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Can we improve on nature? "Super molecules" of factor VIII

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Summary. Treatment of haemophilia A requires frequent infusion of plasma- or recombinant-derived factor VIII. This regimen is limited due to the high cost and inconvenient access to peripheral veins. In addition, patients frequently develop inhibitory antibodies that limit available therapeutic regimens. Two major advances in factor VIII research over the past 15 years were the ability to isolate homogeneous preparations of factor VIII and the isolation of the factor VIII gene that provided for a detailed biochemical and structural characterization of the factor VIII molecule. With an increased understanding of the requirements for factor VIII function, studies have attempted to produce improved factor VIII molecules for replacement therapy. These findings have produced forms of factor VIII that are more efficiently produced, that are less immunogenic, and that have higher specific activity. The future will see the engineering of novel factor VIII molecules with increased therapeutic efficiency while minimizing inhibitor antibody development. In addition, there are now structural models of factor VIII available that should in the future direct development of novel peptidomimetics that may eventually overcome the requirement for replacement therapy with factor VIII protein.

Keywords: Factor VIII, thrombin, activated protein C, inhibitor antibodies.

Haemophilia A is an X-chromosome-linked bleeding disorder affecting 1/5,000 males that results from a deficiency or abnormality in the plasma protein, factor VIII. Although the crucial role of factor VIII in haemostasis was realized in 1937 [1], a detailed biochemical and structural characterization of factor VIII was only initiated within the last 15 years. In the past, treatment of haemophilia A involved frequent infusion of preparations of factor VIII concentrates derived from human plasma. Although this replacement therapy is effective in controlling bleeding episodes, significant problems exist. First, patients are at risk of blood-borne virally transmissible diseases. The risk of virus infection was significantly reduced by monoclonal antibody purification of factor VIII from human plasma and the development of recombinant-derived factor VIII. However, these improvements have greatly increased the cost of treatment. Second, due to the high cost of factor VIII and the limited access to peripheral veins, patients are generally treated episodically on a demand basis as opposed to prophylactically. A consequence of this therapeutic regimen is chronic bleeding into the joints leading to tissue damage later in life. Finally, about 15% of patients develop inhibitory antibodies to factor VIII. It is likely that solutions to the present limitations in haemophilia therapy will result from further advances in our knowledge about factor VIII. Recombinant DNA technology now provides the ability to design specific changes into the factor VIII gene to derive novel and improved forms of factor VIII. The ability to engineer factor VIII with specific alterations has led to a greater understanding of the regulation of factor VIII expression and its activity and now provides avenues to engineer factor VIII to produce improved proteins for therapeutic use.

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Factor VIII structure and function

Factor VIII functions in the intrinsic pathway of blood coagulation as a cofactor to accelerate the activation of factor X by factor IXa that occurs on a phospholipid surface in the presence of calcium ions. The factor VIII amino acid sequence deduced from the cloned cDNA identified that the molecule is synthesized as a single-chain polypeptide having the domain structure A1-A2-B-A3-C1-C2 [2, 3] and upon secretion from the cell is processed to a heterodimer consisting of a carboxy-terminal derived light chain of 80 kDa in a metal-ion

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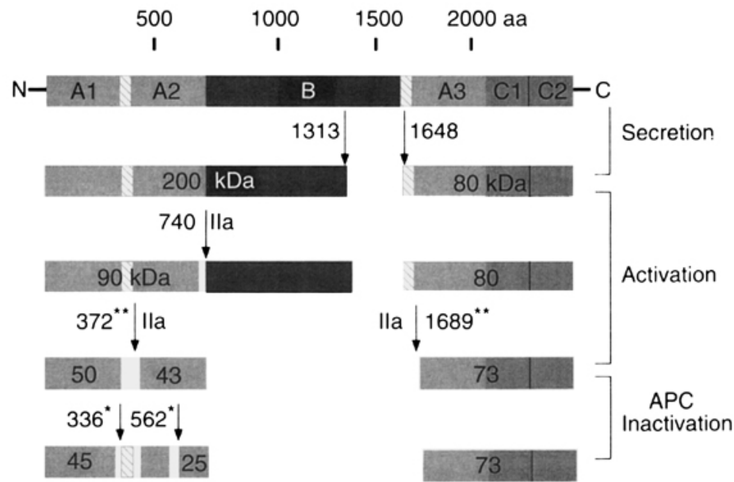

Fig. 1. Domain structure and processing of factor VIII. The structural domains of factor VIII are depicted: A1 domain (1-336), A2 domain (372-740), B domain (740-1648), A3 domain (1690-2020) and the C domains (2020-2332). Three regions rich in acidic amino acid residues between domains A1 and A2, A2 and B, B and A3 are indicated by hashed shading. Intracellularly, factor VIII is cleaved within the B-domain to generate a 200 kDa peptide and the 80 kDa light chain. The two cleavages required for thrombin activation are indicated by **. Sites of activated protein C cleavage are indicated by *.

dependent association with a 200 kDa amino-terminal derived heavy chain fragment (Fig. 1). The domain structure of factor VIII is identical to that of the homologous coagulation factor, factor V [4, 5]. The A domains within factor VIII have 40% amino acid identity with each other and to the A domains of factor V, as well as with the copper binding protein ceruloplasmin [6], suggesting the A domains may be involved in metal ion binding. The C domains exhibit 40% identity to the C domains of factor V, and with proteins that bind glycoconjugates and negatively charged phospholipids [7]. The B-domain is encoded by a single exon and exhibits little homology to the factor V B domain [8, 9]. In plasma, the factor VIII light chain is bound by non-covalent interactions to a primary binding site in the amino terminus of von Willebrand factor (vWF).

The observation that haemophilia A offers protection from ischaemic heart disease [10] and suggestions that elevated factor VIII may be associated with thrombotic disease [11] provides an incentive to understand the mechanism by which factor VIII levels are regulated in plasma. In plasma, vWF circulates as a heterogeneous multimer comprised of 2 to 100 subunits. Although each monomeric vWF molecule contains one factor VIII binding site, *in vitro* binding studies yielded conflicting data for factor VIII:vWF monomer ratios of 1:1 [12], 1:4 [13], 1:10 [14] to as low as 1:70 high affinity binding sites [15, 16]. As different reagents, protein concentrations, and assays were used for these studies, the source for the difference remains unknown. However, the ratio of circulating factor VIII to vWF observed *in vivo* is tightly maintained at 1:50 [17]. Any change in plasma vWF level is coupled with a concordant change in the factor VIII level. The infusion of vWF into vWF deficient patients immediately elevates factor VIII levels to above those observed in normal individuals [17–20]. The presence of vWF increases the plasma half-life of factor VIII from 2–3 h to 12–14 h [21, 22]. Since factor VIII clearance from the circulation is dependent on the FVIII-vWF interaction, it is unlikely that it will be possible to engineer a factor VIII molecule that retains both vWF binding affinity and increased plasma half-life.

In vitro studies demonstrated that vWF regulates factor VIII activity through additional mechanisms: (1) vWF prevents activation of factor VIII by factor Xa [23], whereas it has no effect on activation of factor VIII by thrombin [24, 25]; (2) vWF prevents inactivation of factor VIII by activated protein C [26, 27]; (3) vWF prevents binding of factor VIII to phospholipids [28, 29] and to thrombin-activated platelets [14]; and (4) vWF is required to promote stable accumulation of factor VIII upon secretion into the medium when factor VIII is expressed in mammalian cells in culture [30–32]. Although primary interactive binding sites within the factor VIII light chain (residues 1680–1689 [33, 34] and the C2 domain [35, 36]) and vWF (residues 1–272 [37–39]) have been identified, there are likely multiple contacts that are required to mediate the multitude of effects that vWF has on factor VIII.

In vivo, factor VIII activity is regulated by proteolytic activation as well as inactivation. Upon thrombin activation of factor VIII there is a rapid 30-fold increase and subsequent first-order decay of procoagulant activity. The activation coincides with proteolysis of both the heavy and light chains of factor VIII and release from vWF (Fig. 1) [40–42]. Cleavage within the heavy chain after arginine residue 740 generates a 90 kDa polypeptide that is subsequently cleaved after arginine 372 to yield 50 and 43 kDa polypeptides. Concomitantly, the 80 kDa light chain is cleaved after arginine residue 1689 to generate a 73 kDa polypeptide. Thus, thrombin-activated factor VIII is composed of a heterotrimer of the 50, 43, and 73 kDa fragments [43–45].

Factor VIII and factor VIIIa are both inactivated by

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activated protein C cleavage after residues 336 [40, 46] and/or 562 [47], and this mechanism of inactivation appears physiologically significant since protein C deficiency is associated with thrombotic events [48, 49]. However, the first order decay of procoagulant activity for thrombin-activated factor VIIIa observed *in vitro* does not correlate with any specific proteolytic event [50, 51]. A detailed characterization of thrombin-activated factor VIIIa was hampered due to its marked instability. Protein concentration and pH are important factors for isolation of stable thrombin-activated factor VIIIa [43, 52]. However, presently it is not possible to isolate a stable preparation of human factor VIIIa at physiological pH and concentration that would be suitable for functional analysis in biochemical and biological assay systems. Most data support the conclusion that loss of procoagulant activity after thrombin activation results from a reversible dissociation of the 43 kDa A2-domain polypeptide from the heterotrimer which occurs at physiological pH [44, 45, 52, 53]. The specific activity of porcine factor VIIIa, depending on its concentration, is 2–10-fold higher than human factor VIIIa and this correlates with a lower dissociation rate constant of the A2-domain polypeptide with the thrombin-activated heterotrimer [52, 53].

Potential for improved factor VIII molecules through genetic engineering

Recombinant DNA technology provides unique approaches that may yield improved therapeutic regimens for haemophilia A. These approaches include: (1) alterations that improve factor VIII expression, thereby reducing potential cost of treatment and making prophylactic treatment feasible; (2) alterations that reduce factor VIII immunogenicity, thereby reducing potential for inhibitor antibody development; (3) alterations that improve factor VIII specific activity or increase the half-life of activated FVIII in plasma, thereby reducing the amount of protein required for therapeutic use; and (4) development of oral acting compounds that mimick the action of factor VIII. Each of these approaches will be discussed in turn.

Alterations that improve factor VIII expression

Although most evidence supports that the hepatocyte is the cell type that produces factor VIII *in vivo* [54–58], there are no known established or primary cell lines that express factor VIII. Thus, our knowledge of factor VIII expression is derived from interpretation of results from expression of the cDNA from expression vectors in transfected mammalian cells. Expression of factor VIII in these transfection systems is 2–3 orders of magnitude lower than that observed with other genes using similar vectors and approaches. Studies have identified at least three reasons for the low level of expression [59]: (1) the factor VIII mRNA is inefficiently expressed, (2) the primary translation product is inefficiently transported from the endoplasmic reticulum (ER) to the Golgi apparatus, and (3) high levels of vWF are required in the conditioned medium to promote stable accumulation of factor VIII.

Factor VIII expression upon transfection or infection of cells in culture is limited in the ability to produce factor VIII mRNA and to efficiently secrete factor VIII protein from the cell. One of the most significant observations concerning the ability to improve factor VIII expression was that deletion of the middle 1/3 of the coding region, the B-domain, yielded a molecule that was expressed at significantly greater levels than wild-type factor VIII [60]. The increased expression was attributed to a greater increase in the factor VIII mRNA level and corresponding factor VIII protein synthesis [25]. One of these B-domain deleted forms of factor VIII (termed Refacto) is now presently under study in clinical protocols in humans. Refacto has a 2–3-fold increased specific activity over wild-type recombinant factor VIII. Results of preliminary preclinical and clinical studies were recently presented in August of 1996 [61]. The pharmacokinetic parameters were indistinguishable from wild-type factor VIII upon infusion into animal models as well as human patients. However, there was a slightly greater volume of distribution for the B-domain deleted factor VIII, most likely due to its smaller size. Preliminary clinical studies demonstrated an excellent response upon infusion into 87 previously treated haemophilia A patients with no detectable inhibitor development or adverse reactions. At the time of reporting, 43 previously untreated patients were treated with Refacto with an incidence of inhibitor development not significantly different than studies with recombinant wild-type factor VIII. Although preliminary, these studies suggest that deletion of 1/3 of the factor VIII molecule does not significantly change its *in vivo* pharmokinetic, immunological, or functional properties and demonstrates the feasibilty to produce improved factor VIII molecules. The potential benefits from Refacto include a formulation that will be human serum albumin free, a smaller injection volume, infusions containing less protein, and finally, because of its smaller size, the potential to develop a continuous delivery formulation that can be used prophylactically.

Although proteins can fold into correct tertiary conformations *in vitro* [62], additional factors such as protein chaperones are required to assist protein folding *in vivo*. Our work over the past 10 years demonstrated that the inefficient secretion of factor VIII correlated with interaction with the protein chaperone identified as the immunoglobulin binding protein (BiP) which is the same

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as the glucose-regulated protein of 78 kDa (GRP78) [63] within the lumen of the ER [64, 65]. BiP is a member of the heat-shock protein family which exhibits a peptide-dependent ATPase activity [66] and for which expression is induced by the presence of aberrantly folded protein or unassembled protein subunits within the ER [67, 68]. Factor VIII release from BiP and transport out of the ER required high levels of intracellular ATP [69]. In contrast, the homologous coagulation protein, factor V, did not detectably associate with BiP and did not require high levels of ATP for secretion [70]. Through expression of chimeric cDNAs, it was possible to localize the sequences within factor VIII that inhibit secretion. Exchange of a 110 amino acid region within the A1-domain improved secretion of the molecule and this chimeric protein displayed a reduced interaction with BiP [71]. However, the secreted protein was not active, and this correlated with dissociation between the heavy and light chains. Mutation of single residues within this region identified that a single amino acid change at Phe309 to Ser (the homologous residue present in factor V) improved factor VIII secretion by 3-fold; however, the secreted protein had a specific activity indistinguishable from wild-type factor VIII [72]. In addition, the Phe309Ser mutant factor VIII displayed a reduced requirement for ATP for secretion, suggesting a reduced interaction with BiP. These results demonstrate that mutation of a single residue in factor VIII can influence chaperone interaction to improve the secretion of factor VIII. The findings provide needed information on what sequences are responsible for BiP binding, and will also have practical importance for improving factor VIII expression. The increased expression will facilitate the eventual goal of somatic cell gene therapy for haemophilia A.

Alterations that reduce immunogenicity of factor VIII

One of the major limitations with present replacement thereapy is the development of inhibitor antibodies to factor VIII. Once inhibitor antibodies develop, several strategies are available to provide effective haemostasis. These include the use of porcine factor VIII, bypass therapy with recombinant factor VIIa, and high dose factor VIII with or without immunosuppressive agents in order to induce a state of nonresponsive tolerance. However, it should also be possible to engineer factor VIII to make it less immunogenic. One particular strategy could involve the modification of factor VIII to prevent exposure of antigenic epitopes. For example, covalent modification by polyethylene glycol attachment to lysine residues can both reduce immunogenicity and increase plasma half-life [73]. This is most dramatically demonstrated for modification of adenosine deaminase for the treatment of severe combined immunodeficiency disease [74]. However, to date there are no reported results on the successful modification of factor VIII by polyethylene glycol attachment.

An alternate strategy would be to produce a less immunogenic factor VIII through genetic engineering. This first requires identification of the regions within factor VIII that elicit an immune response and then selective modification of those regions by mutagenesis. Studies have established that the most common factor VIII epitopes that induce inhibitory antibodies are localized to the A2 domain (residues 373–740) and the C2 domain (residues 2173–2332) of factor VIII [75–77]. An additional epitope has also been identified that may be localized to the A3 domain [78, 79]. Since porcine factor VIII can be used to treat inhibitor patients [80, 81], it is likely that the epitopes recognized by anti-human factor VIII inhibitory antibodies are not present in porcine factor VIII. This observation provided the impetus for Lollar and coworkers to prepare human and porcine factor VIII chimeric molecules in order to elucidate what amino acids are responsible for the antigenic differences between human and porcine factor VIII. The results identified a limited number of residues between 484 and 508 within the A2 domain of human factor VIII that contribute significantly to the antibody response [82].

Once critical residues are identified that are responsible for the immunogenicity of human factor VIII, it is possible to alter those residues in the hope of reducing the immunogenicity. In particular, residues that present as strong epitopes for immune response are frequently surface exposed and either positively or negatively charged. By mutation of those amino acids that have charged side chains to alanine, an amino acid that lacks side chains, it may be possible to reduce the immunogenicity of factor VIII. The feasibility of this concept was recently tested by mutation of those residues in the human factor VIII A2 domain that were implicated to elicit inhibitory antibodies to alanine (Fig. 2) [83]. Resultant molecules retained procoagulant activity, but did demonstrate significantly reduced inhibition to an inhibitory monoclonal antibody that reacts with wild-type human factor VIII. This alanine scanning mutagenesis approach identified Tyr487 as a residue that is critical for the recognition by anti-human factor VIII A2-inhibitor antibodies [83]. Once all the immunogenic regions are identified, it may be possible to mutate all the relevant amino acids and yield a molecule with markedly reduced immunogenicity that retains full functional activity.

Alterations that increase the specific activity of factor VIII

The instability of thrombin-activated factor VIII

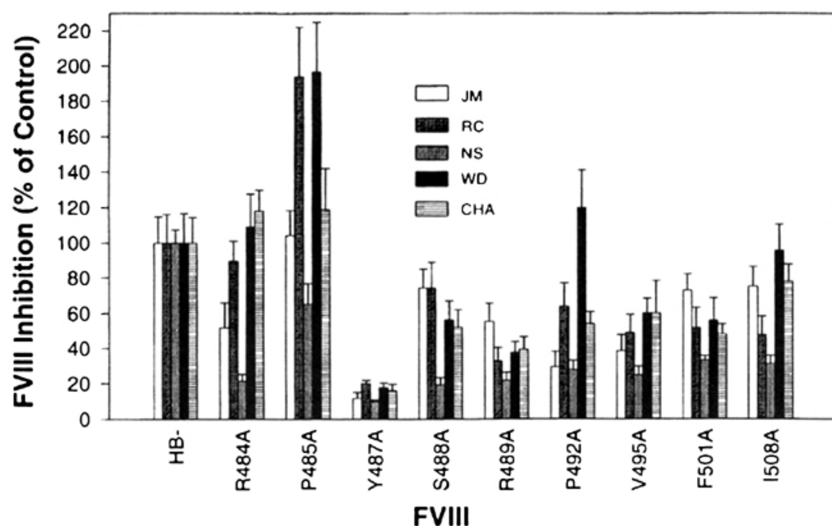

Fig. 2. Alanine-scanning mutagenesis of factor VIII inhibitor epitope. Mutagenesis and expression of B-domain deleted factor VIII was performed and analyzed for inhibition by five different patient inhibitor plasmas as measured by Bethesda assay. For details see [83].

correlates with dissociation of the A2-domain subunit. Therefore, it was considered feasible to derive a more active form of factor VIII if it were possible to minimize dissociation of the A2-domain subunit. To test this hypothesis, experiments were designed to create a form of factor VIII in which the A2-domain was covalently attached to the light chain.

The basis for this approach relied on two observations. First, characterization of cleavage site mutant factor VIII demonstrated that cleavage at residues 372 and 1689 were the only cleavages required for activation of factor VIII activity [84]. Cleavage after 372 was proposed to alter the conformation of the molecule in a manner necessary for cofactor activity [84], whereas cleavage after 1689 was proposed to be required to liberate factor VIIIa from vWF [85] and permit factor VIIIa to interact with negatively charged phospholipids. The second observation was that deletion of residues 741–1689 yielded a molecule (termed 90/73) that displayed significantly reduced binding to vWF and displayed procoagulant activity similar to wild-type factor VIII after treatment with thrombin. Following cleavage by thrombin, the 90/73 factor VIII yielded the 50 kDa/43 kDa/73 kDa heterotrimer that was identical to wild-type factor VIII [86] (Fig. 3A). These observations suggested that if the cleavage between the 740–1690 junction in the 90/73 molecule was prevented, then it should be possible to yield a dimeric factor VIIIa through cleavage by thrombin after residue 372 in which the A2-domain would be covalently attached to the light chain. This molecule may not require cleavage at the amino terminus of the light chain before residue 1690 for activation because it would display significantly reduced binding to vWF. However, when the Arg740 at the junction of the 90kDa and 73 kDa chains was mutated to Lys, the site was not cleaved by thrombin, however the resultant molecule was not active. It was then proposed that the A2-domain may require a spacer so as to attain a conformation that was suitable to develop procoagulant activity. Subsequently, a 54 amino acid spacer from residues 741-794 was inserted and it was observed to yield a molecule that retained factor VIII activity [86]. In order to further increase resistance to inactivation, the resultant molecule was made resistant to cleavage by activated protein C by introducing both Arg336Ile and Arg562Lys mutations. These two mutations were previously shown to inhibit activated protein C inactivation of factor VIII, without affecting its procoagulant activity [87]. The resultant molecule, termed inactivation resistant factor VIII (IR8), had a 5-fold greater specific activity than wild-type factor VIII when measured in a one-stage clotting assay using factor VIII deficient plasma (Fig. 3B). In addition, IR8 displayed 38% of peak activity at 4 hr after activation by thrombin under conditions in which wild-type factor VIII that was completely inactivated after 5 min (Fig. 3C). The results demonstrate the feasibility to produce a form of factor VIII that has elevated specific activity in an *in vitro* clotting assay and that is resistant to inactivation that occurs after thrombin activation and by treatment with activated protein C.

To further characterize the *in vivo* activity of IR8, the factor VIII genetically deficient haemophilic mouse was used [88]. This haemophiliac mouse cannot survive a tail bleed induced by a guillaotine device to remove the last 1 cm of the tail. When as low as 20 ng of wild-type recombinant factor VIII was infused into the mouse tail vein prior to the induced tail bleed, the animal survived (Fig. 4). The reproducibility in survival detected upon recombinant wild-type factor VIII infusion is compromised by difficulty in ensuring the injected factor VIII actually gets into the circulation. However, this model does provide a stringent test for the ability of a given preparation of factor VIII to correct a tail bleed in the

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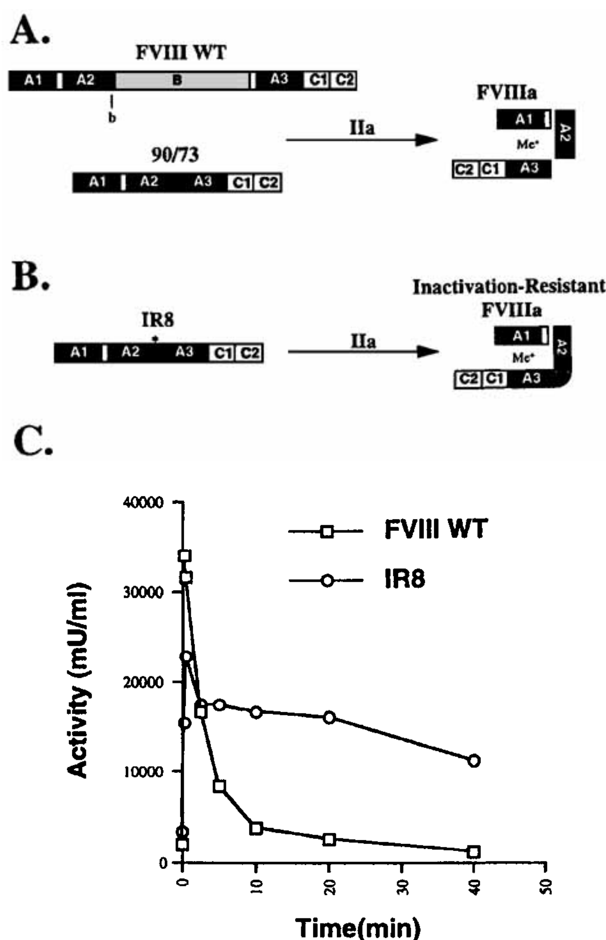
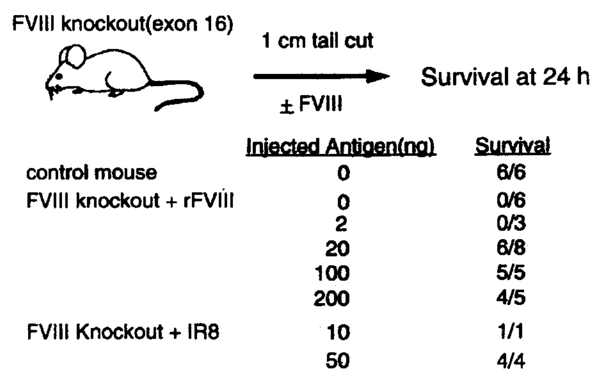


Fig. 3. Inactivation resistant factor VIII. Panel A. Structural domains of factor VIII wild-type (WT) and B-domain deleted factor VIII (90/73) and their predicted factor VIIIa heterotrimeric structure after thrombin (IIa) cleavage. Me+ represents a metal ion necessary for A1 and A3 domain association. Panel B. A representation of IR8 and its predicted heterodimeric subunit structure that results after thrombin activation. *indicates the missense mutation at residue 740 predicting resistance to thrombin cleavage. b indicates 54 amino acids of B-domain retained in the IR8 construct. White boxes represent acidic amino acid rich regions. Panel C. Activation of wild-type and IR8 factor VIII by thrombin. Partially purified proteins (1 nM) were treated with 1 unit/ml thrombin at room temperature and assayed over time for factor VIII activity by the activated partial thromboplastin assay. See [86] for details.

mouse. When IR8 was purified and infused into the haemophilic mouse model, the mouse was able to survive the lethal consequences of the tail bleed. Although we do not know the half-life of IR8 infused into the mouse, it is likely to be significantly shorter than wild-type factor VIII because of its reduced ability to bind vWF. Therefore, these functional data strongly support that the IR8 molecule is at least as effective as wild-type factor VIII in this mouse model system.



	Injected Antigen(ng)	Survival
control mouse	0	6/6
FVIII knockout + rFVIII	0	0/6
	2	0/3
	20	6/8
	100	5/5
	200	4/5
FVIII Knockout + IR8	10	1/1
	50	4/4

Fig. 4. IR8 displays coagulant activity *in vivo*. Purified wild-type recombinant human factor VIII(rFVIII) or IR8 were infused into the tail vein of anaesthetized genetically deficient mice (factor VIII Exon 16 knockout). One minute following infusion a 1 cm terminal section of the tail was cut. Mice were then observed for evidence of effective haemostasis over a 24 h period. Mice that failed to clot received tail cauterization or, where appropriate, were euthanized. Survivors achieved effective haemostasis in the absence of tail cauterization.

The ability to isolate a stable thrombin-activated form of human factor VIIIa will provide a crucial reagent to study the functional significance of VIIIa generation in *in vitro* as well as in *in vivo* studies. The haemostatic efficacy of the more stable thrombin-activated factor VIII heterodimer (IR8) will next be evaluated in a haemophilic dog model [89]. These studies will provide important information concerning the role of the A2-domain dissociation and vWF interaction for factor VIII function *in vivo*. In addition, factor VIII of increased specific activity may have tremendous therapeutic potential by reducing dosage requirements thereby reducing cost of therapy, and reducing the antigenic stimulation to minimize inhibitor antibody development in patients that occurs as a response to factor VIII as a foreign antigen.

Development of oral acting compounds that mimick the action of factor VIII

Our knowledge of the structural requirements for factor VIII activity has dramatically increased since the original isolation of factor VIII protein from human plasma and identification of the factor VIII gene. In addition, a crystal structure of the homologous plasma protein ceruloplasmin is now available [90] that was used to predict the structure of the factor VIII A domains [91]. Additional new insights have come from mutagenesis studies to identify critical regions for factor VIII function and from biochemical analyses to identify interacting regions between factor VIII and factor IXa. Peptide and antibody inhibition data suggest that residues 558–565 and 1778–1840 in factor VIII comprise two sites that interact with the catalytic domain and the first EGF domains of factor IXa, respectively

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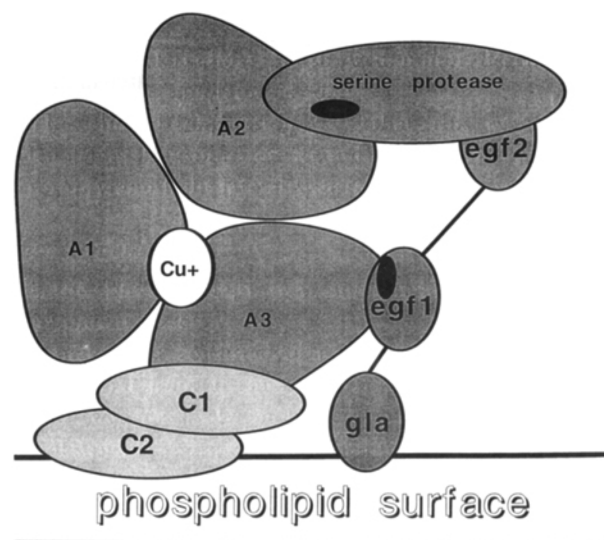

Fig. 5. Model for factor VIIIa interaction with factor IXa. This figure depicts the factor VIII domains A1, A2, A3, C1 and C2 interacting with a phospholipid surface through the C2 domain and the specific interaction of the A2 and A3 domains with factor IXa sites within the serine protease domain and the first epidermal growth factor-like domain. Factor IXa interacts with the phospholipid surface through its region that contains gamma-carboxy glutamic acid residues (gla). Adapted from [91].

[92–95]. With elucidation of the structure of factor IXa [96], it is now feasible to predict how factor VIII may function to enhance the catalytic efficiency of factor IXa. These findings have provided a model where the factor VIII light chain is responsible for complex assembly via the first EGF-like domain in factor IXa, whereas the interaction site with the A2-domain might induce a conformational change within the active site of factor IXa (Fig. 5) [91]. With greater understanding of this conformational change, it may be possible to derive a small peptide, or a peptidomimetic, that mediates the same conformational change. Once a compound is identified, it may be modified to make it more readily delivered by oral administration. These strategies provide enthusiasm for future studies to elucidate the mechanism by which a complex molecule like factor VIII can mediate a specific conformational change in the active site of factor IXa.

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