a tyrosine residue (Tyr1680) that is sulphated as a post-translational modification of the Factor VIII protein [27]. Sulphation of the Tyr1680 residue may be essential for vWF binding.

With regard to the stoichiometry within the Factor VIII–vWF complex, it is of interest to know the precise localization of the sites of interaction. In contrast with the site on Factor VIII, the binding site on vWF has not been characterized in detail. This Factor VIII-binding site has recently been assigned to the N-terminal 272 amino acid residues of the mature vWF subunit [15]. If this site is accessible on all subunits of multimeric vWF, a 1:1 stoichiometry of Factor VIII–vWF binding would be conceivable. Indeed, 1:1 binding has been observed in sedimentation-velocity studies for the pig proteins [28]. However, it is not evident from such studies whether or not the known heterogeneity of vWF introduces multiple binding affinities. For the human proteins, data have been reported suggesting that the Factor VIII/vWF stoichiometry may be as low as 1:25 [29], a value that approximates the Factor VIII/vWF ratio in human plasma [1]. In our studies the high-affinity interaction was restricted to about 1–2% of the subunits of the vWF multimers. The apparent existence of additional binding sites with lower affinity may be explained by heterogeneity in exposition of the Factor VIII-binding site. One factor that may exert a steric effect is the presence of the vWF propeptide, which is adjacent to the Factor VIII-binding domain, and can be detected on a few per cent of the subunits of vWF multimers secreted by endothelial cells [1,14]. Alternatively, multimerization (cf. Fig. 1 inset), which involves cysteine residues located close to the Factor VIII-binding region [13,15], may affect Factor VIII-binding affinity. Further studies will be needed to assess whether or not Factor VIII-binding sites on the free termini differ from those within the vWF multimeric chains.

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