

Novel Factor V C2-Domain Mutation (R2074H) in Two Families with Factor V Deficiency and Bleeding

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Keywords

Factor V deficiency, parahemophilia, inherited coagulation disorders, Factor V, bleeding

Summary

The molecular basis of Factor V deficiency has been defined in few patients only. We report a homozygous nucleotide change (G6395A) in two Tunisian probands with Factor V deficiency and bleeding episodes. This substitution results in the replacement of an arginine (R) by a histidine (H) in amino acid position 2074, located in the Factor V C2-domain. Mutations in this protein domain have not previously been described. Several lines of evidence support that this sequence variant is indeed disease causing: 1) Crystal structures of Factor V and molecular C2-domain modeling studies of H2074 suggest that the conserved R2074 is required for correct folding; 2) Structure-function studies of selective Factor V mutants (R2074A) demonstrate the importance of R2074 for structural stability of the Factor V C2-domain and for cofactor activity (1); 3) In Factor VIII, point mutations in codon 2209, which corresponds to position 2074 in Factor V, cause hemophilia A.

Introduction

Factor V is a large plasma glycoprotein with a molecular weight of approximately 300 kDa (2) and an A1-A2-B-A3-C1-C2 protein structure which is based on amino acid similarity of individual domains (3). In the coagulation cascade, Factor V is activated by thrombin cleavage and separated from its large protein domain B. The resulting heterodimer Factor Va consists of a 110 kDa heavy chain (protein domains A1 and A2) and a 78 kDa light chain (domains A3, C1 and C2), which are non-covalently linked (3, 4). Factor Va is a cofactor to Factor Xa in the prothrombinase complex that facilitates the activation of prothrombin to thrombin.

Identification of the complete Factor V cDNA sequence (5) and subsequent characterization of the gene structure (4) have demonstrated remarkable similarity between Factors V and VIII. Factors V and VIII share approximately 40% amino acid identity in their respective A domains as well as in their corresponding C domains. Moreover, their overall protein structure is identical (4, 6-8).

Clinical manifestations in Factor V deficient patients include epistaxis, ecchymosis, menorrhagia, and hemarthrosis, as well as bleeding after

surgical procedures and dental extractions (9, 10). Rare intracranial hemorrhage has also been described (11-14). Heterozygous individuals are not generally affected despite decreased levels of Factor V. The bleeding diathesis in both studied families is mild. Proband A presented in early childhood with recurrent epistaxis. After the first decade, the symptoms abated and minor surgical procedures such as circumcision have not posed a need for treatment. The proband is one of eight children of consanguineous parents, who are first cousins. None of his siblings appear clinically affected, although one sister with dramatically decreased Factor V levels has a history of recurrent spontaneous abortions at a gestational age of eight weeks (Fig. 1, family A). Proband B and one brother were hospitalized at the age of six years with intractable epistaxis. Since then, the bleeding syndrome in both the proband and her twin brothers has been mild despite very low Factor V levels. It has decreased in severity and frequency of symptoms, with age (Fig. 1, family B).

Factor V deficiency may be diagnosed in the presence of a prolonged prothrombin time (PT) and partial thromboplastin time (PTT), which can be corrected by addition of adsorbed control plasma. Quantitative deficiencies are confirmed by Factor V antigen assays (15).

The current understanding of the genetic basis of Factor V deficiency states is limited. Several previously reported mutations (16-26) are quantitative defects observed in combination with Factor V Leiden (28, 29), leading to "pseudohomozygosity" for activated protein C resistance and hypercoagulability (Table 1). We now report the study of two different consanguineous Tunisian families with clinical Factor V deficiency and report the identification of a novel homozygous missense mutation in the C2-domain of Factor V, in both families.

Materials and Methods

Subjects and Blood Collection

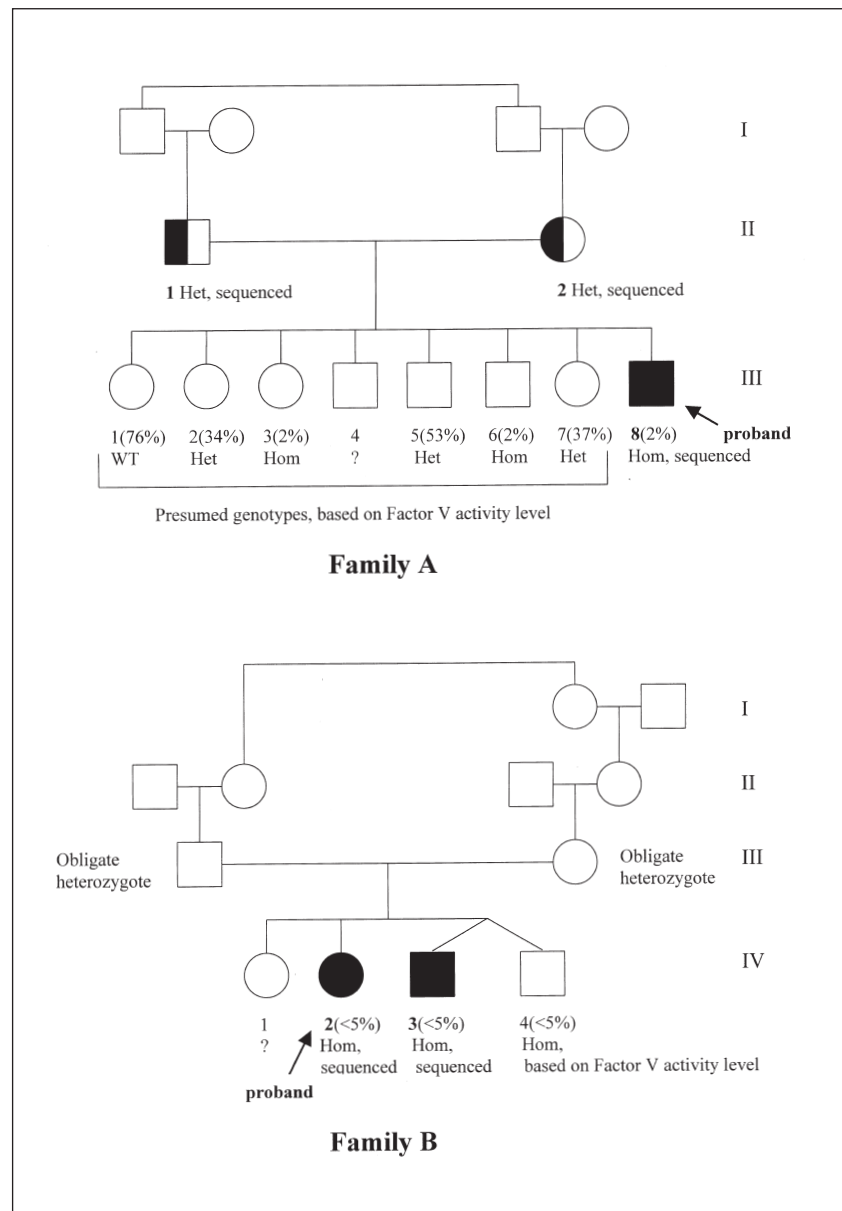
The two probands are patients in the Hematology clinic of Habib Thameur Hospital in Tunis. Both are offspring of consanguineous parents from separate families and have documented Factor V deficiency. Peripheral blood samples were obtained from the probands and consenting relatives who were available for study. The samples were collected in 0.109 M sodium citrate. Plasma was prepared by centrifugation of whole blood at 3000 rpm for 10 min.

Factor V Activity Analysis

Factor V activity studies were performed in 11 individuals using an STA Compact Analyzer (Diagnostica Stago), by measuring the clotting time in the presence of the STA-Neoplastine reagent from the STA-Deficient V kit (Diagnostica Stago). This clotting assay is based on excess presence of all coagulation factors except Factor V, which is derived from the patient sample under investigation. All steps were performed according to the manufacturer's instructions.

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Fig. 1 Abbreviated pedigrees of two consanguineous families with R2074H mutation and Factor V deficiency. Four generations (I, II, III and IV) are depicted to illustrate the source of consanguinity as well as individuals with the G6395A mutation. Sequenced homozygous (Hom) subjects are represented by the black circle and squares, whereas confirmed heterozygosity (Het) is indicated by the half black circle and square. Probands A and B are pointed out by arrows. For clarity, the pedigrees are abbreviated to include only study participants and core family members. Blank squares and circles indicate males and females (respectively) unavailable for molecular genetic evaluation. Individual Factor V activity levels are shown in parentheses. Subjects with bold numbering were included in Factor V activity testing and Factor V mutation analysis (bold).



Factor V Antigen Assays

The Factor V antigen levels were determined using a paired antibody ELISA (Cedarlane Laboratories, Ltd., Hornby, Canada) in both probands, the father of proband A and one brother of proband B. The assay was performed as described by the manufacturer. Briefly, the capture antibody (affinity-purified sheep anti-human FV) was coated onto plates in 50 mM carbonate buffer, pH 9.6. Nonspecific binding was blocked using 1% BSA. Dilutions of normal control plasma and patient plasma were applied to the plates in triplicate. Following incubation and washing, a peroxidase-conjugated sheep anti-human FV detection antibody was added. After additional incubation and washing, antibody binding was detected using O-phenylenediamine (Sigma P6912). A standard curve was constructed from the normal control, and patient antigen concentrations calculated using the standard curve.

PCR Amplification

Genomic DNA was isolated from blood leukocytes according to standard procedures and used for the molecular genetic analysis of the Factor V gene in five consenting subjects. Amplification of genomic DNA was carried out with Amplitaq Gold DNA polymerase 250 Units, 5 U/μl (Perkin Elmer) and the four

2'-deoxynucleotide 5'-triphosphates (Amersham Pharmacia). Primer pairs were designed from intronic sequences that flank individual Factor V exons (Genbank accession numbers AH005274 and Z99572) or generated from reported primer sequences (19, 21). Exons were amplified in their entirety except for the sizeable exon 13, which was amplified in overlapping segments. PCR conditions for all exons are available upon request.

Sequencing Analysis

PCR products were purified by QIAquick PCR purification or QIAquick gel extraction according to the manufacturer's recommendations (Qiagen). Purified samples were sequenced directly with fluorescent terminators (ABI) on an ABI 310 or 377 sequencing instrument (Applied Biosystems).

Subcloning

The 956 bp amplicon of exon 13 segment "e" was subcloned by the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions, using the pCR®2.1-TOPO vector. Following the cloning reaction, TOP10 One Shot™ competent cells were transformed and plated onto Luria-Bertani (LB) agar supplemented to 50 μg/mL with X-GAL (Life Technologies) and to

Table 1 Factor V deficiency mutations

Exon	Domain	Genotypes	Reference
6	A1	C836T; Ala221Val	16
7	A1-A2	A1102T; K310X	25
7	A1-A2	1130 del 8; H351X A5279G; Y1702C	25
10	A2	C1690T; R506X ^{\$}	22
12	A2	T1927C; C585R	25
13	B	C2308T; R712X Factor V Leiden	20
13	B	C2491T; Q773X Homozygous	25
13	B	Del 2833-2834; 900/901X Homozygous	23
13	B	C3571T; R1133X Homozygous	26
13	B	3706 ins TC; 1224X [#] Factor V Leiden	18
13	B	Del 4014-4017; 1303X Homozygous	19
13	B	4291-4294 del C; M1381X Homozygous	25
13	B	4792 [*] ins 4; 1560X Factor V Leiden	21
15	A3	A5279G; Y1702C Factor V Leiden	24
16	A3	G5509A; A1779T Factor V Leiden	17
23	C2	G6395A; R2074H Homozygous	This report

Summary of reported Factor V deficiency genotypes and affected amino acids. Numbering is according to the Factor V cDNA sequence⁵ and the consensus* from the Ad Hoc Committee on Mutation Nomenclature²⁷. "Factor V Leiden" annotation indicates compound heterozygosity for a Factor V deficiency mutation and the Factor V Leiden variant G1691A. #: Numbering revised by Montefusco *et al.*, 2000. \$: also listed in the Human Gene Mutation Database (<http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html>) without genotype/phenotype correlations.

50 µg/mL with carbenicillin (Sigma). After overnight incubation at 37° C, white colonies were picked and expanded for 8 h at 37° C, in 3 mLs of LB broth containing 50 µg/mL carbenicillin. Subsequently, plasmid DNA was isolated and purified using the QIAgen Plasmid Mini kit (QIAgen). Utilizing the plasmid's M13R and T7 priming sites, the plasmids were sequenced with the

BigDye kit for dideoxy-terminator direct sequencing from Applied Biosystems according to protocol. Sequencing products were analyzed on the ABI 310 capillary electrophoresis instrument (Applied Biosystems). Individual sequences were compared and further characterized using GCG SeqWeb (www.gcg.com).

Molecular Modeling

The coordinates of the C2-domain of Factor V (PDBID 1CZT) were used to visualize the interactions of Arg2074 within the structure. Hydrogen bonds were assigned to interatomic distances of less than 3 Å. The alanine and histidine mutations of Arg2074 were analyzed by substitution with the corresponding amino acid, but no energy refinement was carried out. The structure was rendered and the figure produced with the program MIDAS.

Results

Factor V Activity and Antigen Assays

In family A (Fig. 1), Factor V activity levels were determined in seven siblings. In addition to proband A, two other siblings have Factor V levels that are consistent with homozygosity for a Factor V deficiency genotype. Siblings 2, 5, and 7 have moderately decreased Factor V activity (34%, 53%, and 37%, respectively), consistent with heterozygosity for Factor V deficiency. One sister has normal Factor V activity at 76% of the control value (normal range 60-120%). Factor V antigen levels in proband A are 3%, whereas his father has antigen levels of 16%.

Factor V activity was measured in three affected siblings of family B. All have severely decreased Factor V activity (< 5%), in agreement with homozygous Factor V deficiency. Factor V antigen levels in this family are low, as well. Proband B has a level of 6% and her affected sibling (patient IV-3) has antigen levels of 3%. The concordant decrease in activity and antigen levels is consistent with Type 1 Factor V deficiency.

Identification of a Novel Mutation in the C-domain of Blood Coagulation Factor V

Direct genomic DNA sequencing of the 25 Factor V exons and sequencing of the subcloned region of exon 13 revealed a novel non-synonymous G to A transition in nucleotide position 6395 (5) of the Factor V cDNA. This sequence variant, present in the second position of amino acid 2074 (nucleotide 112 of exon 23), changes the wild-type arginine residue to a histidine. It was identified in probands A and B, who were both homozygous 6395A. Genetic studies of participating

Sequence identification	Species	Amino acid sequence	Genbank accession number
Factor V	Human	WEPFR <u>AR</u> LNAQGRVNAW	Z99572
Factor V	Porcine	WEPFR <u>AR</u> LNAQGRVNAW	AF191308
Factor V	Bovine	WEPFL <u>AR</u> LNAQGRVNAW	M81440
Factor V	Murine	WEP <u>SL</u> <u>AR</u> LNAQGRVNAW	U52925
Factor VIII	Human	WSPSK <u>AR</u> LHLQGRSNAW	M88648
Factor VIII	Canine	WSPSQ <u>AR</u> LHLQGRSNAW	AF049489

Comparison of the R2074 residue and surrounding amino acids between human coagulation Factors V and VIII and between human Factor V and Factors V and VIII of other mammals. The underlined segment of three amino acids that contains R2074 is strictly conserved. Amino acid positions are reported according to Genbank annotations (<http://www.ncbi.nlm.nih.gov/>).

Table 2 Alignment of partial human Factor V amino acid sequence with other species and Factor VIII

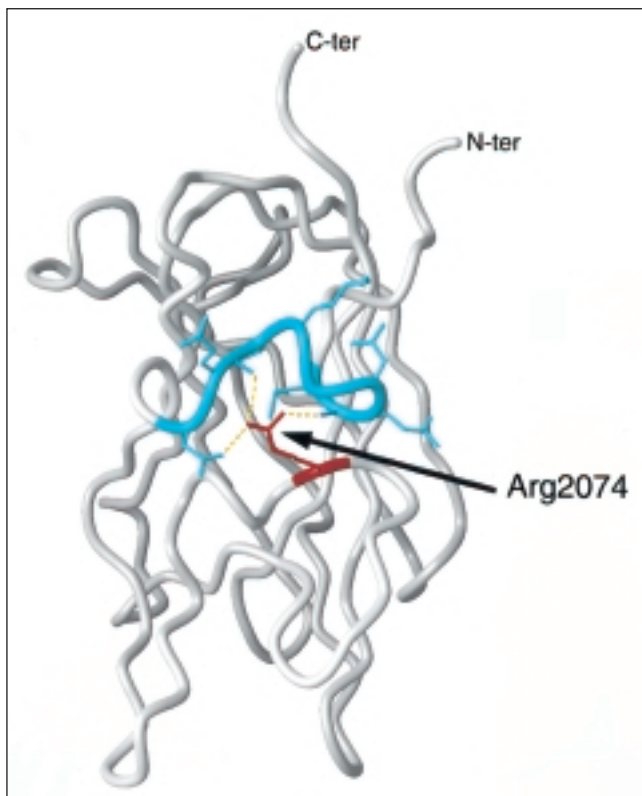


Fig. 2 Molecular modeling of R2074 in the Factor V C2-domain. The predicted interactions of the R2074 residue with surrounding residues in the C2-domain are exhibited in this model, based on the coordinates of the Factor V C2-domain (PDBID 1 CZT). R2074 is most likely to form hydrogen bonds with residues 2044 and 2049, as well as a salt bridge with D2051

family members demonstrated homozygosity for this mutation in IV-3 of family B (Fig. 1) who has clinical features of Factor V deficiency. In agreement with an autosomal recessive pattern of inheritance, the clinically unaffected parents of proband A (II-1 and II-2) are heterozygous.

The newly identified G6395A mutation is located in the C2-domain of Factor V. This region is encoded by exons 23 through 25 and forms the carboxy terminal part of the Factor V protein (4). Comparison of a C2-domain subsection with the corresponding segment of human Factor VIII and with both Factors V and VIII in other species, as submitted to Genbank (30), shows complete conservation of R2074 and the two amino acids (A2073 and L2075) that surround it (31). The aligned stretch of 17 amino acids that border R2074 is 65% conserved with human and canine Factor VIII. It is identical to the porcine Factor V section, 94% conserved in cow and shares 88% amino acid identity with the matching segment in the mouse (Table 2).

In addition to the R2074H mutation, four previously described sequence variants were identified (Whitehead cSNP database; http://waldo.wi.mit.edu/cvar_snps/). Both probands were homozygous C for a G409C transversion in exon 3, which changes an aspartic acid to a histidine (D78H) and has a reported frequency of 5-15%. Homozygosity for two polymorphisms in exon 4 was also present in both probands. The synonymous G495A and G642T variants have reported frequencies of 5-15% (3) (Whitehead cSNP database). Finally, we identified a non-synonymous G1628A polymorphism in exon 10. This variant changes an arginine residue to a lysine in amino acid position 485 and is thought to be clinically innocent. The allele

frequency is 2.9% (19, 32). None of these polymorphisms is associated with Factor V deficiency.

Molecular Modeling of R2074

As shown in Fig. 2, R2074 plays a critical role in stabilizing the C2-domain loop of residues 2044-2051 (shown in cyan), forming a salt bridge with Asp 2051, and hydrogen bonds with the main chain carbonyls of residues 2044 and 2049 (distances all < 3 Angstroms). Alanine substitution (R2074A) results in loss of all of these stabilizing interactions, and would be predicted to destabilize the molecule and alter the conformation. This is consistent with the experimental observation that the R2074A substitution results in loss of monoclonal antibody binding and loss of Factor V function. Similarly, the R2074H mutation results in the possibility of a hydrogen bond only with the main chain carbonyl of residue 2044 and would be predicted to have a similar destabilizing effect on the C2-domain.

Discussion

Factor V deficiency (parahemophilia, OMIM #227400) has been described in both sexes, and occurs without geographic or ethnic preference (9). We have studied two Tunisian probands with mild bleeding episodes and identified homozygosity for the same missense mutation (G6395A) in both families. This mutation occurs in Factor V exon 23, causes amino acid replacement R2074H, and is located in the amino-terminal side of the C2-domain, an integral part of the Factor Va light chain (protein domains A3-C1-C2) (3, 4).

Direct support for G6395A pathogenicity is imparted by homozygosity of probands A and B while both unaffected parents of proband A are carriers. The parents of proband B were not available for our study. In addition, Factor V activity is severely decreased in all three homozygous individuals and only moderately reduced in presumed heterozygotes (Fig. 1). The Factor V antigen assay demonstrates levels well below 10% in both probands, which is consonant with a Type 1 deficiency of the mutant Factor V protein.

Several recent studies provide additional support for a pathologic role of mutations at R2074. Factor VIII, which accelerates Factor X activation through interaction with Factor IXa, is very similar to Factor V in both structure and function. Whereas Factor Va combines with Factor Xa in order to generate the prothrombinase complex, the second C-domain of both activated cofactors V and VIII interacts with cellular surfaces by binding to phospholipids (8, 33). Complete lack of binding was demonstrated in recombinant deletion analysis of the Factor V C2-domain. In association with the hampered binding, marked loss of procoagulant activity was observed (34). Recombinant chimeras with partial substitutions of the C2-domain revealed a phosphatidylserine binding site in the amino-terminal exon 23, where R2074 is located. Binding is inhibited in the presence of natural or monoclonal immunoglobulins that recognize epitopes between amino acid residues 2037 and 2087 (35). The resolution of the Factor V-C2 crystal structure has enabled comparisons between the overall folds of both C1 and C2-domains of Factors Va and VIIIa, which proved to be very similar. Among other residues, R2074 is critical for proper folding and strictly conserved across species, between Factors V and VIII and between C1 and C2 protein domains (36). We have performed a molecular modeling study of R2074 (Fig. 2) which illustrates its position and most likely interactions with nearby residues in the C2 loop. Kim et al. (1) explored the function of individual amino acids in the C2-domain by selective mutagenesis of charged residues to alanine. The individual mutant

R2074A had a specific activity of less than 15% of normal, as measured by determination of thrombin generation in a prothrombinase assay. Binding to phosphatidylserine and phosphatidylcholine was reduced concomitantly. Poor binding of two monoclonal antibodies with non-overlapping C2 epitopes implicated improper folding as a reason for the decreased binding. From these data, the authors concluded that R2074 is necessary for the structural integrity of the domain.

The compromise in structural integrity of the C2 domain that has been predicted to result from R2074 replacement (1) must be placed in light of our *in vivo* findings. The low levels of Factor V antigen in our patients indicate that the mutation does not affect the C2-domain in isolation. Based on the type of mutation, it is most likely that the protein is synthesized, but either secretion or stability of the Factor V protein are severely affected, leading to a concomitant decrease in measured Factor V activity.

Factor VIII mutations in the corresponding position (R2209) include substitutions by glutamine, leucine, and glycine, as well as nonsense mutations (37) (Haemophilia A Mutation Database: <http://europium.csc.mrc.ac.uk>). All lead to hemophilia A, which highlights the pathologic consequence of altering this amino acid. Clinical expression varies from mild to severe, even in patients with identical mutations. In Factor V deficiency, correlation between plasma Factor V levels and bleeding symptoms is not always consistent either, and patients without significant manifestations have been reported (8, 9, 38). The same is evident in the families described in this report. Whereas phenotypic variability between patients may be due to allelic heterogeneity, intrafamilial variability may be explained by epigenetic events and environmental factors. Therefore, genotype-phenotype correlations in Factor V deficiency should be made with caution.

Even though Factor V deficiency is a rare bleeding disorder, it underscores the pathophysiologic complexity of defects in the coagulation pathway. The mutation that we identified in two families of Tunisian extraction and further characterized in this report illustrates the functional importance of conserved arginine residues in Factors V and VIII, and demonstrates the consequences of R2074 replacement in the Factor V C2-domain, *in vivo*.

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Received July 9, 2001 Accepted after resubmission November 21, 2001