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ORIGINAL ARTICLE

Analysis of newly detected mutations in the *MCFD2* gene giving rise to combined deficiency of coagulation factors V and VIII

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Summary. Combined deficiency of coagulation factor V (FV) and factor VIII (FVIII) (F5F8D) is a rare autosomal recessive disorder characterized by mild-to-moderate bleeding and reduction in FV and FVIII levels in plasma. F5F8D is caused by mutations in one of two different genes, *LMAN1* and *MCFD2*, which encode proteins that form a complex involved in the transport of FV and FVIII from the endoplasmic reticulum to the Golgi apparatus. Here, we report the identification of a novel mutation Asp89Asn in the *MCFD2* gene in a Tunisian patient. In the encoded protein, this mutation causes substitution of a negatively charged aspartate, involved in several structurally important interactions, to an uncharged asparagine. To elucidate the structural effect of this mutation, we performed circular dichroism (CD) analysis of secondary structure and stability. In addition, CD analysis was performed on two missense mutations found in previously reported F5F8D patients. Our results show that all analysed mutant variants give rise to destabilized proteins and highlight the importance of a structurally intact and functional MCFD2 for the efficient secretion of coagulation factors V and VIII.

Keywords: circular dichroism, combined FV and FVIII deficiency, LMAN1, MCFD2, mutations

Introduction

Combined deficiency of coagulation factor V (FV) and factor VIII (FVIII) (F5F8D; OMIM 227300) is an inherited human bleeding disorder first described by Oeri *et al.* [1]. Congenital F5F8D is estimated to be extremely rare (1:100 000), affecting males and females in equal numbers. In the general population, increased frequency is associated with consanguineous marriage [2]. Affected individuals are characterized by concomitantly low levels of both FV and FVIII, usually between 5% and 30% of normal values, which leads to a mild-to-moderate bleeding tendency, with normal platelet count, prolonged prothrombin time (PT) and partial thromboplastin time tests (PTT) [3]. The most common clinical features associated with F5F8D are epistaxis, menorrhagia and excessive bleeding during or after trauma, surgery, or labour. Treatment of bleeding episodes requires a source of both FV and FVIII. Replacement of FV is achieved through the use of fresh frozen plasma, and replacement of FVIII is achieved by using Desmopressin, specific FVIII concentrates, or plasma derived FVIII or recombinant FVIII products [4].

F5F8D is caused by mutations in one of two different genes, *LMAN1* and *MCFD2*, mapped respectively to chromosomes 18 (2p21) and 2 (2p16.3) [5,6]. It is now established that the gene products, LMAN1 and MCFD2, form a stable, Ca^{2+}-dependent complex with 1:1 stoichiometry that specifically aids in the transport of glycosylated FV and FVIII in the early secretory pathway [7]. This was confirmed by the recently reported crystal structure of a complex of the carbohydrate binding domain (CRD) of LMAN1 and MCFD2 [8,9].

To date, at least 51 mutations affecting the LMAN1 and MCFD2 proteins have been described, 70% of which are located in the *LMAN1* gene [10]. All *LMAN1* mutations reported to date are null mutations except for two, one that disrupts disulfide bond formation required for oligomerization and one that causes structural destabilization [11–13]. In contrast, 12

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missense mutations have been identified in the *MCFD2* gene [10,14,15]. All these mutations except one, which is found in the binding surface to LMAN1, have been shown to give rise to a disordered or severely destabilized MCFD2 protein [8,16,17].

The 3D-structure of human MCFD2 determined by nuclear magnetic resonance (NMR) methods [16] showed that the protein is built up of two EF-hand motifs and revealed that binding of Ca^{2+} ions to these motifs induces folding from a disordered apo-state. Notable features are that the N-terminus of MCFD2 remains disordered even in the Ca^{2+}-bound state and that the linker region between the two EF-hand motifs is largely unstructured. From the crystal structure of the complex of LMAN1-CRD/MCFD2, the interaction interface of MCFD2 to LMAN1 could be located at the EF hand domain [8,9]. Comparison of the complex structure with the structures of the unbound proteins shows that MCFD2 undergoes conformational changes upon complex formation, while no major structural changes can be detected in LMAN1. This indicates that structural changes in MCFD2 are important for the formation of a functional ternary cargo receptor complex.

Here, we report the identification of a novel mutation Asp89Asn in the *MCFD2* gene in a Tunisian patient and elucidate the structural consequence of this mutation using circular dichroism (CD) analysis. In addition, we performed CD analysis on two missense mutations carried together in previously reported F5F8D patients, Asp81His and Val100Asp, in homozygous state and heterozygous state respectively [18].

Patients and methods

Patient

The patient, a 22-year-old woman of Tunisian origin, is the offspring of a consanguineous marriage. The symptoms are menorrhagia, epistaxis and gingivorrhagia. Laboratory tests show PT and PTT with lowered levels of FV = 10% and FVIII = 19% of normal levels.

Mutation screening

Blood samples were obtained from the patient after consent was signed by her. Genomic DNA was prepared from whole blood using a commercial DNA extraction kit QiaAmp (Qiagen, Crawley, UK). The *LMAN1* and *MCFD2* coding regions, including intron–exon boundaries, were amplified by PCR. Primers and PCR conditions are available upon request. PCR products were directly sequenced with the same primers as used for the amplification reactions. All detected gene variations have been sequenced and confirmed. Mutation nomenclature was assigned according to Human Genome Variation Society guidelines (http://www.hgvs.org). Nucleotide numbering is relative to the initiation codon (ATG) of the GenBank sequence NM_139279 for the *MCFD2* gene. The numbering of the amino acids is according to the Swiss-Prot PZES.

Cloning

Full-length human MCFD2 residues 27–146 (the mature protein, excluding the signal sequence) was cloned as described previously [16]. Amino acid substitutions were introduced using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's recommendations.

Expression and purification

Recombinant wild type MCFD2 and mutant variants were expressed in *Escherichia coli* and purified by Ni-affinity chromatography and gel filtration as described previously [16].

Circular dichroism spectroscopy

Circular dichroism measurements were performed on a JASCO J-810 spectro-polarimeter using a cuvette of 0.1 cm path length. All proteins were at a concentration of 0.1 mg mL^{-1} in 5 mM Bis-Tris buffer pH 7.4 with or without 5 mM $CaCl_2$. Spectra were recorded from 195 to 260 nm at 20°C. Ten scans were averaged and corrected for the buffer spectrum. Thermal unfolding profiles were recorded by monitoring the CD signal at 222 nm between 15 and 90°C using a heating rate of 2°C min^{-1}. Thermal denaturation was reversible in all cases. Melting points (T_m) from experimental data were calculated using GraphPad Prism (GraphPad Software, Inc, San Diego, CA, USA).

Results

Identification of a novel mutation Asp89Asn in exon 3 of MCFD2 in a Tunisian patient

The *LMAN1* and *MCFD2* genes were screened for mutations to identify the cause of F5F8D in a 22-year-old woman of Tunisian origin. The results of direct sequencing demonstrate the presence of a novel mutation (c.265G>A) in exon 3 of *MCFD2* in a homozygous state, while mutations were absent in the *LMAN1* gene (Fig. 1a). In the encoded protein, this mutation causes substitution of a negatively charged aspartate to an uncharged asparagine in the first EF hand, p.Asp89Asn (Figs 1b and 2a).

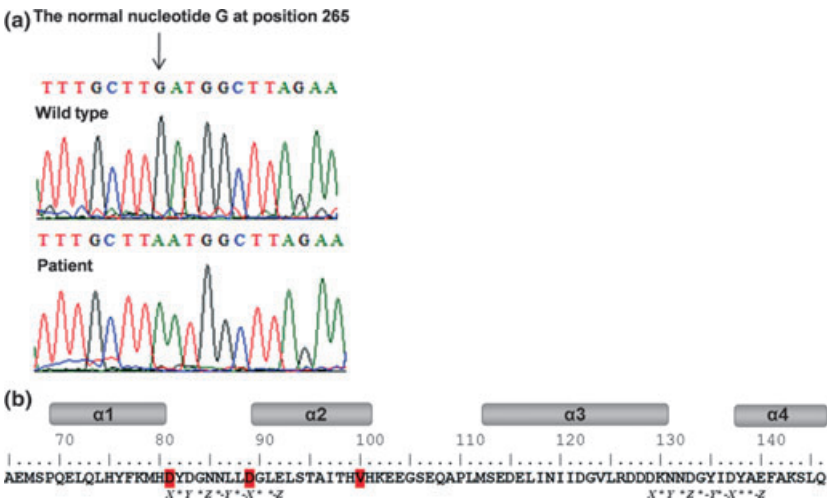

Fig. 1. (a) DNA sequence surrounding nucleotide 265 at MCFD2 exon 3. Wild type: the normal nucleotide (G) is indicated by an arrow. Patient: a homozygous mutation G to A transition at nucleotide 265. (b) Amino acid sequence of the two EF-hand motifs of native human MCFD2. Helical secondary structure is indicated by cylinders above and the conserved EF-hand loop motifs are identified below. Mutant variants analysed in this study are highlighted in red.

Circular dichroism analysis

To clarify the structural effect of the mutation Asp89Asn reported in this study, we performed a CD analysis of secondary structure and stability. In addition, a recently described F5F8D causing mutant variant Asp81His-Val100Asp and the separate effect of the single mutations Asp81His and Val100Asp were analysed (Figs 1b and 2b).

Recombinant wild-type MCFD2 and mutant variants were produced in *E. coli* and purified to homogeneity for the CD analysis. Far-UV CD measurements of the mutant variants, Asp81His and Asp81His-Val100Asp, in the presence of Ca^{2+} both resulted in spectrum profiles similar to the disordered apo state of native MCFD2, indicating that they have lost the ability to bind Ca^{2+} and are disordered (Fig. 2c). The spectra of the mutants, Asp89Asn and Val100Asp, both have features of α-helical secondary structure, with change of sign from negative to positive at 197 nm and minima at 207 and 222 nm, and resemble the Ca^{2+} bound form of native MCFD2. However, the thermal melting profiles of Asp89Asn and Val100Asp yield T_m values of 45 and 44°C respectively, which are significantly lower compared to 59°C for wild type MCFD2 (Fig. 2d). Thus, both these mutations result in reduced structural stability.

Discussion

In the present study, we present a novel mutation (c.265G>A; p.Asp89Asn) in exon 3 of *MCFD2* in a homozygous state. In addition to this mutation, we have previously reported F5F8D patients with two missense mutations carried together, Asp81His and Val100Asp, in homozygous state and heterozygous state respectively [18,19]. Both these mutations were also found in exon 3 of *MCFD2*.

NMR studies have shown that the structural integrity of MCFD2 relies on the ability to bind calcium ions and formation of a small hydrophobic core [16]. The structural destabilization seen in these newly reported mutations in our CD analysis can be associated with these structural features of MCFD2. The residue Asp89 is localized in the first EF-hand motif and forms a hydrogen bond to a water ligand of the Ca^{2} ion. Most likely, the substitution of this aspartate by an asparagine reduces the Ca^{2+} binding affinity. This, in combination with that the side chain of Asp89 caps the N-terminus of the helix after the Ca^{2+} binding loop, presumably gives rise to the structural destabilization demonstrated by the lowered T_m-value. Moreover, Asp89 forms a salt bridge with Lys53 of LMAN1 upon complex formation and substitution to the uncharged asparagine could be expected to lead to reduced binding affinity (Fig. 2a). Recent studies performed in living cells have, however, shown that the mutation Lys53Ala in LMAN1 has no effect on the interaction between LMAN1 and MCFD2. Thus, the main effect of the Asp89Asn mutation is a structural destabilization of MCFD2 and, as a consequence of that, F5F8D. The importance of this residue for the structural integrity of MCFD2 has also been shown in previous CD analysis of the patient mutation Asp89Ala [8,17]. The two other mutations, namely Asp81His and Val100Asp, are also localized within the first EF-hand motif (Fig. 2b). Asp81 is the first residue of the first EF-hand Ca^{2+} binding loop and coordinates Ca^{2+} through the oxygen of the side-chain carboxyl group. This residue is highly conserved and forms several hydrogen bonds that contribute to the specific structural conformation of the EF-hand motif. The substitution of this aspartate to a histidine prevents calcium binding and thereby disrupts the formation of the core structure, giving an explanation to the disordered

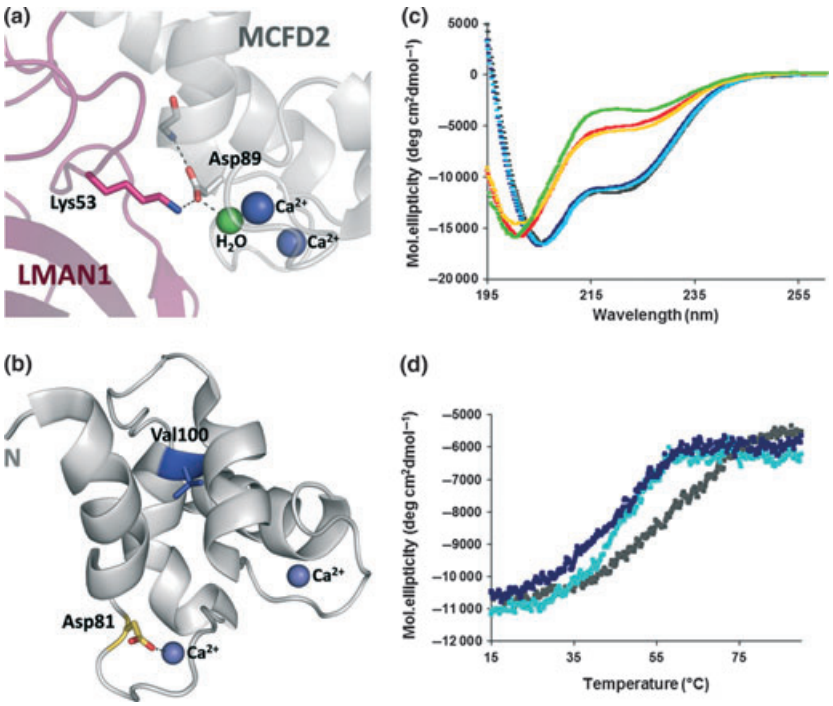

Fig. 2. (a) Close-up view of the crystal structure of the complex of MCFD2 in grey and the CRD of LMAN1 in pink (PDB code 3LCP). The mutant variant Asp89Asn and interacting residues are shown as sticks, with bound Ca^{2+} ions (blue) and water molecules (green) as spheres. Figs 2a, 2b and 3 were prepared with PyMol [20]. (b) NMR structure of MCFD2 in grey (PDB code 2VRG), bound Ca^{2+} ions as spheres (blue) and the positions of the F5F8D-causing mutations are shown as stick models Asp81His (yellow) and Val100Asp (blue). (c) Far-UV CD spectra of MCFD2 and mutant variants. Asp81His (yellow), Asp81His-Val100Asp (red), Val100Asp (blue), Asp89Asn (cyan), folded wild type (grey) and the Ca^{2+} free apo form (green). (d) Thermal unfolding experiments performed monitoring the CD signal at 222 nm. Mutant data are shown with the same colour coding as in (c) and folded Ca^{2+} bound wild type in grey.

spectrum profiles of the mutant variants, Asp81His and Asp81His-Val100Asp. A previously analysed mutant variant with an amino acid substitution at the same position, Asp81Tyr, resulted in a similar disordered spectrum profile, highlighting the significance of this residue in folding of MCFD2 to its correct structure [8].

The mutation Val100Asp is not a confirmed disease-causing mutation, but we wanted to see the effect of this mutation as it has been found in a heterozygous state together with Asp81His in a homozygote state. In the NMR structure of MCFD2 Val100 is part of the hydrophobic core (Fig. 2b) and substitution of this valine with a charged aspartate disrupts the hydrophobic core and has a destabilizing effect, as indicated by the lowered T_m. Interestingly, Val100 is part of a region involving helix 2 and the proceeding loop that is not seen in the electron density in the crystal structure of the LMAN1-CRD/MCFD2 complex, indicating that this region is highly flexible. Moreover, in the NMR structure of uncomplexed MCFD2, helix 2 is slightly bent and four residues longer than in the complex (Fig. 3). Thus, Val100 is part of a region that undergoes conformational changes upon complex formation. It is possible that these structural changes in MCFD2 are needed for the formation of a stable ternary cargo receptor complex. However, further experiments are needed to provide evidence for this hypothesis.

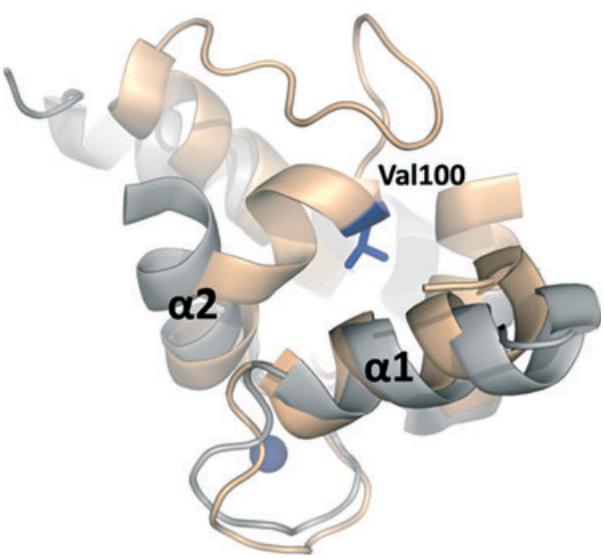

Fig. 3. Superposition of the MCFD2 structure determined by NMR (light brown) and MCFD2 in complex (grey). Val100 shown as sticks in blue at the end of helix 2.

In conclusion, we have shown that the mutant variants, Asp89Asn, Asp81His, Val100Asp and Asp81His-Val100Asp, give rise to destabilized proteins, which may prevent formation of a functional LMAN1/MCFD2 transport receptor complex, thus leading to inefficient transport of FV and FVIII.

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Authors' Contributions

H.Elmahmoudi, E.Wigren and Y.Lindqvist designed the research study, analysed the data and wrote the paper; H. Elmahmoudi, E. Wigren and A.Jlizi performed the research; A.Laatiri and E.Gouider performed clinical evaluation of the patient; A.Elgaaied, E.Gouider and Y.Lindqvist supervised the project.

Disclosures

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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