Journal of Thrombosis and Haemostasis, **1**: 922–930

REVIEW ARTICLE

The future of recombinant coagulation factors

E. L. SAENKO,* N. M. ANANYEVA,* M. SHIMA,† C. A. E. HAUSER‡ and S. W. PIPE§

*Department of Biochemistry, Jerome H. Holland Laboratory for the Biomedical Sciences, American Red Cross, Rockville, MD, USA; †Department of Pediatrics, Nara Medical University, Nara 634–8522, Japan; ‡Department of Obstetrics and Gynecology, Medical University of Lubeck, Germany; and §Department of Pediatrics, University of Michigan, Ann Arbor, MI, USA

Summary. Hemophilias A and B are X chromosome-linked bleeding disorders, which are mainly treated by repeated infusions of factor (F)VIII or FIX, respectively. In the present review, we specify the limitations in expression of recombinant (r)FVIII and summarize the bioengineering strategies that are currently being explored for constructing novel rFVIII molecules characterized by high efficiency expression and improved functional properties. We present the strategy to prolong FVIII lifetime by disrupting FVIII interaction with its clearance receptors and demonstrate how construction of human-porcine FVIII hybrid molecules can reduce their reactivity towards inhibitory antibodies. While the progress in improving rFIX is impeded by low recovery rates, the authors are optimistic that the efforts of basic science may ultimately lead to higher efficiency of replacement therapy of both hemophilias A and B.

Keywords: bioengineering, hemophilia, recombinant factor VIII, recombinant factor IX, replacement therapy.

Evolution of replacement therapy for hemophilia A

Advancements in treatment of hemophilia A, a severe X chromosome-linked bleeding disorder, have paralleled the evolution of insights into the pathophysiology of the disease and appreciation of congenital or acquired deficiency of a coagulation factor (F)VIII as the major cause of hemophilia A [1]. FVIII plays a key role in the intrinsic pathway of blood coagulation, which maintains the coagulation process by generating activated FX and, subsequently, thrombin more efficiently than tissue factor-dependent pathway [2].

Based on the observation that clotting times in hemophilia could be corrected by plasma, whole blood transfusions were first utilized for replacement therapy of hemophilia A. Later, replacement therapy progressed from the use of plasma in the 1940s to the development of plasma concentrates in the 1950s.

Correspondence: Evgueni L. Saenko, Jerome H. Holland Laboratory for the Biomedical Sciences, American Red Cross, 15601 Crabbs Branch Way, Rockville, MD 20855, USA.
Tel.: +1 301 738 0743; fax: +1 301 738 0499; e-mail: saenko@usa.redcross.org

Received 20 October 2002, accepted 19 December 2002

Hemophilia A patients further benefited from fractionation of cryoprecipitates in the mid-1960s that were enriched in FVIII and von Willebrand factor (VWF), and preparation of freeze-dried FVIII in 1968 suitable for long-term storage.

With the elucidation of the FVIII gene structures and rapid development of recombinant DNA technologies, several groups successfully expressed FVIII in mammalian cells [3–6] and clinical use of recombinant (r)FVIII began in 1989. Recombinant FVIII has become an advantageous new alternative to plasma-derived products as its development over the past two decades has significantly increased the availability of factor replacement, has reduced the risk of transmission of blood-borne pathogens and provided opportunity to treat patients prophylactically. 'Second-generation' rFVIII products are now available with marked reductions in human albumin content and 'third-generation' rFVIII products, free of any human protein exposure, are currently being tested in clinical trials. Although progress with gene transfer of FVIII should be acknowledged [7–9], replacement therapy is still the mainstay of hemophilia A care.

Development of improved FVIII molecules for replacement therapy

The high costs associated with producing rFVIII can be divided approximately equally between expression and purification from mammalian cell lines, the lyophilization process and final packaging. Expression of rFVIII within current mammalian expression systems is two to three orders of magnitude lower than that for other recombinant proteins expressed via similar strategies [10]. Several mechanisms have been identified that limit FVIII expression that include: inefficient expression of FVIII mRNA, inefficient folding of the primary translation product within the endoplasmic reticulum (ER) and a requirement for facilitated transport from the ER to the Golgi apparatus (Fig. 1). Upon intravenous infusion of rFVIII, its plasma half-life is limited by a requirement for association and stabilization within plasma by VWF. In addition, its cofactor activity is limited due to instability of the thrombin-activated form (FVIIIa) and inactivation by proteases such as activated protein C. Finally, for up to 25–30% of patients with hemophilia A, inhibitor antibodies render any infused rFVIII ineffective by a variety of mechanisms. Having now identified the limitations in current rFVIII technology, the challenge for bioengineering strategies is to overcome these limitations and construct rFVIII

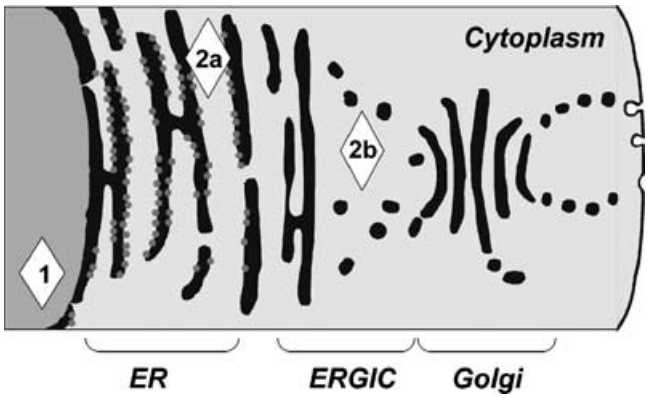

Fig. 1. Intracellular mechanisms limiting FVIII expression. FVIII mRNA is expressed in the nucleus (1). This inefficient expression can be partially overcome by deleting the exon encoding a bulky B domain. Additionally, FVIII expression can be improved by substituting the introns encoding transcriptional silencers for sequences enhancing transcription of FVIII cDNA. FVIII secretion involves the release of FVIII primary translation product from the ER (2a) and subsequent transport of FVIII protein from the ER to the Golgi apparatus (2b). FVIII release from the ER is controlled by a protein chaperone BiP, which retains misfolded protein molecules in the ER. Efficient FVIII transport to the Golgi apparatus requires FVIII interaction with a mannose-binding chaperone within the ER-Golgi intermediate compartment (2b).

molecules that would have enhanced expression, efficient secretion, prolonged plasma half-life, stability of the thrombin-activated form and reduced antigenicity and immunogenicity [11,12]. Whether novel forms of rFVIII can achieve all of these characteristics or whether individual molecules will need to be designed tailored for specific indications is not yet known. The following summarizes current state of the art technology in the bioengineering of rFVIII.

Bioengineering for more efficient expression of FVIII mRNA

FVIII is synthesized as a 2332 amino acid residues-long molecule (~300 kDa) consisting of three homologous A domains, two homologous C domains and the unique B domain, which are arranged in the order A1-A2-B-A3-C1-C2 [4,5]. Prior to its secretion into plasma, FVIII is processed intracellularly to a series of metal ion-linked heterodimers produced by cleavage at the B-A3 junction and by a number of additional cleavages within the B domain. These cleavages generate a variably sized (90–200 kDa) heavy chain (HCh) consisting of the A1 (1–336), A2 (373–719) and B domains (741–1648) and the 80 kDa light chain (LCh) composed of the domains A3 (1690–2019), C1 (2020–2172) and C2 (2173–2332). The C-terminal portions of the A1 (337–372) and A2 (720–740) domains and N-terminal portion of the A3 domain (1649–1689) contain a high proportion of negatively charged residues and are called acidic regions, *a1*, *a2* and *a3*, respectively.

The FVIII A and C domains have important procoagulant functions including the regions responsible for interaction with FIXa and FX, VWF and the phospholipid surface. The B domain, constituting approximately 38% of the primary cDNA sequence of FVIII, is encoded by a single exon and contains a large number of asparagine (N)-linked oligosaccharides and serine/threonine-linked oligosaccharides [13,14]. The discovery that the B domain can be removed without loss of FVIII procoagulant activity [15] was a breakthrough in improving the yield of rFVIII. B-domain deleted (BDD)-rFVIII was observed to yield approximately 20-fold higher expression of the primary translation product than wild-type FVIII by the same expression vector [16,17] and this could be attributed to more efficient expression of the FVIII mRNA (Fig. 1, 1). BDD-rFVIII has a similar biochemical profile and comparable pharmacokinetic parameters to those of wild-type FVIII and plasma-derived FVIII [17,18]. Among rFVIII products which are currently on the market, BDD-rFVIII is represented by ReFacto (Wyeth/Genetic Institute, Boston, MA, USA) approved for licensure in Europe in 1999 and in the USA in 2000. Clinical studies show that BDD-rFVIII provides a safe, well tolerated and effective treatment of hemophilia A when given as on-demand therapy, in routine or intermittent prophylaxis, or during surgery. To date, over 60 million units of ReFacto™ have been infused in more than 200 patients. Importantly, rates of inhibitor formation to BDD-rFVIII in previously untreated and treated patients proved to be similar to that observed for full-length rFVIII [19,20] demonstrating that, despite such a major modification of the FVIII protein, this bioengineered form of FVIII was not more immunogenic.

Another approach to increase the yield of FVIII mRNA has been recently suggested by Plantier *et al*. [21]. A truncated intron 1 sequence of FIX was inserted in BDD-FVIII cDNA in place of either intron 1, 12 or 13 and also as a combination of introns 1 and 12 or introns 1 and 13. FVIII intron 1 was targeted because the corresponding intron of FIX provides effective secretion of FIX [22,23]. Introns 12 and 13 in the FVIII gene were previously shown to code for a transcriptional silencer, which represses expression of FVIII [24]. Simultaneous substitution of FVIII introns 1 and 13 for the truncated FIX intron 1 led to a significant, 13-fold increase in FVIII secretion that was associated with a dramatically higher level of FVIII mRNA accumulation in the cell [21]. This FVIII minigene may represent a particular interest to improve production of rFVIII, Fig. 1, 1.

Bioengineering for more efficient FVIII secretion

Reduced ER chaperone interactions

Several studies have identified that secretion of FVIII requires its dissociation from the protein chaperone BiP, which resides in the ER and controls the transport of FVIII primary translation product to the Golgi compartment by retaining misfolded protein molecules in the ER [25,26], (Fig. 1, 2a). BiP possesses a peptide-stimulated ATPase activity. FVIII release from BiP and transport out of the ER requires high levels of intracellular ATP and ATP hydrolysis by BiP [27,28]. A 110-amino acid region within the A1 domain of FVIII was identified which inhibited its secretion [29]. A putative BiP-binding site was localized to a hydrophobic β-sheet within this 110 amino acid region, in which 7 of 11 amino acid residues are Leu or Phe. FV

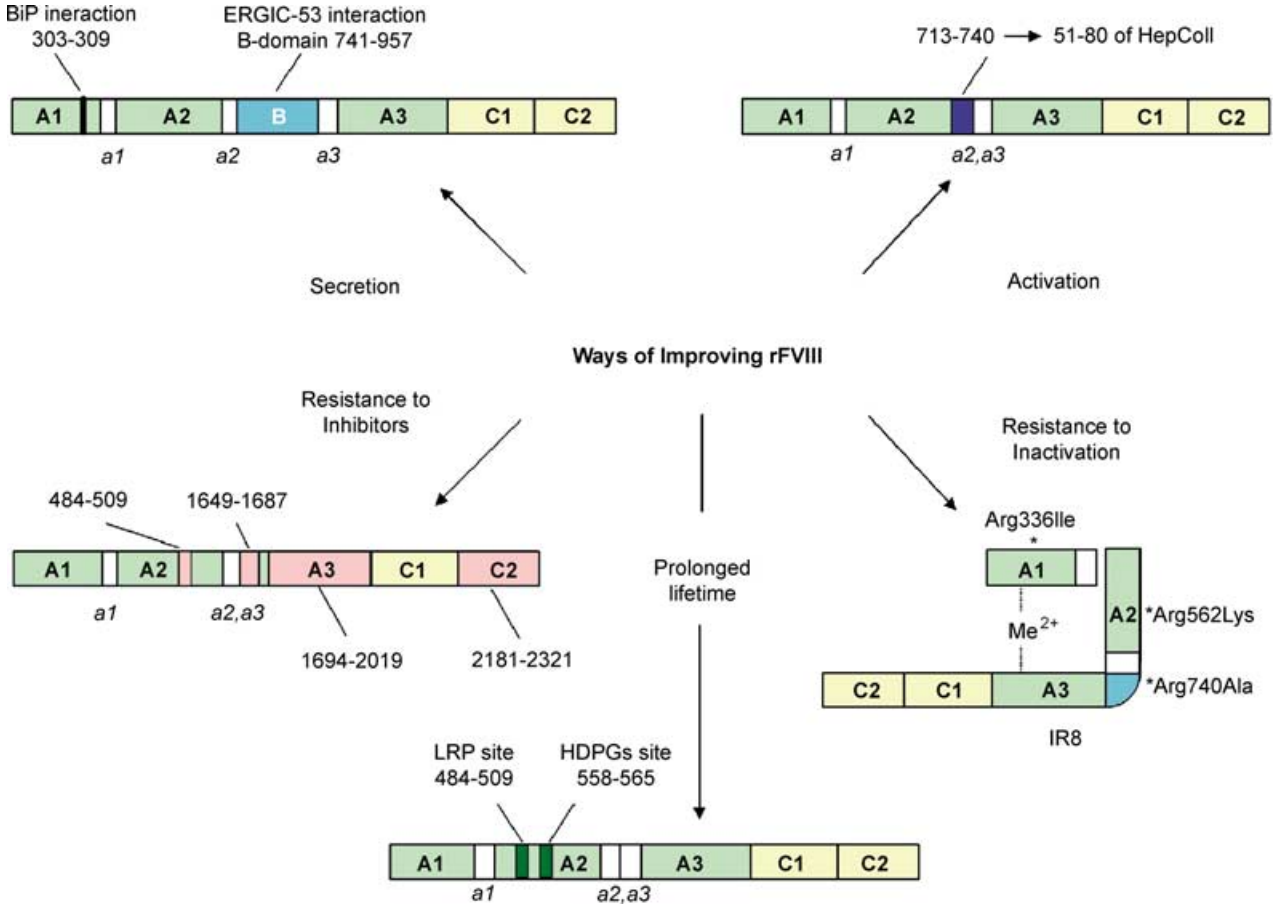

Fig. 2. Approaches to improve secretion and functional characteristics of rFVIII. Secretion: Mutations within the A1 region 303–309 of FVIII disrupt the putative binding site for the ER chaperone BiP (black) and in this way facilitate FVIII release from the ER. The maximal increase in FVIII secretion has been achieved with a double mutation of Phe309Ser/Leu303Glu. Inclusion of the B domain region 741–967 (light blue) into the BDD-FVIII cDNA construct improves FVIII secretion that may be due to interaction of this glycosylated region with a mannose-binding chaperone ERGIC-53, which mediates FVIII transport from the ER to the Golgi apparatus. Activation: Improved activation of FVIII is achieved by replacing FVIII region Asp713-Arg740 for the region Ile51-Leu80 of a thrombin inhibitor heparin cofactor II (dark blue). Resistance to inactivation: In inactivation-resistant FVIII (IR8), the B domain region 794–1689 is deleted (remaining B domain region 741–793 is shown in light blue) and a thrombin cleavage site at Arg740 (star) is mutated. Activation of IR8 occurs via a single cleavage at Arg372. In activated IR8, the A2 subunit is covalently bound to the light chain preventing non-proteolytic inactivation due to spontaneous dissociation of A2. Mutations at Arg336 and Arg562 (stars) provide resistance of IR8 to proteolytic inactivation by APC. Prolongation of FVIII lifetime: Mutations of amino acid residues within FVIII regions 484–509 and 558–565 (dark green) are expected to disrupt FVIII interaction with its clearance receptors, LRP and HSPGs, respectively, and in this way prolong FVIII lifetime in the circulation. Resistance to inhibitors: Substitution of inhibitory epitopes within the A2, A3, C2 and *a3* regions (shown in pink) of FVIII proved to be necessary and sufficient to achieve maximal reduction in FVIII reactivity towards most of inhibitor-containing plasmas from hemophilia A patients.

has the identical domain structure to FVIII, and these proteins share ~40% amino acid identity between their respective A and C domains. Curiously, FV is 20-fold more efficiently expressed compared to FVIII in mammalian expression systems and does not interact with BiP. When the 110 amino acid region of FVIII bordered by residues 226–336 was replaced for the homologous residues from FV, the FV/FVIII hybrid exhibited a 5-fold increase in the efficiency of secretion most likely due to a decreased binding to BiP. However, the generated FV/FVIII hybrid did not retain a cofactor function. Subsequently, Swaroop *et al.* performed site-directed mutagenesis within the hydrophobic β-sheet within this 110 amino acid region of the FVIII A1 domain (illustrated in Swaroop Fig. 2) and demonstrated that a single mutation of Phe309 to Ala or the homologous residue from FV (Ser) enhanced secretion of functional FVIII by 3-fold, and the double mutation Phe309-Ser/Leu303Glu yielded even higher levels of secreted FVIII [30]. Importantly, both single and double mutants of FVIII retained full cofactor function.

Improved ER-Golgi transport. As stated above, the BDD-rFVIII modification allowed achieving a 20-fold increase in mRNA levels and expression of FVIII primary translation product [17,31]. However, despite this dramatic difference in accumulation of mRNA, secretion of BDD-rFVIII from the cell was observed to be only ~2-fold higher compared to full-length wild-type FVIII [31]. This surprising observation suggested that the rate of ER-Golgi transport for the BDD-rFVIII molecule was actually reduced compared to B domain containing FVIII. More insight into this has been gleaned from the

characterization of the molecular defect underlying the majority of patients with combined deficiency of FV and FVIII. This rare, autosomal recessive bleeding disorder was first described by Oeri *et al*. in 1954 [32] and patients suffering from this disorder have plasma levels of FV and FVIII (both antigen and activity) in the range of 5–30 U dL^{-1}. Positional cloning demonstrated that in two thirds of the patients the disease results from null expression of a protein that serves as a marker for the ER-Golgi intermediate compartment (ERGIC) known as ERGIC-53. ERGIC-53 is a homo-hexameric transmembrane lectin exhibiting mannose-selective binding that is proposed to operate as a transport receptor for glycoproteins in the early secretory pathway, targeting them to COPII-coated vesicles budding from the ER for trafficking to the Golgi compartment (Fig. 1, 2b). Since the B domains of FV and FVIII contain the majority of the asparagine-linked oligosaccharide structures, it was proposed that the mannose residues presented by the FVIII B domain mediate the intracellular trafficking of FVIII via this ER-Golgi facilitated transport mechanism. This has led to reconsideration of the functional role of the B domain within the secretory pathway and may be responsible for the observed impaired rate of secretion with BDD-rFVIII. Subsequent studies have demonstrated that the B domain mediates FVIII interaction with ERGIC-53 that is at least in part dependent on its carbohydrate content [33]. Recent work was set out to determine the optimal oligosaccharide content required for optimal rate of secretion. When as little as 226 amino acid residues of the N-terminal portion of the B domain was added to a BDD-rFVIII construct (Fig. 2), this B domain variant retained equal efficiency to BDD-rFVIII in synthesis of the primary translation product (indicating efficient mRNA accumulation) yet secretion of this rFVIII protein was increased approximately 10-fold compared to BDD-rFVIII (consistent with improved efficiency of ER-Golgi transport) [34].

Bioengineering for improved FVIII activity

While the above described strategies are aimed at improving the efficiency of rFVIII expression, replacement therapy of hemophilia A may also benefit from improving the functional properties of FVIII.

Improved efficiency of activation. To exert its cofactor function, FVIII requires activation by two major physiological activators – thrombin and FXa. The activation coincides with proteolysis of both the heavy and light chains of FVIII. Cleavage within the HCh after Arg740 generates a 90-kDa polypeptide that is subsequently cleaved after Arg372 to yield the 50 kDa A1 and 43 kDa A2 fragments. Concomitantly, the 80 kDa LCh is cleaved after Arg1689 to generate a 73-kDa A3-C1-C2 fragment. In activated FVIII, the A1 and A3 domains retain the metal ion–mediated interaction, whereas the A2 subunit is weakly associated with the A1/A3-C1-C2 heterodimer through electrostatic interactions. Upon thrombin activation of FVIII there is a rapid 50-fold increase and subsequent first-order decay of procoagulant activity.

Voorberg *et al*. took advantage of the fact that efficient activation of FVIII by thrombin is dependent on the presence of amino acid sequence Asp713-Arg740 and replaced this region of FVIII for amino acid sequence Ile51-Leu80 of the thrombin inhibitor heparin cofactor II (Fig. 2), as this sequence is important for the inhibitory function of heparin cofactor II toward thrombin [35]. The hybrid molecule des-(868–1562)-FVIII-HCII had an increased sensitivity to thrombin, was more readily activated and effectively reduced the clotting time of FVIII-deficient plasma.

Resistance to inactivation. Thrombin-activated FVIII (FVIIIa) is an unstable heterotrimer susceptible to proteolytic inactivation by activated protein C (APC), FIXa or FXa. In particular, APC cleaves FVIIIa after residues 336 and 562, however, the physiological significance of these cleavages *in vivo* is unknown. In addition, FVIIIa exhibits a first-order decay of procoagulant activity *in vitro* that does not correlate with any specific proteolytic event. Rather, loss of procoagulant activity after thrombin activation *in vitro* results from a reversible dissociation of the 43 kDa A2 subunit from the heterotrimer that occurs at physiological pH. Recent studies have also shown that mutations in FVIII that decrease the stability of the FVIIIa heterotrimer result in hemophilia A. Therefore, A2 subunit dissociation is another factor limiting the procoagulant activity of FVIIIa *in vivo*.

Strategies for increasing FVIIIa activity in circulation include inhibition of FVIIIa proteolytic inactivation by APC, FIXa or FXa by mutating the FVIII residues which are the targets for these proteases. While several reports on generating such mutants have been published, further functional characterization of the mutants or better understanding of their respective inhibitory mechanisms are required to determine any prospect of these strategies in prolonging FVIIIa activity *in vivo*. For example, single mutations at either Arg336 or Arg562 in FVIII were insufficient for complete resistance to APC-mediated inactivation, whereas the double mutant Arg 336Ile/Arg562Lys proved to be resistant [36]. Since Arg336 is also a target for FIXa, FVIII mutant Arg336Ile would be expected to be resistant to FIXa-catalyzed cleavage at the A1 site. Analysis of this mutant in a FXase decay assay demonstrated that its effect was manifested only at high FIXa concentrations, whereas at low FIXa concentrations the predominant mechanism of FXase decay was dissociation of FVIIIa subunits rather than FIXa-catalyzed proteolysis of the A1 subunit [37].

Experimental evidence supports that A2 subunit dissociation limits the procoagulant activity of FVIIIa *in vivo*. In order to address this limitation, an inactivation-resistant FVIII (IR8) was genetically engineered which is not susceptible to dissociation of the A2 domain subunit and proteolytic inactivation by APC and therefore has a prolonged cofactor activity [38]. In designing IR8, B domain residues 794–1689 were deleted and Arg740 was replaced by alanine, which eliminated the thrombin-cleavage sites at Arg740 and Arg1689. As a result, FVIII activation by thrombin occurs via a single cleavage after Arg372. This leads to generation of a FVIIIa dimer that retains the A2 domain

covalently attached to the light chain thus preventing its spontaneous dissociation. Additionally, missense mutations at APC inactivation cleavage sites provided resistance to further proteolysis of FVIIIa (Fig. 2). The specific activity of IR8 proved to be 5-fold higher than that of wild-type FVIII, and 38% of peak activity was retained even after 4 h *in vitro*, whereas wild-type FVIII, under similar conditions was inactivated by thrombin after 10 min.

Bioengineering for prolonged FVIII plasma half-life

Receptor-mediated clearance of FVIII. A breakthrough in understanding the mechanisms responsible for FVIII turnover was achieved when two groups independently found that FVIII catabolism is mediated by low-density lipoprotein receptor-related protein (LRP) [39–41], a hepatic clearance receptor with a broad ligand specificity [42]. A remarkably wide spectrum of LRP ligands includes a number of proteins involved in blood coagulation and fibrinolysis, such as FIXa [43], FXa [44], a complex of thrombin with antithrombin III [45], tissue factor pathway inhibitor [46], plasminogen activators and their complexes with plasminogen activator inhibitor [42].

Conclusions about involvement of LRP in FVIII catabolism were based on the findings that FVIII was able to bind to purified LRP (K_d 60 nM [39]; K_d 116 nM [40]); that it was efficiently internalized and degraded by various LRP-expressing cell lines; and that FVIII half-life in the circulation in a mouse model could be significantly prolonged (3.3-fold) by blocking LRP by its classical antagonist, a 39-kDa receptor-associated protein [40,47]. Two distinct FVIII binding sites are involved in interaction with LRP – one within the A2 domain of HCh [40] and the other one within the C2 domain of LCh [39]. The A2 domain LRP-binding site has been mapped to the region 484–509 [40]. While the C2 site of FVIII has not been finely mapped, it most likely overlaps with the site responsible for binding to VWF, since an anti-C2 domain monoclonal antibody which inhibits FVIII binding to LRP also inhibits its binding to VWF [39,48].

In circulation, where FVIII is normally present as a tight non-covalent complex with VWF, only the A2 LRP-binding site would be exposed and would mediate FVIII catabolism. This is consistent with experimental findings that catabolism of the isolated A2 subunit and of FVIII from its complex with VWF by LRP-expressing cells shows similar kinetics [49].

An *in vivo* situation where the C2 site is exposed and has functional significance occurs in patients with severe von Willebrand disease who lack plasma VWF. Indeed, in such patients infused FVIII was shown to have an approximately 3-times shorter half-life [50,51] in comparison with normal individuals [52] and hemophilia A patients, who have normal levels of VWF [53]. Another condition leading to exposure of the C2 site is dissociation of FVIII from VWF upon proteolytic activation to FVIIIa. It is likely that exposure of both the A2 and C2 LRP-binding sites in heterotrimeric FVIIIa (A1/A2/A3-C1-C2) may be important for LRP-mediated regulation of FVIIIa levels at the site of a coagulation event [54].

Further studies on FVIII catabolism have revealed that as with many LRP ligands, LRP-mediated clearance of FVIII from its complex with VWF is facilitated by cell-surface heparan sulfate proteoglycans (HSPGs), one of the major glycoprotein components of the extracellular matrix [49]. Cooperation of LRP and HSPGs in FVIII clearance was confirmed by the simultaneous blocking of these two receptors, which led to a more significant prolongation of FVIII half-life in mice (5.5-fold) than the blocking of LRP alone (3.3-fold). The region of FVIII involved in binding to HSPGs was localized within the A2 domain residues 558–565 [49]. The current knowledge about FVIII catabolism implicates the initial binding of FVIII/VWF complex to HSPGs, which concentrate the complex on the cell surface and present it to LRP, followed by LRP-mediated catabolism of FVIII.

Prolongation of FVIII lifetime by disrupting LRP- and HSPGs-binding sites. Progress in understanding the mechanisms of FVIII catabolism suggests a direction for prolongation of FVIII half-life in the circulation by mutating LRP- and HSPGs-binding sites and generating FVIII mutant(s) with disrupted interactions with clearance receptors. Generation of such molecules may be based on the principle of ligand binding to LRP and HSPGs. These interactions are mediated by positively charged residues within the ligands and the negatively charged residues of the structural repeats of the extracellular domain of LRP [42] or the negatively charged sulfate and carboxyl groups of the glycosaminoglycan chains of HSPGs [55]. According to the most recent three-dimensional model of FVIII [56], the LRP-binding site within the A2 domain (residues 484–509) contains six positively charged residues, three Lys at positions 493, 496 and 499 and three Arg at positions 484, 489 and 490, which are tightly clustered and form a prominently exposed cationic patch on the A2 surface. Two Lys residues at positions 556 and 570 and Arg571 are exposed within the HSPGs-binding site (residues 558–565) or in immediate proximity to it, and outside residues Lys380, Lys523, Lys659 and Lys661 are spatially close to the 558–565 region. These basic residues, which are potentially responsible for FVIII interactions with LRP and HSPGs, represent prospective targets for site-directed mutagenesis (Fig. 2). They can be substituted either by neutral amino acids such as Ala or Ser or, alternatively, by oppositely charged residues (Glu), which may result in a more pronounced effect due to repulsion between the mutated residue and the corresponding negatively charged residues of LRP and HSPGs. The feasibility of the mutagenic approach has been previously shown for other LRP ligands, including the complex of urokinase-type plasminogen activator with plasminogen activator inhibitor-1 [57] and α_2-macroglobulin [58]. A similar approach has also been proven effective in reducing interaction of the protease nexin 1 with HSPGs and its subsequent LRP-mediated catabolism [59]. Since in addition to electrostatic, hydrophobic and hydrogen-bond interactions were shown to contribute to ligand binding to LRP [42], several hydrophobic residues (such as Leu491, Leu498 and

Ile508) or residues carrying hydroxyl groups (Thr481, Tyr487 and Ser488) could also be included into mutagenesis as they are spatially close to the LRP-binding site. In order to achieve maximal prolongation of FVIII half-life in the circulation, it may be worthwhile to combine mutations reducing FVIII interactions with both LRP and HSPGs. Suppression of FVIII interaction with its catabolic receptors LRP and HSPGs can potentially prolong FVIII lifetime in circulation and thus increase the efficacy of FVIII replacement therapy of hemophilia A.

Bioengineering to reduce FVIII antigenicity

Another important issue to be considered in engineering improved rFVIII is reduction of antigenicity of FVIII protein. Inhibitory antibodies to FVIII (inhibitors) develop in approximately 25% of hemophilia A patients in response to repeated infusions of FVIII products, thus reducing the efficacy of hemophilia A therapy. The major targets for inhibitor binding are the A2 and C2 domains of FVIII [60]. It was found that human inhibitors have limited cross-reactivity with porcine FVIII [61]. Therefore, it would be expected that substitution of major inhibitory epitopes in human rFVIII for porcine sequences would result in FVIII hybrids which are substantially less reactive with inhibitory antibodies. Barrow *et al*. constructed seven B-domainless hybrid FVIII molecules with various porcine substitutions within the A2, A3, C2 and *a3* of human FVIII [62]. It was found that substitution of A2 amino acids 484–508, *a3* amino acids 1649–1687, A3 residues 1694–2017, and C2 residues 2181–2321 resulted in a maximal reduction of FVIII antigenicity with respect to most of 23 inhibitory antibody plasmas tested (Fig. 2).

The current status of replacement therapy of hemophilia B

Hemophilia B is defined as an X-chromosome-linked inherited bleeding disorder due to subnormal levels of coagulation FIX and in its clinical manifestation resembles hemophilia A. As in hemophilia A, bleeding episodes in hemophilia B had originally been treated with fresh frozen plasma and during the last 30 years with FIX concentrates [63]. The cDNA for FIX was cloned in the early 1980s by two different groups in Oxford and Seattle, and the entire 34 kb FIX gene was subsequently sequenced [64], which launched expression of recombinant FIX (rFIX) for replacement therapy of hemophilia B [65]. As with rFVIII, preparation of rFIX minimized the risk of transmission of viral or other pathogenic contaminants from human blood, which is an impediment with plasma-derived products [66].

Production of rFIX requires the use of mammalian cells as hosts since FIX synthesis involves complex post-translational modifications, such as glycosylation, γ-carboxylation, β-hydroxylation, sulfation, phosphorylation and processing of the N-terminus [67–69]. While there are several rFVIII products for treatment of hemophilia A, there is just one rFIX approved for treatment of hemophilia B. This rFIX (BeneFIX^{TM}) is produced by Wyeth/Genetics Institute and is expressed in Chinese hamster ovary cells [65,70] cultured in a medium which does not contain any blood or plasma products. BeneFIX has been shown to have high purity and specific activity of $>200\,IU\,mg^{-1}$ [71,72]. The structure of BeneFIX is similar to that of plasma-derived FIX, whereas post-translational modifications of rFIX are similar but not identical to those of plasma-derived FIX [72]. BeneFIX protein exists in three isoforms, which are indistinguishable in regard to phospholipid binding, endothelial cell binding, or activation of FX and have similar clotting activities [73]. Many post-translational modifications of rFIX include N- and O-glycosylation. While the structure of the O-glycans appears to be the same in plasma-derived and recombinant FIX, the N-glycans in rFIX are generally more complex and contain a broader range of fucosylation, linkages and N-acetyllactosamine structures [72,74]. These differences in post-translational modifications, however, do not affect the higher-order structure of rFIX. Pharmacokinetic and efficacy studies with rFIX in previously treated patients were satisfying, and the frequency of formation of inhibitory antibodies (3–5%) was also comparable to that for plasma-derived FIX. Unfortunately, the *in vivo* recovery of rFIX proved to be substantially lower than that of plasma-derived FIX, which is most likely associated with differences in post-translational modifications [73]. Moreover, recovery of rFIX proved to show a wide patient-related variability [75].

While with FVIII several directions for improving the yield and therapeutic properties of recombinant protein have been clearly outlined in bioengineering, the unfavorable low recovery rate of rFIX seems to impede the progress in the development of next generation rFIX products. In this respect, it is worth mentioning the laboratory finding that rFIX production level can be increased 8-fold by including three ATG triplets as a translation initiation signal [23]. Additional improvement of rFIX production was achieved by introducing a truncated form of FIX intron 1 into the wild-type FIX cDNA construct. It was shown that the presence of intron 1 in FIX cDNA strongly enhanced accumulation of FIX mRNA in the nucleus most likely due to better protection of such transcripts from random degradation. In its turn, this led to 8–15-fold increase in rFIX production both *in vitro* and *in vivo* [22,76,77].

An impressive progress has been also achieved in gene therapy of hemophilia B [7–9,78]. Taking into consideration current concerns related to possible irreversible changes in the patient's genome, the authors believe that gene therapy of hemophilia B at its best will serve as an alternative approach to FIX replacement therapy offering a choice to a patient.

Concluding remarks

Replacement therapy remains the mainstay for treatment of hemophilia A and B. The substantial progress made in understanding the mechanisms of FVIII biosynthesis, structure-functional relationship and catabolism over the past two decades has provided targets for bioengineering strategies to improve rFVIII (Fig. 2). These strategies include achieving higher expression

levels of FVIII mRNA, more efficient secretion of FVIII protein based on the identification of FVIII domains providing its interaction with intracellular chaperones, and development of FVIII molecules with improved properties such as an increased sensitivity to activation, resistance to inactivation or prolonged lifetime of FVIII in the circulation. As introduction of desired mutations or other changes may alter antigenicity of the FVIII molecule, this issue has to be carefully experimentally addressed. Another and already developed direction in improving properties of FVIII molecule is reduction of FVIII antigenicity towards inhibitory antibodies, which represent a serious complication in hemophilia A therapy. This is achieved by generating hybrid FVIII molecules in which major epitopes of inhibitory antibodies are replaced for homologous regions of porcine FVIII, far less reactive with human anti-FVIII inhibitory antibodies. Although no clinical trials have yet been initiated with novel FVIII molecules described in the present review, preclinical work on further biochemical characterization and investigations in small animal models are ongoing. It is conceivable that the enumerated improvements to FVIII constructs may be combined, which is expected to permit achieving sustained and sufficiently high circulatory levels of functionally active rFVIII in hemophilia A patients with or without inhibitors. While recombinant FIX is widely used for treatment of hemophilia B, the concept for improvement of rFIX molecules remains to be developed. In our optimistic view, there are good chances that improvements to rFVIII and rFIX suggested by basic science may ultimately enter the clinical practice and make treatment of hemophilia A and B more efficient and less costly.

Acknowledgements

We express our gratitude to Dr Diana Kouiavskaia (The Holland Laboratory, American Red Cross, USA) for her valuable help in preparation of this review.

References

1 Brinkhous K. Clotting defect in hemophilia: Deficiency in a plasma factor required for platelet utilization. *Proc Soc Exp Biol Medl* 1947; **66**: 117.

2 Mann KG. Biochemistry and physiology of blood coagulation. *Thromb Haemost* 1999; **82**: 165–74.

3 Gitschier J, Wood WI, Goralka TM, Wion KL, Chen EY, Eaton DH, Vehar GA, Capon DJ, Lawn RM. Characterization of the human factor VIII gene. *Nature* 1984; **312**: 326–30.

4 Toole JJ, Knopf JL, Wozney JM, Sultzman LA, Buecker JL, Pittman DD, Kaufman RJ, Brown E, Shoemaker C, Orr EC, Amphlett GW, Foster WB, Coe ML, Knutson GJ, Fass DN, Hewick RM. Molecular cloning of a cDNA encoding human antihaemophilic factor. *Nature* 1984; **312**: 342–7.

5 Vehar GA, Keyt B, Eaton D, Rodriguez H, O'Brien DP, Rotblat F, Oppermann H, Keck R, Wood WI, Harkins RN, Tuddenham EGD, Lawn RM, Capon DJ. Structure of human factor VIII. *Nature* 1984; **312**: 337–42.

6 Wood WI, Capon DJ, Simonsen CC, Eaton DL, Gitschier J, Keyt B, Seeburg PH, Smith DH, Hollinghead P, Wion KL, Delwart E, Tuddenham EGD, Vehar GA, Lawn RM. Expression of active human factor VIII from recombinant DNA clones. *Nature* 1984; **312**: 330–7.

7 Monahan PE, White IIGC. Hemophilia gene therapy. *Update Curr Opin Hematol* 2002; **9**: 430–6.

8 High K. Gene-based approaches to the treatment of hemophilia. *Ann N Y Acad Sci* 2002; **961**: 63–4.

9 High KA. Gene transfer as an approach to treating hemophilia. *Circ Res* 2001; **88**: 137–44.

10 Lynch CM, Israel DI, Kaufman RJ, Miller AD. Sequences in the coding region of clotting factor VIII act as dominant inhibitors of RNA accumulation and protein production. *Hum Gene Ther* 1993; **4**: 259–72.

11 Chuah MK, VandenDriessche T, Morgan RA. Development and analysis of retroviral vectors expressing human factor VIII as a potential gene therapy for hemophilia A. *Hum Gene Ther* 1995; **6**: 1363–77.

12 Kaufman RJ, Pipe SW. Can we improve on nature? 'Super molecules' of factor VIII. *Haemophilia* 1998; **4**: 370–9.

13 Hironaka T, Furukawa K, Esmon PC, Fournel MA, Sawada S, Kato M, Minaga T, Kobata A. Comparative study of the sugar chains of factor VIII purified from human plasma and from the culture media of recombinant baby hamster kidney cells. *J Biol Chem* 1992; **267**: 8012–20.

14 Kumar HP, Hague C, Haley T, Starr CM, Besman MJ, Lundblad RL, Baker D. Elucidation of N-linked oligosaccharide structures of recombinant human factor VIII using fluorophore-assisted carbohydrate electrophoresis. *Biotechnol Appl Biochem* 1996; **24**: 207–16.

15 Toole JJ, Pittman DD, Orr EC, Murtha P, Wasley LC, Kaufman RJ. A large region (⩾95 kDa) of human factor VIII is dispensable for *in vitro* procoagulant activity. *Proc Natl Acad Sci USA* 1986; **83**: 5939–42.

16 Dorner AJ, Bole DG, Kaufman RJ. The relationship of N-linked glycosylation and heavy chain–binding protein association with the secretion of glycoproteins. *J Cell Biol* 1987; **105**: 2665–74.

17 Pittman DD, Alderman EM, Tomkinson KN, Wang JH, Giles AR, Kaufman RJ. Biochemical, Immunological, and *in vivo* Functional Characterization of B-Domain-Deleted Factor VIII. *Blood* 1993; **81**: 2925–35.

18 Sandberg H, Almstedt A, Brandt J, Castro VM, Gray E, Holmquist L, Lewin M, Oswaldsson U, Mikaelsson M, Jankowski MA, Bond M, Scoble HA. Structural and functional characterization of B-domain deleted recombinant factor VIII. *Semin Hematol* 2001; **38**: 4–12.

19 Courter SG, Bedrosian CL. Clinical evaluation of B-domain deleted recombinant factor VIII in previously untreated patients. *Semin Hematol* 2001; **38**: 52–9.

20 Courter SG, Bedrosian CL. Clinical evaluation of B-domain deleted recombinant factor VIII in previously treated patients. *Semin Hematol* 2001; **38**: 44–51.

21 Plantier JL, Rodriguez MH, Enjolras N, Attali O, Negrier C. A factor VIII minigene comprising the truncated intron I of factor IX highly improves the *in vitro* production of factor VIII. *Thromb Haemost* 2001; **86**: 596–603.

22 Kurachi S, Hitomi Y, Furukawa M, Kurachi K. Role of intron I in expression of the human factor IX gene. *J Biol Chem* 1995; **270**: 5276–81.

23 Enjolras N, Rodriguez MH, Plantier JL, Maurice M, Attali O, Negrier C. The three in-frame ATG, clustered in the translation initiation sequence of human factor IX gene, are required for an optimal protein production. *Thromb Haemost* 1999; **82**: 1264–9.

24 Hoeben RC, Fallaux FJ, Cramer SJ, van den Wollenberg DJ, van Ormondt H, Briet E, van der Eb AJ. Expression of the blood-clotting factor-VIII cDNA is repressed by a transcriptional silencer located in its coding region. *Blood* 1995; **85**: 2447–54.

25 Dorner AJ, Wasley LC, Kaufman RJ. Increased synthesis of secreted proteins induces expression of glucose- regulated proteins in butyrate-treated Chinese hamster ovary cells. *J Biol Chem* 1989; **264**: 20602–7.

26 Dorner AJ, Wasley LC, Kaufman RJ. Overexpression of GRP78 mitigates stress induction of glucose regulated proteins and blocks secretion of selective proteins in Chinese hamster ovary cells. *EMBO J* 1992; **11**: 1563–71.

27 Dorner AJ, Wasley LC, Kaufman RJ. Protein dissociation from GRP78 and secretion are blocked by depletion of cellular ATP levels. *Proc Natl Acad Sci USA* 1990; **87**: 7429–32.

28 Tagliavacca L, Wang Q, Kaufman RJ. ATP-dependent dissociation of non-disulfide-linked aggregates of coagulation factor VIII is a rate-limiting step for secretion. *Biochemistry* 2000; **39**: 1973–81.

29 Marquette KA, Pittman DD, Kaufman RJ. A 110-amino acid region within the A1-domain of coagulation factor VIII inhibits secretion from mammalian cells. *J Biol Chem* 1995; **270**: 10297–303.

30 Swaroop M, Moussalli M, Pipe SW, Kaufman RJ. Mutagenesis of a potential immunoglobulin-binding protein-binding site enhances secretion of coagulation factor VIII. *J Biol Chem* 1997; **272**: 24121–4.

31 Pittman DD, Marquette KA, Kaufman RJ. Role of the B domain for factor VIII and factor V expression and function. *Blood* 1994; **84**: 4214–25.

32 Oeri J, Matter M, Isenschmid H, Hauser F, Koller F. Angeborener mangel an faktor V (parahaemophilie) verbunden mit echter haemophilie A bein zwei brudern. *Med Probl Paediatr* 1954; **1**: 575.

33 Pipe SW, Morris JA, Shah J, Kaufman RJ. Differential interaction of coagulation factor VIII and factor V with protein chaperones calnexin and calreticulin. *J Biol Chem* 1998; **273**: 8537–44.

34 Pipe SW, Miao H, Tendulkar R, Kaufman RJ. Asparagine-linked glycosylation sites within the B domain of coagulation factor VIII improve secretion efficiency. *Blood* 2001; **98**: 705a(Abstract No. 2947).

35 Voorberg J, van Stempvoort G, Bos JM, Mertens K, van Mourik JA, Donath MJ. Enhanced thrombin sensitivity of a factor VIII-heparin cofactor II hybrid. *J Biol Chem* 1996; **271**: 20985–8.

36 Amano K, Michnick DA, Moussalli M, Kaufman RJ. Mutation at either Arg336 or Arg562 in factor VIII is insufficient for complete resistance to activated protein C (APC)-mediated inactivation: implications for the APC resistance test. *Thromb Haemost* 1998; **79**: 557–63.

37 Fay PJ, Beattie TL, Regan LM, O'Brien LM, Kaufman RJ. Model for the factor VIIIa-dependent decay of the intrinsic factor Xase: role of subunit dissociation and factor IXa-catalyzed proteolysis. *J Biol Chem* 1996; **271**: 6027–32.

38 Pipe SW, Kaufman RJ. Characterization of a genetically engineered inactivation-resistant coagulation factor VIIIa. *Proc Natl Acad Sci USA* 1997; **94**: 11851–6.

39 Lenting P, Neels JG, van den Berg BM, Clijsters PFM, Meijerman DWE, Pannekoek H, van Mourik JA, Mertens K, Zonneveld A-J. The light chain of factor VIII comprises a binding site for low density lipoprotein receptor-related protein. *J Biol Chem* 1999; **274**: 23734–9.

40 Saenko EL, Yakhyaev AV, Mikhailenko I, Strickland DK, Sarafanov AG. Role of the low density lipoprotein-related protein receptor in mediation of factor VIII catabolism. *J Biol Chem* 1999; **274**: 37685–92.

41 Turecek PL, Lenting PJ, van Mourik JA, Binder B, Mihaly J, Denis C, Wagner D, Dorner F, Schwarz HP. Low density lipoprotein receptor-related protein (LRP) mediates the clearance of factor VIII in vWf-deficient mice. *Blood* 1999; **94**: 647a.

42 Neels JG, Horn IR, Van den Berg BMM, Pannekoek H, van Zonneveld A-J. Ligand–receptor interactions of the low density lipoprotein receptor-related protein, a multi-ligand endocytic receptor. *Fibrinolysis Proteolysis* 1998; **12**: 219–40.

43 Neels JG, van den Berg BM, Mertens K, Pannekoek H, Zonneveld A-J, Lenting P. Activation of factor IX zymogen results in exposure of a binding site for low-density lipoprotein receptor-related protein. *Blood* 2000; **96**: 3459–65.

44 Narita M, Rudolph AE, Miletich JP, Schwartz AL. The low-density lipoprotein receptor-related protein (LRP) mediates clearance of coagulation factor Xa *in vivo*. *Blood* 1998; **91**: 555–60.

45 Kounnas MZ, Church FC, Argraves WS, Strickand DK. Cellular internalization and degradtion of antithrombin III-thrombin, heparin cofactor II-thrombin, and alpha1-antitrypsin-trypsin complexes is mediated by the low density lipoprotein receptor-related protein. *J Biol Chem* 1996; **271**: 6523–9.

46 Warshawsky I, Broze GJ Jr, Schwartz AL. The low density lipoprotein receptor-related protein mediates the cellular degradation of tissue factor pathway inhibitor. *Proc Natl Acad Sci USA* 1994; **91**: 6664–8.

47 Turecek PL, Schwarz HP, Binder BR. *In vivo* inhibition of low density lipoprotein receptor-related protein improves survival of factor VIII in the absence of von Willebrand factor. *Blood* 2000; **95**: 3637–8.

48 Ananyeva N, Kouiavskaia D, Shima M, Saenko E. Catabolism of the coagulation factor VIII. Can we prolong lifetime of fVIII in circulation? *Trends Cardiovasc Med* 2001; **11**: 252–7.

49 Sarafanov AG, Ananyeva NM, Shima M, Saenko EL. Cell surface heparan sulfate proteoglycans participate in factor VIII catabolism mediated by low density lipoprotein receptor-related protein. *J Biol Chem* 2001; **276**: 11970–9.

50 Tuddenham EG, Lane RS, Rotblat F, Johnson AJ, Snape TJ, Middleton S, Kernoff PB. Response to infusions of polyelectrolyte fractionated human factor VIII concentrate in human haemophilia A and von Willebrand's disease. *Br J Haematol* 1982; **52**: 259–67.

51 Lethagen S, Berntorp E, Nilsson IM. Pharmacokinetics and hemostatic effect of different factor VIII/von Willebrand factor concentrates in von Willebrand's disease type III. *Ann Hematol* 1992; **65**: 253–9.

52 Over J, Sixma JJ, Bruine MH, Trieschnigg MC, Vlooswijk RA, Bieser-Visser NH, Bouma BN. Survival of ^{125}iodine-labeled factor VIII in normals and patients with classic hemophilia. Observations on the heterogeneity of human factor VIII. *J Clin Invest* 1978; **62**: 223–34.

53 Fijnvandraat K, Berntorp E, Ten Cate JW, Johnsson H, Peters M, Savidge G, Tengborn L, Spira J, Stahl C. Recombinant, B-domain deleted factor VIII (r-VIII SQ): pharmacokinetics and initial safety aspects in hemophilia A patients. *Thromb Haemost* 1997; **77**: 298–302.

54 Neels JG, Bovenschen N, Zonneveld A-J, Lenting P. Interaction between factor VIII and LDL receptor-related protein. *Trends Cardiovasc Med* 2000; **10**: 8–14.

55 Mann DM, Romm E, Migliorini M. Delineation of the glycosaminoglycan-binding site in the human inflammatory response protein lactoferrin. *J Biol Chem* 1994; **269**: 23661–7.

56 Stoilova-McPhie S, Villoutreix BO, Mertens K, Kemball-Cook G, Holzenburg A. Three-Dimentional structure of membrane-bound coagulation factor VIII. modeling of the factor VIII heterodimer within a three-dimentional density map derived by electron crystallography. *Blood* 2002; **99**: 1215–23.

57 Rodenburg KW, Kjoller L, Petersen HH, Andreasen PA. Binding of urokinase-type plasminogen activator-plasminogen activator inhibitor-1 complex to the endocytosis receptors alpha2-macroglobulin receptor/low-density lipoprotein receptor-related protein and very-low-density lipoprotein receptor involves basic residues in the inhibitor. *Biochem J* 1998; **329**: 55–63.

58 Howard GC, Yamaguchi Y, Mirsa UK, Gawdi G, Nelsen A, DeCamp DL, Pizzo SV. Selective mutations in cloned and expressed α_2-macroglobulin receptor binding fragment alter binding to either the α_2-macroglobulin signaling receptor or the low density lipoprotein receptor-related protein/α_2-macroglobulin receptor. *J Biol Chem* 1996; **271**: 14105–11.

59 Knauer MF, Kridel SJ, Hawley SB, Knauer DJ. The efficient catabolism of thrombin-protease nexin 1 complexes is a synergistic mechanism that requires both the LDL receptor-related protein and cell surface heparins. *J Biol Chem* 1997; **272**: 29039–45.

60 Saenko EL, Ananyeva NM, Kouiavskaia DV, Khrenov AV, Anderson JA, Shima M, Qian J, Scott D, Haemophilia A. effects of inhibitory antibodies on factor VIII functional interactions and approaches to prevent their action. *Haemophilia* 2002; **8**: 1–11.

61 Koshihara K, Qian J, Lollar P, Hoyer LW. Immunoblot cross-reactivity of factor VIII inhibitors with porcine factor VIII. *Blood* 1995; **86**: 2183–90.

62 Barrow RT, Healey JF, Gailani D, Scandella D, Lollar P. Reduction of the antigenicity of factor VIII toward complex inhibitory antibody plasmas using multiply-substituted hybrid human/porcine factor VIII molecules. *Blood* 2000; **95**: 564–8.

63 Thompson AR, Cheng S-H. Characterization of factor IX defects in hemophilia B. In: *Methods in Enzymology.* Colowick, SP, Kaplan, NO, eds. San Diego: Acad Press, Inc. 1993.

64 Yoshitake S, Schach BG, Foster DC, Davie EW, Kurachi K. Nucleotide sequence of the gene for human factor IX (antihemophilic factor B). *Biochemistry* 1985; **24**: 3736–50.
65 Kaufman RJ, Wasley LC, Furie BC, Furie B, Shoemaker CB. Expression, purification, and characterization of recombinant gamma-carboxylated factor IX synthesized in chinese hamster ovary cells. *J Biol Chem* 1986; **261**: 9622–8.
66 Roth DA, Kessler CM, Pasi KJ, Rup B, Courter SG, Tubridy KL. Human recombinant factor IX. safety and efficacy studies in hemophilia B patients previously treated with plasma-derived factor IX concentrates. *Blood* 2001; **98**: 3600–6.
67 Suttie JW. Mechanism of action of vitamin K. synthesis of gamma-carboxyglutamic acid. *CRC Crit Rev Biochem PG* 1980; **8**: 191–223.
68 Fernlund P, Stenflo J. Beta-hydroxyaspartic acid in vitamin K-dependent proteins. *J Biol Chem* 1983; **258**: 12509–12.
69 Kurachi K, Davie EW. Isolation and characterization of a cDNA coding for human factor IX. *Proc Natl Acad Sci USA* 1982; **79**: 6461–4.
70 Balland A, Faure T, Carvallo D, Cordier P, Ulrich P, de la Fournet BSH, Lecocq JP. Characterisation of two differently processed forms of human recombinant factor IX synthesised in CHO cells transformed with a polycistronic vector. *Eur J Biochem* 1988; **172**: 565–72.
71 Harrison S, Adamson S, Bonam D, Brodeur S, Charlebois T, Clancy B, Costigan R, Drapeau D, Hamilton M, Hanley K, Kelley B, Knight A, Leonard M, McCarthy M, Oakes P, Sterl K, Switzer M, Walsh R, Foster W. The manufacturing process for recombinant factor IX. *Semin Hematol* 1998; **35**: 4–10.
72 Bond M, Jankowski M, Patel H, Karnik S, Strang A, Xu B, Rouse J, Koza S, Letwin B, Steckert J, Amphlett G, Scoble H. Biochemical characterization of recombinant factor IX. *Semin Hematol* 1998; **35**: 11–7.
73 White GC, Beebe A, Nielsen B. Recombinant factor IX. *Thromb Haemost* 1997; **78**: 261–5.
74 Agarwala KL, Kawabata S, Takao T, Murata H, Shimonishi Y, Nishimura H, Iwanaga S. Activation peptide of human factor IX has oligosaccharides O-glycosidically linked to threonine residues at 159 and 169. *Biochemistry* 1994; **33**: 5167–71.
75 Ewenstein BM, Joist JH, Shapiro AD, Hofstra TC, Leissinger CA, Seremetis SV, Broder M, Mueller-Velten G, Schwartz BA. Pharmacokinetic analysis of plasma-derived and recombinant F IX concentrates in previously treated patients with moderate or severe hemophilia B. *Transfusion* 2002; **42**: 190–7.
76 Zheng B, Qiu XY, Tan M, Xing YN, Lo D, Xue JL, Qiu XF. Increment of hFIX expression with endogenous intron 1 *in vitro*. *Cell Res* 1997; **7**: 21–9.
77 Rodriguez MH, Enjolras N, Plantier JL, Rea M, Leboeuf M, Uzan G, Bordet JC, Negrier C. Expression of coagulation factor IX in a haematopoietic cell line. *Thromb Haemost* 2002; **87**: 366–73.
78 Kaufman RJ. Advances toward gene therapy for hemophilia at the millennium. *Hum Gene Ther* 1999; **10**: 2091–107.