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Factor VIII Procoagulant Protein Interacts with Phospholipid Vesicles Via its 80 kDa Light Chain

G. Kemball-Cook, S. J. Edwards, K. Sewerin*, L.-O. Andersson*, and T. W. Barrowcliffe

From the National Institute for Biological Standards and Control, Potters Bar, Herts., UK and KabiVitrum AG*, Stockholm, Sweden

Key words

Factor VIII – Phospholipid – Antibodies – Immunocomplexes

Summary

In a previous report, we detailed the fractionation of polyclonal human anti-Factor VIII:C into a component directed exclusively against the phospholipid-binding site on Factor VIII (PL-site antibody) and another directed at other sites (non-PL-site antibody). The location on the F.VIII molecule of its PL-binding site has now been studied by two different methods using this fractionated ^{125}I-labelled anti-F.VIII:C Fab'.

The first method was modified from that of Weinstein et al. (Proc Natl Acad Sci USA 1981; 78: 5137–41), involving electrophoresis of F.VIII peptide-^{125}I-Fab' A/F.VIII immunocomplexes in SDS-polyacrylamide gels. PL-site antibody reacted with F.VIII peptides of apparent M_r approximately 80 kDa and sometimes 160 kDa in plasma and concentrate, but not with larger peptides. Non-PL-site antibody, however, reacted with a range of peptides of apparent M_r 90 kDa to 280 kDa. In addition, when purified F.VIII containing heavy and light chains (HC + LC), and isolated LC peptides were analysed, PL-site antibody bound to LC peptides whereas non-PL-site antibody did not.

The second method used the antibody pools in immunoradiometric assays (IRMA's) of purified F.VIII peptides. Both labels measured similar amounts of F.VIII:Ag in a sample of purified F.VIII containing both HC and LC; on assaying an HC preparation, however, PL-site label measured only 2% of F.VIII:Ag found by non-PL-site label, indicating that PL-binding sites are absent in HC preparations.

These results indicate that F.VIII binds to PL via its 80 kDa light chain.

Introduction

The interaction between Factor VIII protein and negatively-charged phospholipid (PL) vesicles has been shown to be essential for the in vitro expression of the cofactor activity of Factor VIII (1, 2). However, despite progress in elucidating the structure and function of Factor VIII (3–8), the locus of the molecule responsible for its interaction with PL, and probably with the activated platelet surface, is unknown.

Recently we have described the preparation of labelled human anti-F.VIII:C Fab' directed exclusively at the PL-binding site(s) on the F.VIII molecule (PL-site antibody); in addition a preparation of F.VIII-binding Fab' fragments directed against other epitopes (non-PL-site antibody) was produced (9).

This paper describes the use of these two antibodies in identifying the peptide(s) bearing the PL-binding site. Two techniques were used: the first a modified version of the method of Weinstein et al. (10), where immunocomplexes of F.VIII with the labelled antibodies are separated by differences in relative molecular mass (M_r) in SDS-polyacrylamide gels and autoradiographed. Qualitative analysis of the F.VIII peptides is possible, even in highly impure samples such as plasma, and the usefulness of this method in examination of different F.VIII products has been recently underlined (11). The second method used was the fluid phase immunoradiometric assay (IRMA), which has already been used in conjunction with the two antibody pools to assess PL-bound F.VIII in concentrates (12).

Materials and Methods

^{125}I-labelled anti-F.VIII:C Fab' PL-site and non-PL-site antibody pools were prepared from anti-F.VIII:C haemophilic plasma CC8000, and each batch was checked for specificity by IRMA as previously described (9). Briefly, ^{125}I-Fab' from inhibitor plasma was incubated with a mixture of F.VIII/vWF complex and excess PS:PC (1:1) vesicles. Fab' not bound to F.VIII-PS:PC was separated by gel filtration and further incubated with F.VIII/vWF in the absence of added PS:PC vesicles. The bound Fab' from these two incubations was recovered from the immune complexes by acid treatment and gel filtration to provide two pools of anti-F.VIII:C antibody. This PL-site antibody binds only to the PL-binding site on F.VIII:Ag, while non-PL-site antibody reacts with other epitopes. When PL-site Fab' was used in an IRMA to measure F.VIII:Ag in the presence of excess PS:PC vesicles, less than 2% of added antigen was measured: the binding of non-PL-site Fab' to antigen was unaffected by added PS:PC. In all, fifteen fractionations have been performed, of which 13 produced antibody pools of proven specificity. Three separate preparations of antibody were used in the studies described here.

Factor VIII antigen (F.VIII:Ag) standard for immunoradiometric assays was the 4th British Working Standard for Factor VIII, Concentrate (NIBSC 83/591), prepared from an intermediate purity concentrate supplied by the Blood Products Laboratory, Elstree (BPL).

Highly-purified Factor VIII peptides were isolated by immunoaffinity chromatography and FPLC on Mono Q gel (7), resulting in various preparations of HC alone, whole F.VIII containing both HC and LC and possessing F.VIII:C activity, and LC alone. The identity and purity of the peptides was confirmed by both SDS-PAGE analysis and Western blotting using polyclonal antibodies to F.VIII:C (7).

Factor VIII concentrates used were Koate HT (Miles Laboratories, Slough) and NHS concentrate type 8Y (BPL).

Human thrombin semi-purified, was an NIBSC preparation (82/570, 700 u/mg protein).

Thrombin inhibitor I-2581 was obtained from KabiVitrum, Stockholm.

Molecular weight markers for use in non-reduced Laemmli SDS-PAGE were the HMW calibration kit 330–36 kDa (Pharmacia, Milton Keynes) and SDS-6H HMW mixture 205–29 kDa (Sigma, Poole).

Sample treatment buffer for electrophoresis was 62 mM Tris–10% (v/v) glycerol–2% (w/v) SDS–0.001% (w/v) bromophenol blue, pH 6.8.

Slab gels (3–9% with 3% stacking gel) were cast in a Mighty Small Multiple Gel Caster (Biotech Instruments Ltd., Luton) using the buffer system of Laemmli (13): the gels were run in a Mighty Small Slab Gel Unit (Biotech).

Fixing and destaining of gels was in 10% (v/v) glacial acetic acid–40% methanol–50% water: gels were stained for protein with a 0.2% (w/v) solution of Coomassie brilliant blue dye R-250 (Hopkins & Williams, Chadwell Heath) in fixative.

Correspondence to: Dr. G. Kemball-Cook, Division of Haematology, National Institute for Biological Standards and Control, South Mimms, Potters Bar, Herts. EN6 3QG, UK

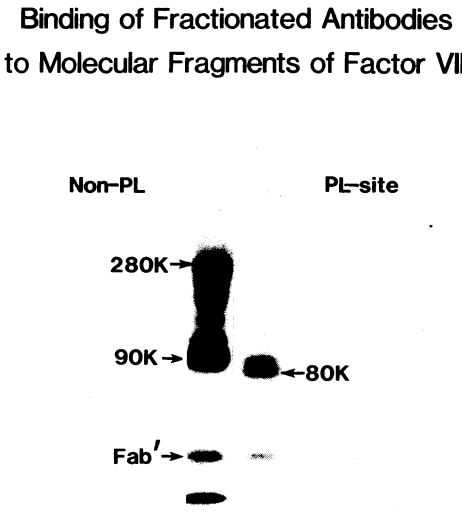

Fig. 1 Autoradiograph of SDS-polyacrylamide gel showing F.VIII peptide-^{125}I-Fab' immunocomplexes obtained on incubation of F.VIII concentrate (HT Koate) with either non-PL-site or PL-site labelled antibody. Indicated approximate M_r's are after subtraction of 50 kDa (= 1 Fab' molecule) and sample migration was from top to bottom

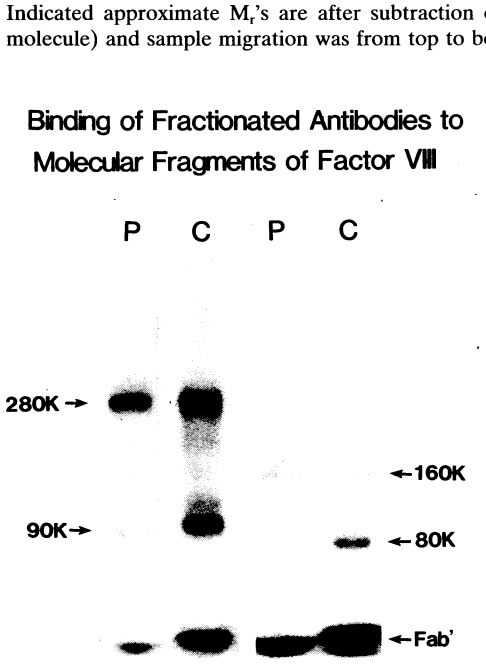

Fig. 2 Autoradiograph of SDS-polyacrylamide gel showing F.VIII peptide-^{125}I-Fab' patterns obtained with normal plasma (P) or F.VIII concentrate (Elstree 8Y, C) complexed with both non-PL-site and PL-site antibody

Vacuum-drying of gels was performed using a Model 443 slab gel drier (Bio-Rad, Watford).

Autoradiography of dried gels was carried out at −70° C for between 1 and 14 days in Protex cassettes fitted with a Philips Ultra S intensifying screen, using Kodak X-Omat XS-1 film (Everything X-Ray Ltd., Watford).

Preparation of samples for SDS-PAGE generally, F.VIII-containing samples were diluted (if necessary) to between 0.5 and 3.0 iu/ml F.VIII:Ag, then 10 µl were mixed with 10 µl of ^{125}I-Fab' (1,000–15,000 cpm) and 4 µl of 9% (w/v) PEG 4000 (Sigma P-3640) and incubated for 2 hr at 37° C to allow formation of F.VIII-^{125}I-Fab immunocomplexes. 20 µl of sample buffer were added and incubation continued for a further 30 minutes, after which samples (25–30 µl approx.) were loaded onto the gel.

Electrophoresis was carried out at 20 mA per gel until the tracking dye reached within 1 cm of the bottom of the gel.

Thrombin treatment of Factor VIII concentrate concentrates were diluted to 2 iu/ml F.VIII:C, then either buffer or human thrombin (final concentration 0.01 u/ml) was added. After incubation for 20 min at 37° C, excess thrombin was neutralised by addition of I-2581 (final concentration 50 mM) and the sample was mixed with ^{125}I-Fab' as described above.

Calculations of relative molecular mass (M_r) of visualised peptide bands on autoradiographs were performed by comparison with molecular weight markers on dried Coomassie-stained gels, with subtraction of 50 kDa (= M_r of one Fab' molecule) to give the mass of the peptide alone.

Immunoradiometric assays using the two antibody pools were performed as described previously (14).

IRMA dilution buffer for all samples was 50 mM Tris–150 mM NaCl–0.02% NaN_3 pH 7.4 containing 1 mg/ml non-immune human IgG as carrier (Blood Products Laboratory, Elstree).

Results

Electrophoresis of Immunocomplexes

Where estimates of peptide M_r are given below, these should be taken as approximate. This gel technique uses non-denaturing conditions in the absence of reducing agents and, in addition, the molecular mass of one Fab' fragment (approximately 50 kDa) has been subtracted from that calculated after comparison with mobility of molecular weight markers. Unlike conventional SDS-PAGE, in which accurate estimates of peptide size are obtained, the value of this method is in the visualisation of peptide patterns with antibodies of different specificities.

Analysis of F.VIII concentrates and plasma. Fig. 1 shows the autoradiograph obtained from mixing a Factor VIII concentrate (Koate HT) with either the non-PL-site or PL-site antibody before SDS-PAGE. The pattern of radiolabelled peptide-Fab' complexes was quite different in each case: PL-site antibody reacted with two peptide bands of apparent M_r approximately 80–90 kDA, whereas non-PL-site antibody reacted with a range of at least five peptides between M_r 90–280 kDa. When CRM-ve severe haemophilic plasma was tested in this system, no peptide-Fab' bands were observed with either antibody.

A comparison of peptide reactivity in normal plasma and F.VIII concentrate (BPL 8Y) is shown in Fig. 2. Again, the non-PL-site antibody complexed with peptides between 90–280 kDa, with a marked predominance of higher M_r peptides in the plasma sample compared with concentrate. The PL-site antibody showed similar reactivity with plasma and concentrate, labelling two peptides of apparent M_r approximately 80 kDa and 160 kDa. These analyses of plasmas and concentrates were found to be very reproducible from gel to gel and over three preparations of fractionated antibody, with the exception that, while the 80 kDa band was always seen, the appearance of this 160 kDa band was variable, depending on the batch of PL-site antibody used in the experiments.

The effect of trace thrombin treatment (0.01 u/ml) of F.VIII concentrate (BPL 8Y) before SDS-PAGE analysis is shown in Fig. 3. After 20 min of F.VIII activation, non-PL-site antibody detects only peptides of approximately M_r 90 kDa and 50 kDa, indicating proteolysis of the peptides of higher M_r and generation of a degradation product of smaller size. Estimation of apparent M_r by electrophoretic mobilities is not precise enough with this technique to indicate whether this degradation product is the 52 kDa or 43 kDa peptide seen on thrombin treatment of purified F.VIII (7). The pattern of peptides detected by PL-site antibody is much less altered, the 160 kDa band appearing resistant to the quantity of thrombin used, and the 80 kDa band increasing

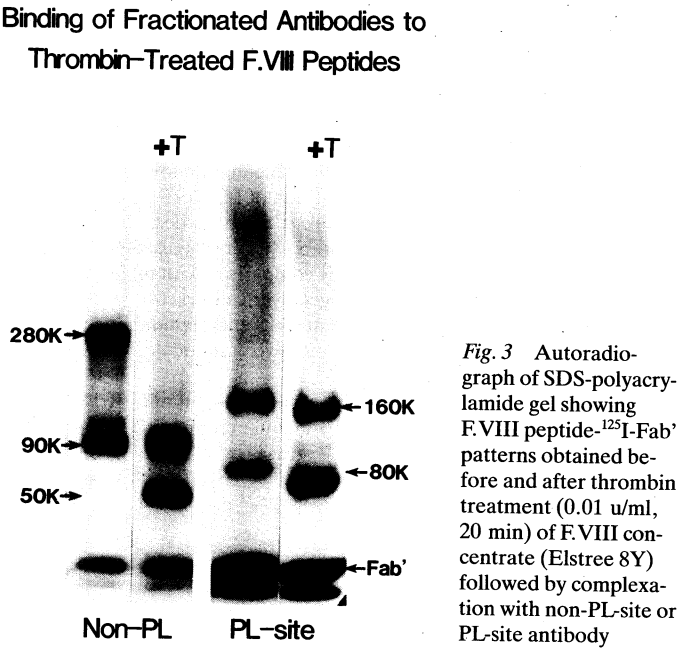

Fig. 3 Autoradiograph of SDS-polyacrylamide gel showing F.VIII peptide-^{125}I-Fab' patterns obtained before and after thrombin treatment (0.01 u/ml, 20 min) of F.VIII concentrate (Elstree 8Y) followed by complexation with non-PL-site or PL-site antibody

slightly in mobility. The antigenic site recognised by the PL-site antibody in this peptide is preserved following thrombin treatment.

Taken together these SDS-PAGE results on plasma and concentrates indicate that PL-site antibody reacts with a restricted portion of the F.VIII molecule which resembles the 80 kDa light chain (LC) portion, while non-PL-site antibody is reactive with a range of heavy chain (HC)-like peptides. In addition, the patterns generated on trace thrombin treatment resemble those seen on thrombin activation of purified F.VIII when analysed by SDS-PAGE in reducing gels (3–8). The doublet seen on incubation of F.VIII concentrate with PL-site antibody (Fig. 1) may represent undegraded and thrombin-cleaved LC species: the upper band is unlikely to be the result of a reaction between the antibody and the 90 kDa HC species since there is no reaction with larger HC peptides.

Analysis of purified F.VIII peptides. Immunoisolated F.VIII was further purified by ion exchange on a Mono Q column followed by gel filtration (7) to produce preparations of highly purified F.VIII consisting of both HC and LC, and LC alone. Together with a CRM-ve severe haemophilic plasma, these were analysed by SDS-PAGE, again using both antibody pools. The results are shown in Fig. 4. While PL-site antibody reacted with peptides of approximately M_r 70–90 kDa in both whole F.VIII (HC + LC) and LC preparations, non-PL-site antibody detected peptides predominantly in the F.VIII (HC + LC) sample, with only a very slight reaction to LC peptides. This faint reaction may be due either to contamination of the non-PL-site antibody with a small proportion of PL-site label, or to the presence in the former of a population of antibodies directed at antigenic sites on the LC peptide distinct from the PL-binding site.

It is highly unlikely that the reaction of PL-site antibody with F.VIII (HC + LC) as seen in Fig. 4 is due to binding to HC rather than to LC, since there is no reaction with the higher M_r forms of HC as seen with non-PL-site Ab (see also Fig. 1).

It therefore appears that PL-site-directed Fab' binds to LC peptide(s) while non-PL-site Fab' reacts predominantly with HC peptides. The doublet seen on reaction of purified LC with PL-site antibody probably represents binding of antibody to both undegraded and degraded species of LC, as seen by Andersson et al. (7) during preparation of this type of purified material.

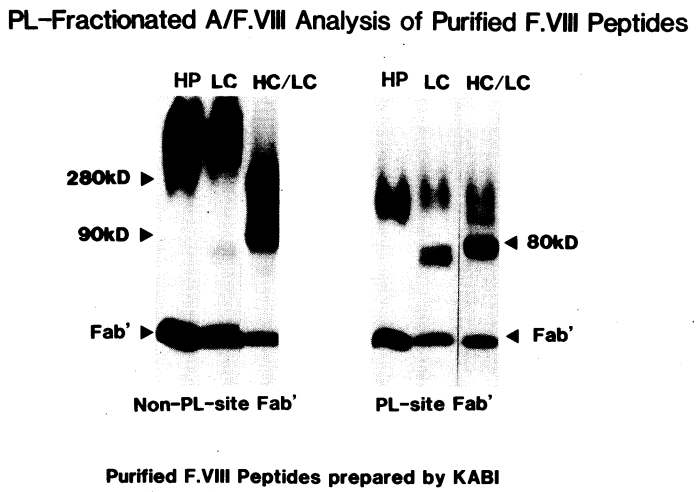

Fig. 4 Autoradiograph of SDS-polyacrylamide gel showing F.VIII peptide-^{125}I-Fab' patterns obtained by complexing of severe haemophilic plasma (HP), whole F.VIII (HC + LC) and light chain alone (LC) with non-PL-site or PL-site antibody

No specific bands in the region 80–280 kDa were seen in the CRM-ve severe haemophilic plasma sample; however, diffuse darkening of the autoradiograph at a position above M_r 250 kDa was often seen in F.VIII-deficient samples due to PEG-enhanced self-association of ^{125}I-Fab' monomers to form high M_r

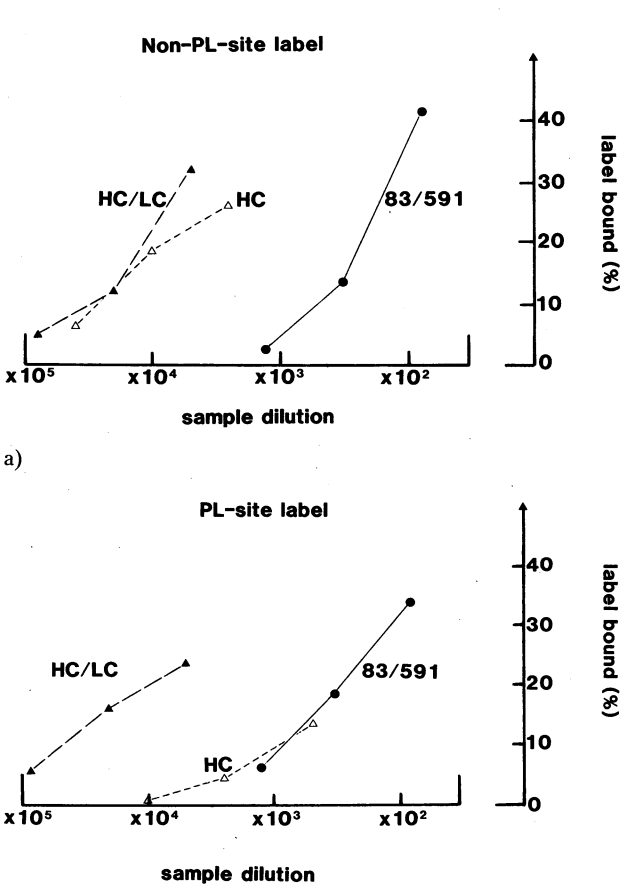

Fig. 5 IRMA assays of purified whole F.VIII (HC + LC) and HC peptides against 4th BWS Factor VIII Concentrate (NIBSC 83/591): a) with non-PL-site ^{125}I-Fab' as tracer; b) with PL-site ^{125}I-Fab' as tracer

Table 1 Approximate F.VIII:Ag levels in purified F.VIII peptides

	Tracer	
	Non-PL-site	PL-site
HC + LC	250 iu/ml	200 iu/ml
HC	200 iu/ml	5 iu/ml

aggregates. If PEG was omitted from the sample buffer, or if F.VIII:Ag levels of >0.5 iu/ml in the sample were present, this nonspecific association was reduced or abolished.

Immunoradiometric Assays (IRMA's) of Purified F.VIII

Highly purified preparations of F.VIII (HC + LC) and HC peptides were assayed by IRMA against the 4th British Working Standard for Factor VIII, Concentrate (NIBSC 83/591) using both of the antibody pools as tracer. Unfortunately, purified LC peptides were unavailable for this part of the study. The dose-response curves obtained are shown in Figs. 5a and 5b.

Using the non-PL-site label, the antibody-binding curves were similar for both whole F.VIII (HC + LC) and HC, indicating that both these samples contained similar levels of the epitopes recognised by this antibody pool (Fig. 5a). With the PL-site directed antibody, however, a quite different result was obtained: the HC + LC mixture gave a strong response as with non-PL-site Fab', but HC had a fifty-fold lower response, clearly showing that this latter sample lacked the epitope recognised by PL-site antibody (Fig. 5b). The approximate F.VIII:Ag levels measured by these assays are shown in Table 1.

These IRMA's demonstrate that, although purified F.VIII containing a mixture of purified heavy and light chains of F.VIII possesses PL-binding sites, purified heavy chains do not: thus the light chain of Factor VIII must carry this functional site.

Discussion

The intention of the work presented here was to localise the PL-binding region of the F.VIII protein to one or more of the F.VIII peptides, by means of the two immunological techniques utilising labelled antibodies of well-defined specificity (9).

The electrophoretic technique, although qualitative, has the great advantage that samples of widely-differing purity may be examined after minimal processing; thus peptide patterns obtained with normal or deficient plasmas were compared directly with those from F.VIII concentrates and highly purified F.VIII peptides. Further, the dependence of the results on epitope specificity of the labelled antibody ensures that only the peptides of interest are visualised. The interpretations made are dependent on the assumption that there is predominantly a 1:1 stoichiometry of binding between F.VIII peptides and Fab' fragments: this has been shown in our laboratory and elsewhere (10) by experiments in which the ratios between peptides and labelled Fab' have been varied widely with no change in the pattern following autoradiography.

A drawback preventing precise analysis of the molecular mass of a peptide visualised by this technique is that the gels are run under mild non-denaturing and non-reducing conditions (since immunocomplexes would dissociate otherwise); hence the M_r's are apparent and approximate only, and tend to be more inaccurate as peptide size increases. However, the strong similarities seen (with or without thrombin treatment) between both PL-site-identified peptides with F.VIII:LC, and non-PL-site peptides with F.VIII:HC patterns on reduced gels provide strong evidence for PL-site location. In addition, the analysis of highly purified F.VIII fragments by the electrophoretic method provides further confirmation of this conclusion, with PL-site antibody binding to a sample containing LC alone.

PL-site antibody did not react with the range of higher M_r peptides present in plasma or highly-purified F.VIII (Figs. 1 and 4), indicating that it is not directed at HC peptides. The results from thrombin treatment of F.VIII concentrate indicate that the PL-binding site is probably conserved following cleavage of the 80 kDa LC to its lower M_r form.

More quantitative evidence for this epitope assignment was provided by the immunoradiometric assays of purified F.VIII peptides: clearly an HC preparation was almost completely deficient in PL-binding sites, while purified whole F.VIII containing both HC and LC was not; some of this work has appeared elsewhere (28). Further studies of direct F.VIII binding to PL vesicles comparing isolated LC and HC peptides are intended: it has not been possible to carry out direct comparisons between both isolated HC and LC in this study.

Apparent homology between human Factors VIII and V has been noted: in cofactor activity (15), native structure (16, 17) and in the sizes of their thrombin-cleaved subunits (18). So far, PL-binding studies of human F.V are not well advanced, but studies using the bovine protein have confirmed that the PL-binding site of the thrombin-activated protein (90 kDa HC + 80 kDa LC) is on the LC peptide (19–21) which corresponds structurally to the 80 kDa LC of human F.VIII. Thus the assignment of the PL-binding site to the light chain of human F.VIII continues the striking similarities between the two proteins.

The higher M_r peptide with an apparent mass of 160 kDa visualised by some preparations of PL-site antibody may be a thrombin-resistant remnant of initial proteolytic processing of the F.VIII native single chain molecule. Toole et al. (22) reported an LC-containing peptide of approximate M_r 160 kDa formed by cleavage of native F.VIII at Arg 1313 inside the B region of factor VIII, and this may be the peptide seen here. Alternatively, the band may consist of a complex between LC and two Fab' fragments: since LC contains a duplicated C domain (4) each of which may contain PL-binding sites, the domains might each bind one Fab' fragment.

Finally, it should be noted that these studies have been performed with antibodies fractionated by the use of a complex of Factor VIII with artificial PL vesicles (9); these antibodies are therefore only those directed against binding-sites on Factor VIII for such PL vesicles. It has been shown for activated Factor V that the binding of the protein for platelets is considerably stronger than for artificial PL vesicles (23, 24), implying that other interactions besides that of PL with LC binding site are important for the binding of activated F.V. Further, it has been reported that assembly of the F.IXa-F.VIII complex is more rapid in the presence of washed aggregated platelets than with phospholipid vesicles (25, 26). Therefore, although a recent report (27) has confirmed that the binding of F.VIII to activated platelets is via the PL head-groups, it is likely that other interactions between Factor VIII and a platelet surface are important in its binding, in addition to that between a PL surface and the PL-binding site identified here.

Note

A report has just been published (29) suggesting that the carboxyterminal (LC) region of F.VIII mediates F.VIII binding to phosphatidyl serine coated onto ELISA plates.

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