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Disorders of Hemostasis

Molecular characterization of factor X deficiency associated with borderline plasma factor X levels

Borderline plasma factor X (FX) levels might complicate the diagnosis of FX deficiency. An asymptomatic individual with 73% FX activity was identified to be heterozygous for the Val342Ala mutation. Expression studies suggested that this substitution is responsible for a CRM+ FX variant with normal activation but modestly reduced catalytic function.

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Factor X (FX) deficiency is a rare coagulation disorder, inherited as an autosomal recessive trait and characterized by a variable clinical presentation which correlates poorly with the laboratory phenotype.[1] A severe bleeding phenotype is usually associated with homozygous or doubly heterozygous conditions.[2] Heterozygous FX deficiency is generally asymptomatic, and in most cases is identified incidentally during pre-operative screening. The diagnosis of heterozygous FX deficiency and an estimate of the prevalence of this deficiency can be furher complicated by FX levels borderline to the normal range. Borderline levels may represent a general problem for the diagnosis of both hemorrhagic and thrombotic risk conditions. We have previously addressed this issue in asymptomatic FVII deficiency by exploiting a molecular genetic approach.[3]

In this study, we investigated a 51-year old man presenting with 73% FX activity in a prothrombin (PT)-based assay (normal range 77-123%) during a pre-surgery screen. FX antigen levels were not available.

DNA sequencing of the FX gene[4] revealed that the subject was heterozygous for an alanine (GCG) to valine (GTG) substitution at position 342 (cDNA numbering).[5]

Val342 (c160, chymotrypsin numbering) is conserved in FVII, FIX and FXII and is substituted by Ile in protein C. The presence of topologically equivalent mutations in FIX (Val to Phe) and protein C (Ile to Phe) associated with coagulation deficiencies supported the causative role of the Val342Ala substitution. To assess the effect of the Val342Ala mutation on FX function, the 342AFX variant was expressed in human embryonic kidney 293 cells (HEK293) and studied.

Construction of the expression vector, transient transfection of HEK293, determination of antigen levels and functional assays of the r342A-FX variant in conditioned medium were conducted as previously described.[6] The forward primer for FX cDNA mutagenesis was 5'GCTCAAGATGCTGGAGGCGCCCTACGTGGACCG3'. Antigen levels of r342A-FX (418±154 ng/mL) in medium were not significantly different from those of recombinant wild type (rWt)-FX (593±214 ng/mL), thus suggesting that the Val342Ala change does not impair FX biosynthesis or secretion.

Table 1. Activity of the r342A-FX in FX depleted plasma.

Thrombin generation activity	
extrinsic activation	52±4
intrinsic activation	50±6
Amidolytic activity	
extrinsic activation	64±3
Coagulant activity	
PT-based assay	35

Values (% of rWt-FX) are reported as mean ± standard deviation.

In a PT-based assay, the r342A-FX coagulant activity (Table 1) was 35% of rWt-FX activity and was consistent with that measured in the plasma of the heterozygous subject.

Activation of r342A-FX in conditioned medium with increasing concentrations of FVIIa-tissue factor (TF) complex was estimated through Western blot analysis (Figure 1A). At the lowest concentration of activator used the amount of FXaα form was comparable for both variants, thus suggesting normal activation of the r342A-FX molecule. Accordingly, a similar increase in FXa fluorogenic activity of both recombinant proteins was observed at increasing concentration of FVIIa/TF complex (Figure 1B).

The r342A-FX activity was further studied towards thrombin or a peptidyl substrate in FX depleted plasma to reproduce the *in vivo* conditions more precisely. Reduced thrombin generation activity was observed upon both extrinsic (52±4%) and intrinsic activation (50±6%) (Table 1). Once activated by the extrinsic pathway, r342A-FX showed a similarly reduced activity (64±3%) towards a peptidyl fluorogenic substrate. These findings suggested that the mutation was responsible for a dysfunctional FX variant. However, to confirm a conventional CRM+ phenotype the FX antigen evaluation in plasma would be required.

The reduction in activity by both activation pathways, and the parallel reduction in amidolytic and thrombin generation activity predict that the Val342Ala substitution alters the active site rather than the macromolecular interactions in the prothrombinase complex. These experimental observations are supported by the position of Val342, far from exosites known to participate in factor Va interactions.[7] The reduced generation of the γ-form (Figure 1A), deriving from the autocatalytic activity of the r342A-FXaβ form, might further reflect the partially compromised catalytic function of this variant.

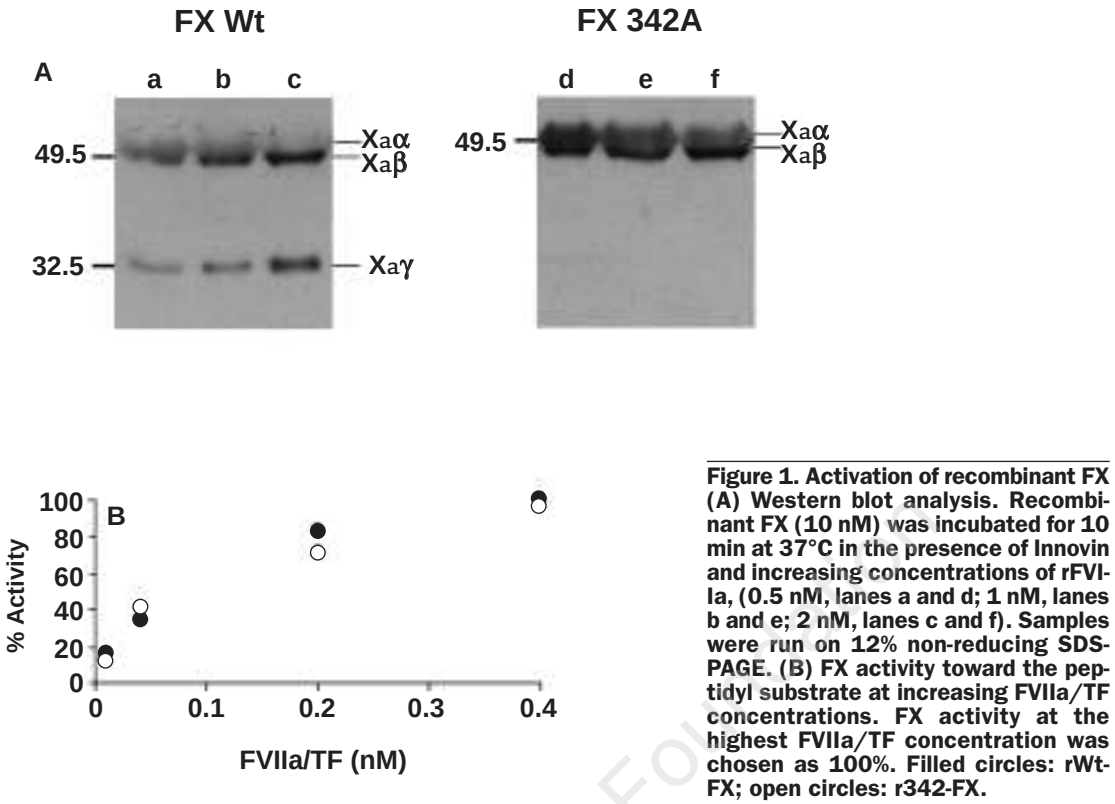

Figure 1. Activation of recombinant FX (A) Western blot analysis. Recombinant FX (10 nM) was incubated for 10 min at 37°C in the presence of Innovin and increasing concentrations of rFVIIa, (0.5 nM, lanes a and d; 1 nM, lanes b and e; 2 nM, lanes c and f). Samples were run on 12% non-reducing SDS-PAGE. (B) FX activity toward the peptidyl substrate at increasing FVIIa/TF concentrations. FX activity at the highest FVIIa/TF concentration was chosen as 100%. Filled circles: rWt-FX; open circles: r342-FX.

In the FXa crystallographic structure,[8] Val342 belongs to a surface β-sheet of the catalytic domain and engages three H-bonds which, in the 342Ala FX molecular model, are partially rearranged. Interestingly, changes in H-bonding in this region, produced by the substitution of the adjacent Pro343 in the FX Friuli (Pro343Ser), caused severe impairment of the catalytic activity as well as of activation.[9] H-bonding involving the highly conserved Pro343 was maintained in the 342Ala FX model, thus potentially explaining the milder effect of the conservative Val342Ala substitution. In conclusion, this study on a new causative mutation in the catalytic domain of FX contributes to defining the heterogeneous molecular basis of FX deficiency[10] and indicates that borderline low FX levels might reflect the presence of a true genetic defect. Functional characterization of this mutation, responsible for remarkable FX residual activity, predicts an asymptomatic clinical phenotype, even in the homozygous or doubly heterozygous condition.

Mirko Pinotti, Monia Monti, Marcello Baroni, Giovanna Marchetti, Francesco Bernardi

Department of Biochemistry and Molecular Biology, University of Ferrara, Italy

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Key words: borderline FX levels, heterozygous FX deficiency, naturally occurring FX variant, recombinant FX.

Correspondence: Professor Francesco Bernardi, Dipartimento di Biochimica e Biologia Molecolare, via L. Borsari 46, 44100 Ferrara, Italy. Phone: international +39.0532.291443. Fax: international +39.0532.202723. E-mail ber@dns.unife.it

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