Functional basis of protection against age-related macular degeneration conferred by a common polymorphism in complement factor B

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Abbreviations. AMD: age-related macular degeneration; AP: alternative pathway; CP: classical pathway; Bb: larger cleavage subunit of factor B; Ba: smaller cleavage subunit of factor B; fB: factor B; fD: factor D; fH: factor H; rfB: recombinant fB; CReg: complement regulator; SCR: short consensus repeat; vWF-A: von Willebrand factor-A; SPR: surface plasmon resonance; RU: resonance unit; $K_D$: dissociation equilibrium constant
Mutations and polymorphisms in complement genes have been linked with numerous rare and prevalent disorders, implicating dysregulation of complement in pathogenesis. The three common alleles of fB encode Arg (fB32R), Gln (fB32Q) or Trp (fB32W) at position 32 in the Ba domain. The fB32Q allele is protective for age-related macular degeneration, the commonest cause of blindness in developed countries. fB variants purified from plasma of homozygous individuals were tested in haemolysis assays. The protective variant fB32Q had decreased activity compared to fB32R. Biacore comparison revealed markedly different proenzyme formation; fB32R bound C3b with four-fold higher affinity, and formation of activated convertase was enhanced. Binding and functional differences were confirmed with recombinant fB32R and fB32Q; an intermediate affinity was revealed for fB32W. To confirm contribution of Ba to binding, affinity of Ba for C3b was determined. Ba-fB32R had three-fold higher affinity compared to Ba-fB32Q. We demonstrate that the disease-protective effect of fB32Q is consequent upon decreased potential to form convertase and amplify complement activation. Knowledge of the functional consequences of polymorphisms in complement activators and regulators will aid disease prediction and inform targeting of diagnostics and therapeutics.


**Introduction**

Dysregulation of the alternative pathway (AP) of complement is associated with numerous pathologies, including age-related macular degeneration (AMD), atypical hemolytic uremic syndrome (aHUS), rheumatoid arthritis, Dense Deposit Disease (DDD) and lupus nephritis (1). The AP ‘ticks over’ continuously in plasma, enabling rapid response to pathogen, and also amplifies the other complement activation pathways (2). The critical AP activation step is cleavage of C3 to C3b. The C3-cleaving enzyme, or convertase, is formed through the Mg^{2+}-dependent binding of factor B (fB) to C3b, forming the proenzyme, C3bB. fB within the complex is then cleaved by a plasma serine protease, factor D (fD), releasing an amino terminal fragment, Ba (comprising 3 short consensus repeats; SCR); this fragment takes no further part in the complement cascade. Bb, comprising a serine protease domain and a von Willebrand Factor Type A (vWF-A) domain, remains bound to C3b, and is an active serine protease capable of cleaving further C3. Convertase-generated C3b can itself form more C3bBb, providing amplification of activation. As the C3b clusters around the surface-bound convertases a C5-cleaving enzyme is formed (C3bBbC3b), C5b is generated and the lytic pathway proceeds, with formation of the membrane attack complex (MAC) (3).

To protect against the tendency of the AP to rapidly amplify, host cells express an armoury of complement regulatory proteins (CReg) which inhibit convertases or prevent MAC formation on their surfaces (4, 5). These are either membrane-associated (CD55, CD46, CD59) or fluid phase (factor H (fH), C4b-binding protein) proteins. Dysregulation of the AP is brought about by loss-of-function/expression mutations in CReg or gain-of-function mutations in components, both scenarios result in uncontrolled complement activation on self-surfaces and subsequent tissue damage and/or inflammation. The best characterised disease of AP dysregulation is aHUS,
where loss-of-function mutations in the CReg or gain-of-function mutations in components lead
to increased complement activation at cell surfaces, resulting in renal damage (6, 7).

More remarkably, common polymorphisms in AP components and CReg have also been linked
to disease. Of particular interest are three common polymorphic variants of fB that differ at
position 32 in the Ba domain (aa 7 in the mature protein; \(fB_{32R}, fB_{32Q}\) and \(fB_{32W}\); rs12614,
rs641153)(8-10). \(fB_{32R}\) is the most frequent allele in Caucasians (allele frequency 0.79) and was
originally described as fB-S (‘slow’, defined by electrophoretic mobility); the fB-F (‘fast’) allele
was further defined as fB-FA (\(fB_{32Q}\); allele frequency 0.05) and fB-FB (\(fB_{32W}\); allele frequency
0.16) (11). fB-S and fB-F, have long been associated with various pathologies, including
susceptibility to pathogenic infection, for example with Trypanosoma cruzi, where fB-S is
protective in cardiomyopathy associated with Chagas’ disease (12). FB-S is reported to be over-
represented in multiple sclerosis (13), and weakly associated with Type I diabetes mellitus,
where it is present in a HLA haplotype strongly linked to disease (14). However, the most
striking example of linkage of common variants in fB to disease is in AMD, the commonest
cause of irreversible blindness in the elderly in the Western world (15).

The hallmark lesion of AMD is the development of drusen, lipoproteinaceous deposits located
between the retinal pigment epithelium (RPE) and Bruch’s membrane (16). Deposition of
drusen is followed by extensive atrophy of the RPE and overlaying photoreceptor cells
(geographic atrophy, GA), or aberrant choroidal neovascularization (CNV). CNV under the
macula is the primary cause of blindness. Although the pathogenesis of AMD is still unclear,
inflammatory responses are implicated (17, 18). AMD is a multifactorial disease, influenced by
age, ethnicity, environmental and genetic risk factors (19). Two major AMD susceptibility loci
(1q31, CFH, and 10q26, LOC387715/HTRA1) that independently contribute to disease risk have
been recently identified by candidate region linkage and whole genome association analyses (20-
Linkage to CFH, the gene encoding fH, was followed by reports describing association of AMD with polymorphic variations in other complement genes, fB, C3 and the factor H related genes CFHR1 and CFHR3 (24-28). Remarkably, while most AMD-associated polymorphisms are linked to increased disease risk, several replication studies have demonstrated that the fB32Q variant confers significant protection from development of AMD (26, 27, 29).

The risk allele in LOC387715 causes destabilisation of the mRNA and subsequent reduced expression of the encoded protein, a mitochondrial protein normally expressed at high levels in the retinal photoreceptor cells (30). A functional consequence increasing risk of AMD is thus plausible. In contrast, no functional explanation of the association of any complement polymorphism with AMD has yet been provided. Here we demonstrate the functional mechanism underlying the association of fB variants with pathology. Using purified and recombinant fB variants, we demonstrate that the fB32Q variant, identified by genetic association studies to be protective in AMD, is less efficient at forming the amplifying AP convertase. This likely protects from development of pathology in AMD and perhaps other chronic inflammatory diseases by dampening complement activation, but may predispose to infection due to reduced amplification activity.

Results

Purification of fB variants and differential activity in haemolysis assays

In order to investigate whether the R32Q polymorphism in fB influenced function, fB was purified from plasma of a donor identified by genotyping to be R32Q heterozygote. The two variants were separated by anion exchange chromatography at pH 6.0 (Figure S1) and individually gel filtered on Superdex 200 equilibrated in CFD. Fractions containing pure, monomeric fB were identified and used immediately for functional analyses. In order to compare functional activity of the two variants, fB was immunodepleted from human serum
(NHSΔfB) and used as a source of all other complement components. Rabbit erythrocytes were incubated with NHSΔfB and different concentrations of fB variants, lysis was measured (Figure S2). The fB32Q variant was less haemolytically active than fB32R, at least two-fold more protein was required to achieve equivalent lysis.

To confirm and extend these data, fB was purified by affinity chromatography and gel filtration from plasma of 6 individuals identified by genotyping to be homozygote for fB32R (three individuals), fB32Q (two individuals) and fB32W (one individual). The three variants showed consistent differences in hemolytic activities, with fB32Q the least lytic and fB32R the most lytic (Figure 1). Calculated EC50s were, for the fB32Q individuals 35.6 and 43.5nM, for the fB32R individuals 15.4, 12.6 and 13.9nM, and for the fB32W individual 17.9nM. The difference in means between fB32R and fB32Q was statistically significant (two-tailed unpaired T test; p<0.0038).

**Formation of AP convertase by fB32R, fB32Q and fB32W variants**

We previously used SPR (Biacore) to monitor proenzyme (C3bB) formation and convertase activation (C3bBb) in real-time. In order to dissect the mechanisms underlying differential hemolytic activities of fB32R and fB32Q, fB, purified from homozygote donors (inset Figure 2), was gel filtered into Biacore buffer and analysed immediately. Proenzyme formation was analysed by SPR by flowing fB over the C3b-immobilised surface in the presence of Mg²⁺, without fD. FB32R was more efficient in forming proenzyme, with higher levels of C3bB32R formed at identical fB concentrations. Decay was rapid for each variant. We have previously shown that several points of contact and conformational transitions are involved in the interaction between fB and C3b which is best modelled using a ‘two-state transition’ model (31). Analysis of sensorgrams (Figure 2), revealed a four-fold higher affinity of binding for fB32R over
fB32Q; $K_D$ calculated as a mean of three independent experiments using ‘two state reaction’
model: fB32R: 0.17±0.03μM, fB32Q: 0.74±0.25μM).

To analyse formation of the convertase C3bBb, fD was included with the fB variants. Although
the kinetics of convertase formation by fB32R and fB32Q were similar, because fB32Q bound with
lower affinity, lower levels of convertase were formed (Figure 3A).

To investigate the half-lives of the activated convertase, purified fB variants were flowed over a
C3b surface in the presence of fD. Dissociation rates (kd) are not dependent on concentration;
therefore, decay curves were normalised in the y-axis. There was an obvious gradation of
enzyme formation (fB32R > fB32W > fB32Q), while rates of decay of the three enzymes were
identical (Figure 3B), as expected because Ba, containing the variant amino acid, is released
from the convertase, and cannot influence decay of Bb from C3b.

**Formation of AP convertase by recombinant fB32R, fB32Q and fB32W variants**

In order to confirm these kinetic data, we expressed recombinant (r) forms of fB differing only at
position 32, rfB32R, rfB32Q and rfB32W in CHO cells and purified from supernatant by affinity
chromatography and gel filtration (Figure 4A, inset). Analysis of proenzyme formation by SPR
revealed higher affinity binding of rfB32R compared to rfB32Q confirming results obtained with
the native proteins (Figure 4A). The third variant, rfB32W revealed an intermediate efficiency of
binding. Kinetic profiles differed slightly when compared to native protein, likely due to
different glycosylation, but measured affinities were similar for native and recombinant.
Formation of activated convertase using recombinant fB variants confirmed that rfB32R
generated most convertase and rfB32Q the least, with rfB32W intermediate (Figure 4B); decay rates
of Bb were identical for convertases generated by each recombinant protein.
Isolation of Ba and binding to C3b

The above data imply that variation in Ba influences proenzyme formation and the amount of enzyme formed. FB binds C3b through a multi-point contact, involving Ba and the vWF-A domain. In order to confirm differential binding of the variant Ba domains to C3b, Ba fragments were generated from purified fB32R and fB32Q by incubating fB and fD with C3b immobilised on Sepharose. Bb was removed on an anti-Bb affinity column, Ba was ‘polished’ by gel filtration and used immediately in SPR binding studies with immobilized C3b (Figure 5A). In contrast to fB, binding was very weak, necessitating high concentrations of Ba to determine binding affinity. Ba purified from fB32R bound C3b with three-fold higher affinity than Ba generated from fB32Q (Figure 5B,C).

Effect of ionic strength on convertase formation

In order to dissect out the differences between the fB variants, enzyme formation and Ba binding were analysed in low salt concentrations. To confirm that the differential binding occurred at physiological salt, proenzyme and convertase formation by fB32R and fB32Q were analysed in HEPES-buffered saline (150mM). The fB32R variant again showed a 3-4-fold higher affinity in proenzyme formation compared to fB32Q, with resultant enhanced convertase formation (Figure S2).

Discussion

The common allotypes of fB affect risk of disease, the most striking example being AMD where fB32Q is protective (OR 0.32; 95%CI: 0.16-0.65; unpublished data from Spanish cohort) (26, 27). The variant amino acid (aa) in fB is just 7aa from the amino terminus (25 aa leader sequence removed) within the Ba domain; a location that is paradoxical in that Ba is not part of the activated convertase and cannot therefore affect enzyme activity (Figure 6). The crystal structure of fB demonstrated that Ba was folded back onto the Bb domain, with SCR2 and SCR3
of Ba packed tightly into an antiparallel dimer capped by SCR1, this amino-terminal SCR likely hindering access of C3b to the vWF-A domain in Bb (32). It was suggested that initial binding of the three SCRs to C3b displaced them from the vWF-A and serine protease domains, allowing access of C3b, and triggering structural rearrangements that enabled proteolytic cleavage of fB by fD. The amino terminus of fB is unstructured and the crystal structure commences at Q$_{34}$ (aa 9 in the mature protein) (32); Q$_{34}$ (and by inference, the variant aa R$_{32}$/Q$_{32}$) is in close proximity to two residues in the vWF-A domain (D$_{279}$ and F$_{286}$) known to affect proenzyme formation (7) (Figure 6). The location of the variant aa in fB, and data indicating a binding interaction between Ba and C3b (33), led us to hypothesise that the polymorphism would affect proenzyme formation.

To test the hypothesis fB variants were isolated from plasma. In hemolysis assays using rabbit erythrocytes as target they showed differential activities, with fB$_{32Q}$ being the least lytic (Figure 1). The underlying mechanism was dissected using SPR. When proenzyme formation was analysed, fB$_{32Q}$ bound C3b with a 4-fold lower affinity compared to fB$_{32R}$; the fB$_{32W}$ variant had an intermediate affinity (Figure 2). These data were confirmed using recombinant fB variants (Figure 4). The influence of the polymorphism in the Ba domain on C3b binding was confirmed by isolating the variant Ba and directly comparing binding to C3b (Figure 5). Although the affinity of Ba for C3b is weak, avidity effects from simultaneous multi-site binding in the Ba and Bb domains likely explains the striking effect of variation in Ba on apparent affinity of fB.

When effects of fB variants on formation of the activated convertase, C3bBb were studied, fB$_{32R}$ generated 2.5-fold more enzyme than fB$_{32Q}$ under the experimental conditions (Figure 3). These data show that fB$_{32Q}$ is less efficient at maintaining amplification in the AP and provide a mechanism for the observation that fB$_{32Q}$ protects from pathology in AMD.
Previous investigations into functional differences between the fB variants have produced variable results. Early studies using haemolytic overlay assays indicated that fB32Q had either decreased or equivalent functional activity compared to fB32R (34-36). These data were complicated by variable plasma concentrations of fB and differences in expression levels of the allele products, making quantitative comparisons difficult. A later study by Horiuchi et al. analysed the functional activity of recombinant forms of fB32R and fB32Q and concluded that there was no functional difference (37). In this study, convertase was assembled on the surface of C3b-coated sheep erythrocytes using native properdin and fD, and recombinant fB (quantified by ELISA in cell culture supernatant). We have shown that the R32Q polymorphism influences the efficiency of proenzyme formation, rather than the activity of the convertase itself, therefore the functional difference would only be revealed if the assay reported by Horiuchi et al. was developed at a point where formation of activated convertase was incomplete and not all the fB was cleaved to Bb and stabilised by properdin. In our studies, all proteins were highly purified and polished by gel filtration into assay buffer immediately prior to each assay, the resultant sensitive assays have enabled us to establish beyond doubt functional differences between the variants.

The fB32Q variant is apparently only protective in AMD and not in other diseases of complement dysregulation (eg. DDD, aHUS). The reasons for disease specificity are unclear. Several studies have demonstrated elevated plasma levels of complement activation products (including C3a, C3d, Ba, SC5b-9) in AMD compared to controls, indicating systemic dysregulation of complement and implying that AMD is a systemic disease with local manifestation in the retina (38, 39). This systemic component may amplify the effects of the fB polymorphism, explaining the disease specificity. Comparative expression levels from the fB alleles in controls and AMD are not yet available; early studies using crossed immunoelectrophoresis and radial immunodiffusion assays demonstrated a trend for higher expression from the fB32Q allele in
normal individuals (38-40), although as with many complement components the range around
the mean was large. Decreased plasma levels of the fB32R allele product may indicate decreased
expression, or increased catabolism due to AP tickover or chronic inflammatory processes.

The triggers for drusen accumulation and retinal pathology in AMD are unknown, but
autoimmune mechanisms have been suggested; lipid moieties in the rod photoreceptor
membranes become photo-oxidised over time, forming omega-(2-carboxyethyl)pyrrole (CEP)
adducts that may elicit an immune response, generating autoantibodies that trigger local
inflammatory processes, including complement activation (40, 41). Damaged tissue and
accumulation of extracellular insoluble debris is in itself inflammatory, likely driving
complement activation and immune responses in AMD, as in other chronic pathologies such as
Alzheimer’s disease, where damaged neurons and deposits of amyloid β peptide drive
complement activation (42).

We here explain the mechanism by which a common polymorphism in a complement
component protects an individual from AMD. The R32Q polymorphism in fB (rs12614,
rs641153) affects risk of AMD by altering AP activity. Variations in C3 (R102G) and CFH
(I62V, Y402H) are also linked to increased risk of AMD, although the functional consequences
of these changes have not been characterised; early reports that fH402H bound less well to CRP in
drusen have not been substantiated (43). Because fB32Q is less efficient at amplifying the AP, it
protects from AMD and perhaps other chronic inflammatory pathologies; however, it may have
the opposite effect in infection, where fB32Q may predispose to disease due to sub-optimal AP
activation on pathogens. Indeed, the association of variants of multiple complement components
or CReg with disease (for example: C3, fB, fH in AMD; fH, fI, fB, C3, CD46 in aHUS) suggest
that particular combinations of variants, or ‘complotypes’, may combine to influence systemic
complement activity in an individual, thus affecting risk of chronic inflammatory damage and
susceptibility to infection. The multi-locus risk model of AMD, suggests that the effects of common variants are additive and the model can identify individuals whose lifetime risk of AMD ranges from less than 1% to more than 50% (20-28). Specific variant sets of complement components and CReg can thus dramatically influence outcome, an observation that is increasingly important as the population ages, likely impacting not only in AMD, but also in other chronic inflammatory diseases such as Alzheimer’s disease and atheroma. Understanding of the mechanisms underlying these associations is an essential prerequisite to the design of therapies that target complement activation in these diseases; for example, our data suggest that anti-Ba antibodies may have potential in the clinic, particularly for those individuals who carry high risk combinations of complement components and CReg.

**Materials & Methods**

**Genotyping**

Healthy volunteers were screened for mutations/polymorphisms in the CFB gene by DNA sequencing of PCR amplified fragments. Genomic DNA was prepared from peripheral blood cells according to standard procedures. Each exon of the CFB gene was amplified from genomic DNA using specific primers derived from the 5’ and 3’ intronic sequences as described (7). Sequencing was performed in an ABI 3730 sequencer using a dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA).

**Preparation of complement components and activation fragments**

C3 and factor B were purified from normal human serum by classical chromatography (C3) or affinity chromatography (factor B); details are given in SI Methods. Ba was purified as a by-product of AP activation as detailed in SI Methods. All proteins used for Biacore studies were gel-filtered into the appropriate Biacore buffer before experimentation.
Production of recombinant fB

A cDNA encoding full length fB32R was introduced in the eukaryote expression vector pCI-Neo (Promega, Madison, WI), and R32Q and R32W aa substitutions introduced using QuickChange site-directed mutagenesis kit (Stratagen, La Jolla, CA) and appropriate primers. All fB cDNA clones were fully sequenced to confirm fidelity. CHO cells, maintained in Ham-F12 medium (GIBCO-BRL, Carlsbad, CA) supplemented with 10% fetal calf serum, L-glutamine (2 mM final concentration), penicillin and streptomycin (10 U/ml and 100 μg/ml), were plated in p60 plates at 5 x 10^5 cells per well. Transfections were carried out one day later with 10 μg of the pCI-Neo constructs and 24 μl of Lipofectin (Invitrogen) in a total volume of 1 ml of medium per well. Transfected cells were selected in G418 sulfate (Geneticin; GIBCO-BRL, Carlsbad, CA) at 500 μg/ml, cloned by limiting dilution and clones producing the highest levels of recombinant fB (rfB) as assessed by ELISA (7), were expanded for production. rfB was purified from tissue culture supernatant by affinity chromatography on anti-Bb as described above. Eluate was concentrated and rfB was polished by gel filtration on Superdex200 in Biacore buffer.

Haemolysis assays

NHS was depleted of fB by flowing over the JC1 anti-Bb affinity column in CFD. Run-through was collected and undiluted fractions pooled and stored at -80°C. Use of this standard fB-depleted serum eliminated any variation in haemolysis due to polymorphisms or concentration differences in other complement components and regulators. Rabbit erythrocytes (Erb), washed in CFD (Complement Fixation Diluent, Oxoid) and resuspended to 2% (v/v), were mixed with fB depleted NHS (NHSΔfB; 1/38 final dilution; 50μl cells, 25μl diluted NHSΔfB) and 50μl of a dilution of purified, gel-filtered fB. CFD permits activation of all pathways of complement activation, therefore lysis resulted from direct activation of the AP on the surface of Erb or from AP-mediated amplification of the other pathways. After 45 minutes, cells were pelleted by
centrifugation and haemoglobin release was measured by absorbance at 415nm. Control incubations included 0% lysis (buffer only) and 100% lysis (0.1% Nonidet-P40). Percent lysis =100*(A415 test sample-A415 0% control)/(A415 100% control-A415 0%control). The log_{10} of fB concentration (final concentration in the incubation) was plotted on the x-axis, and % lysis on the y-axis. Non-linear regression was used to fit the curves (GraphPad Prism) and EC50 was calculated as the concentration of fB giving a response half way between background (no fB) and maximum.

**Biosensor Analysis**

All analyses were carried out on a Biacore T100 (GE Healthcare). C3b was amine coupled to the sensor chip as instructed by the manufacturers (NHS/EDC coupling kit). In the example illustrated in figures 2 and 3, 880RU C3b are immobilised, although replicate experiments used differing amounts. In figure 4, 458RU C3b are immobilised. For kinetic analyses a CM5 (carboxymethylated dextran) chip was used and data collected at 25°C at a flow rate of 30µl/minute; data from a reference cell was subtracted to control for bulk refractive index changes. Samples were injected using the KINJECT command to ensure accurate association kinetics. To analyse proenzyme formation, fB variants were flowed across the C3b surface at different concentrations; the surface was regenerated with EDTA containing buffer as previously described (31). To analyse formation of the activated convertase, fD was incuded at 1µg/ml and the surface was regenerated using soluble recombinant CD55 (31) (gift of Dr Susan Lea, Oxford, UK). In the case of Ba, no regeneration step was required. Due to the low affinity of the Ba interaction, high levels of C3b (4058RU) were immobilised and steady state analysis was used to quantitate affinity. Data were evaluated using Biaevaluation T100 evaluation software (Version 1.1), global fitting was used to determine kinetic parameters that fitted all curves (differing concentrations of fB) within an experiment. Concentration of analytes was assessed using absorbance at A_{280}, molarities were calculated using the following extinction coefficients
(molecular masses and coefficients obtained using Protean software, DNAStar): factor B (1.43), Ba (1.74).

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References


Figure Legends

Figure 1. Haemolytic activity of the fB variants. The variant fB proteins were purified from plasma of six donors known to be homozygous for fB32R (3 donors; circles), fB32Q (2 donors, squares), or fB32W (1 donor, triangles). Rabbit erythrocytes were incubated with fB-depleted serum and different concentrations of the purified fB variants. Lysis was developed for 45 minutes and haemoglobin release was used to calculate percent lysis. Data points represent mean±SD of three determinations. The log10 of fB concentration was plotted against % lysis and curves were fitted using non-linear regression analysis in order to calculate the EC50.

Figure 2. SPR analysis of proenzyme formation by native fB. The variant fB proteins were purified from the plasma of donors known to be homozygous for fB32R or fB32Q, representative preparation is illustrated (reducing SDS-PAGE gel stained with Commissie Blue R250). FB was flowed over the surface of the C3b-coated chip at concentrations between 460nM and 7nM in Biacore buffer (10mM HEPES pH7.4, 50mM NaCl, 1mM MgCl2, 0.005% surfactant P20). Sensorgrams from fB32R are solid lines and fB32Q are dotted lines; identical concentrations are illustrated for the two proteins.

Figure 3. SPR analysis of activated convertase formation by native fB. (A) The variant fB proteins were purified from the plasma of donors known to be homozygous for fB32R or fB32Q. FB was flowed over the surface of the C3b-coated chip in the presence of 1μg/ml fD at concentrations between 460nM and 7nM in Biacore buffer (10mM HEPES pH7.4, 50mM NaCl, 1mM MgCl2, 0.005% surfactant P20). Sensorgrams from fB32R are solid lines and fB32Q are dotted lines; identical concentrations are illustrated for the two variants. Arrows indicate sensorgram generated by either variant at 115nM for comparison. Kinetic information from two independent experiments (different surface and preparation of fB each time) were analysed using
‘two state reaction model’ and data were identical for both experiments. $K_D$: $fB_{32R}: 2.9 \text{nM}$, $fB_{32Q}: 7.1 \text{nM}$ ($\chi^2$ in replicate experiments was between 6 and 13). Data were also analysed using a 1:1 Langmuir model; although the fit was not as good, kinetic analysis revealed similar affinities ($3.2 \text{nM}, fB_{32R}; 7.7 \text{nM}, fB_{32Q}$) ($\chi^2$ in replicates was between 7 and 32). (B) All three variants were purified from plasma of homozygous donors and flowed over a C3b surface at two concentrations (156, 39nM). Sensorsgrams from $fB_{32R}$ are solid lines, $fB_{32Q}$ are dotted lines and $fB_{32W}$ are in gray. Data were normalised in the y axis and sensorsgrams overlaid.

**Figure 4. SPR analysis of enzyme formation by recombinant fB.** The variant rFB proteins were purified from supernatant, representative preparation is illustrated (reducing SDS-PAGE gel stained by silver). (A) rFB was flowed over the C3b-coated chip at concentrations between 233nM and 4nM in Biacore buffer (10mM HEPES pH7.4, 50mM NaCl, 1mM MgCl$_2$, 0.005% surfactant P20). Sensorsgrams from rFB$_{32R}$ are solid lines, rFB$_{32Q}$ are dotted lines and rFB$_{32W}$ are in gray; identical concentrations are illustrated for the three variants. Arrows indicate sensorgram generated by each variant at 233nM for comparison. $K_D$ calculated using ‘two state reaction’ model: rFB$_{32R}$: 0.06\(\mu\text{M}\), rFB$_{32W}$: 0.07\(\mu\text{M}\), rFB$_{32Q}$: 0.12\(\mu\text{M}\). (B) rFB was flowed over the C3b-coated chip at concentrations between 233nM and 4nM in the presence of 1\(\mu\text{g/ml fD}\). Sensorsgrams from rFB$_{32R}$ are solid lines, rFB$_{32Q}$ are dotted lines and rFB$_{32W}$ are in gray; identical concentrations are illustrated for the three variants. $K_D$ calculated using ‘two state reaction’ model: rFB$_{32R}$: 2.7nM, rFB$_{32W}$: 6.0nM, rFB$_{32Q}$: 9.5nM.

**Figure 5. SPR analysis of Ba binding to C3b.** (A) The variant fB proteins were purified from plasma of donors known to be homozygous for fB$_{32R}$ or fB$_{32Q}$, and Ba was isolated. (B) Ba was flowed over the surface of the C3b-coated chip at concentrations between 25\(\mu\text{M}\) and 0.4\(\mu\text{M}\) in Biacore buffer (10mM HEPES pH7.4, 50mM NaCl, 1mM MgCl$_2$, 0.005% surfactant P20). Sensorsgrams from Ba$_{32R}$ are solid lines and Ba$_{32Q}$ are dotted lines; identical concentrations are
illustrated for the two variants. (C) Steady state analysis of the data indicate the affinities for C3b in these buffer conditions are: $K_D$ $\text{Ba}_{32R}$: 14.4μM, $K_D$ $\text{Ba}_{32Q}$: 46.0μM. For $\text{Ba}_{32Q}$ a sample at 40μM was included because the affinity was markedly lower than $\text{Ba}_{32R}$.

**Figure 6. Structural analysis of the fB R32Q amino acid substitution.** The crystal structure of human fB shows the SCRs of Ba folded back across the vWF-A domain (left) (Milder et al., 2007, ref 32, Protein Data Bank accession number 2OK5). Amino acid Q34 is illustrated in the structure (right), this is two residues downstream of Q32 and lies in close proximity to D279 and F286, alterations in which are known to affect proenzyme formation (C3bB). The molecular graphic was generated using PyMOL Molecular Graphics System (DeLano, 2002, http://www.pymol.org).