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DETERMINATION OF THE DOMAINS OF FACTOR VIII ESSENTIAL FOR PRO-COAGULANT ACTIVITY. M.Ph. Verbeet[1], R.F. Evers[1], A. Leyte[1], H.L. Lamain[2], A.J.J. van Ooyen[2], J.A. van Mourik[1] and H. Pannekoek[1]. [1]Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands and [2]Gist Brocades, Delft, The Netherlands.

Factor VIII (FVIII) consists of an obvious domain structure that can be represented as A1-A2-B-A3-C1-C2 (Vehar et al., 1984, Nature 312, 337). In order to determine the domains involved in the procoagulant activity of FVIII, we constructed mutant FVIII cDNAs containing deletions in the coding sequence of the full-length molecule. In one of the mutants a large part of the B domain is deleted. In another one we made a deletion in the B domain that extends beyond the thrombine cleavage site. We used pSV-2 derived expression vectors and COS-1 cells in a transient expression system for the full-length and mutant recombinant proteins. Conditioned media (CM) were harvested.

In accordance with the described mutants of recombinant FVIII (Toole et al., 1986, PNAS 83, 5939), we demonstrated an increase in activity in the CM for these mutants as compared to the full-length activity. We also found that the specific activity of the mutants is similar to that of plasma FVIII. So, shorter chains lead to an increased amount of procoagulant protein.

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THE FUNCTIONAL DOMAINS OF COAGULATION FACTOR VIII

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The functional domains of Factor VIII have been investigated using site-directed mutagenesis to probe the effect of thrombin cleavage on pro-cofactor/cofactor activity. We have previously shown that clotting activity is obtained upon coexpression of the amino terminal (92 kDa) heavy chain and carboxyl terminal (80 kDa) light chain proteolytic cleavage products as individual, secreted proteins without the 909 amino acid central region (Burke et.al., J. Biol. Chem. 261, 12574, 1986). In the present work the thrombin cleavage sites in the heavy and light chains previously characterized by others (D. Eaton et.al., Biochemistry 25, 505, 1986) have been modified to remove these sites and the mutagenized gene reassembled into separate expression vectors for the two chains. Coexpression of wild type and mutant proteins in COS-7 cells has been characterized by coagulant activity, immunological assays specific for each of the two chains, and radioimmuno-precipitations. Alteration of the thrombin cleavage site in the heavy chain (Arg-372->ΔArg-372) leads to loss of coagulant activity, whereas another mutant Arg-372->Lys-372 shows 20-fold reduced activity. Radioimmunoprecipitations and RIA data show that this is not a reflection of reduced synthesis or increased degradation of the mutant polypeptides. These results suggest that Arg-372 is required for the efficient folding, assembly, or proteolytic activation of Factor VIII.

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THE PHOSPHOLIPID-BINDING SITE OF FACTOR VIII IS LOCATED ON THE 80 kD LIGHT CHAIN. G. Kemball-Cook (1), S.J. Edwards (1), K. Sewerin (2), L.-O. Andersson (2) and T.W. Barrowcliffe (1). National Institute for Biological Standards and Control, Hampstead, London, U.K. (1) and KabiVitrum AB, Stockholm, Sweden (2).

The binding of Factor VIII (F.VIII) peptides to phospholipid (PL) vesicles has been studied by two different methods involving the use of fractionated anti-F.VIII:C ^{125}I-Fab'. As previously reported, ^{125}I-Fab' was fractionated by immunoadsorption with F.VIII-PL complexes into two pools: one binding only to PL-binding sites on F.VIII:Ag (PL-site antibody), the other directed against other antigenic sites (non-PL-site antibody).

The first technique used was a modification of the method of Weinstein et al. (Proc.Natl.Acad.Sci.USA, 78, 5137-5141, 1981), and involved incubation of the two anti-F.VIII ^{125}I-Fab' pools with F.VIII-containing samples, followed by electrophoretic separation of the complexes on the basis of size in non-denaturing SDS gels: this technique allows qualitative analysis of antibody reactive peptides in highly impure samples. Non-PL-site pool reacted with a range of peptides with apparent M_r 90 kD up to 280 kD, a similar pattern to that of 'heavy chain' (HC) peptides of F.VIII seen on SDS-PAGE under reducing conditions; the PL-site antibody, however, reacted only with peptides at apparent M_rs of 80 kD and sometimes 150 kD, but not with bands of higher M_r, a pattern more consistent with binding to light chain (LC) peptides. The same patterns with the two labels were seen in both plasma and F.VIII concentrate.

The second approach employed the two labels described above in direct immunoradiometric assays (IRMA's) on purified human F.VIII peptides prepared by immunoaffinity chromatography and ion exchange on Mono Q gel. Both PL-site and non-PL-site labels measured similar amounts of F.VIII in a sample containing both HC and LC peptides; however, on assaying a sample containing purified HC peptides alone, PL-site antibody measured only 2% of F.VIII:Ag found by non-PL-site label, indicating that PL-binding sites present in samples containing both HC and LC are absent in HC alone.

Results from both these immunological methods indicate that the 80 kD LC peptide of F.VIII carries the PL-binding site.

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THE INTERACTION OF PURIFIED FACTOR VIII WITH PLATELETS.

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Factor VIII is known to interact with Factors IXa and X to generate activated Factor X. A requirement for phospholipid in this reaction suggests that this "tenase" protein complex is assembled on a membrane surface. As a first step in studying the involvement of Factor VIII in this process, we wished to determine whether purified Factor VIII could interact directly with platelets. Factor VIII utilized in these experiments was purified from heparinized blood by a six-stage procedure including cryoprecipitation, polyethylene glycol precipitation, Affi-Gel Blue, Aminohexyl, polyelectrolyte E5 and immunoaffinity chromatography. This yielded a single-chain high molecular weight species of approximately 260,000 (specific activity 5,200 units/mg). This homogeneous protein was then radiolabelled with Na^{125}I by a procedure which allowed the retention of approximately 60-80% of the procoagulant activity of Factor VIII. The kinetics of binding of ^{125}I-Factor VIII to washed platelets at physiological concentration (approximately $3x10^8$/mL) was examined. Our results showed that for Factor VIII concentrations between 0.38 and 3.0 ng/mL there was a linear uptake of radiolabelled Factor VIII, whereas for concentrations above 10 ng/mL only a slight increase in uptake occurred. To further define the association of purified Factor VIII with the platelet membrane, we also labelled Factor VIII with a bifunctional, photoactivatable cross-linking reagent, N-[4-(p-azido-m-[^{125}]iodophenylazo)benzoyl]3-aminopropyl-N^1-oxysuccinimide ester. Analysis by PAGE showed that this reagent reacts predominantly with residues in the light chain or near the C-terminal portion of Factor VIII. When mixed with thrombin-stimulated platelets, the cross-linked Factor VIII molecule was shown to transfer greater than 80% of the ^{125}I label to a polypeptide of M.W. 80,000-90,000 isolated from platelet lysates. Autoradiographs of the labelled platelet preparations demonstrated that other minor polypeptides were radiolabelled. These experiments suggest that Factor VIII interacts closely with a platelet membrane protein which could represent a binding site for Factor VIII.