EF-hand domains of MCFD2 mediate interactions with both LMAN1 and coagulation factor V or VIII

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Combined deficiency of factor V and factor VIII (F5F8D) is a bleeding disorder caused by mutations in either LMAN1 or MCFD2. LMAN1 (ERGIC-53) and MCFD2 form a Ca2+-dependent cargo receptor that cycles between the endoplasmic reticulum (ER) and the ER-Golgi intermediate compartment for efficient transport of FV/FVIII from the ER to the Golgi. Here we show that the C-terminal EF-hand domains are both necessary and sufficient for MCFD2 to interact with LMAN1. MCFD2 with a deletion of the entire N-terminal non-EF hand region still retains the LMAN1-binding function. Deletions that disrupt core structure of the EF-hand domains abolish LMAN1 binding. Circular dichroism spectroscopy studies on missense mutations localized to different structural elements of the EF-hand domains suggest that Ca2+-induced folding is important for LMAN1 interaction. The EF-hand domains also mediate the interaction with FV and FVIII. However, mutations in MCFD2 that disrupt the tertiary structure and abolish LMAN1 binding still retain the FV/FVIII binding activities, suggesting that this interaction is independent of Ca2+-induced folding of the protein. Our results suggest that the EF-hand domains of MCFD2 contain separate binding sites for LMAN1 and FV/FVIII that are essential for cargo receptor formation and cargo loading in the ER. (Blood. 2010;115:1081-1087)

Introduction

Combined deficiency of factor V and factor VIII (F5F8D) is an autosomal recessive disorder. Patients with F5F8D show mild to moderate bleeding symptoms. Levels of FV and FVIII in plasma generally fall to between 5% and 30% of the normal.1-3 Genetic studies identified mutations in LMAN1 (lectin, mannose binding 1) or MCFD2 (multiple coagulation factor deficiency protein 2) as the cause of F5F8D.4-5 LMAN1 (also known as ERGIC-53) is a type 1 transmembrane protein with homology to leguminous lectins6,7 that cycles between the endoplasmic reticulum (ER) and the ER-Golgi intermediate compartment (ERGIC).8-10 Previous studies show that LMAN1 exists as hexamers in cells,11 and it forms a protein complex with MCFD2 with a 1:1 stoichiometry.12 Missense mutations of MCFD2 found in F5F8D patients disrupt its interaction with LMAN1,5,13 suggesting that LMAN1-MCFD2 complex formation is important for efficient FV and FVIII secretion.

The ER luminal side of LMAN1 contains a mannose-binding carbohydrate recognition domain (CRD) and a series of 4 α-helices that are predicted to form coiled-coil domains. The crystal structure of the CRD is similar to Ca2+-dependent leguminous lectins and the ER chaperone calnexin, with 2 Ca2+-binding sites.11,14 FVIII can interact with the LMAN1-MCFD2 complex in a Ca2+-dependent manner,12 suggesting that the LMAN1-MCFD2 complex is a cargo receptor, which directly binds and recruits FV and FVIII in the ER for packaging into ER exit vesicles. The cytoplasmic tail of LMAN1 contains a diphenylalanine ER exit motif,16 which binds to the Sec24 subunit of COPII coat proteins that are responsible for generating cargo-containing transport vesicles that bud from the ER.17,18 FV- and FVIII-containing vesicles are then moved to the ERGIC and eventually to the cis-Golgi. The cytoplasmic tail of LMAN1 also has a dileucine ER retrieval signal that directs the protein into the COPI-coated retrograde vesicles that can bring the LMAN1-MCFD2 complex back to the ER, presumably on releasing FV/FVIII cargo in the ERGIC.8 MCFD2 is a 16-kDa soluble protein with an N-terminal sequence of unknown structure and 2 calmodulin-like EF-hand domains at the C terminus.5 MCFD2 lacks sorting signals and is localized to the ERGIC via its interaction with LMAN1.3 MCFD2 can also bind FVIII independent of LMAN1 and the glycosylation state of FVIII.13 A recently solved NMR structure revealed that in the absence of Ca2+ or with EF-hand domain mutations, MCFD2 largely exists in a disordered apo state.19 In the presence of Ca2+, the EF-hand domains form a structure similar to that of calmodulin. In contrast, the N-terminal sequence of the molecule remains disordered even in the Ca2+-bound state. The linker region between the 2 EF-hand domains is also largely unstructured. Despite our knowledge on the individual structures of LMAN1 and MCFD2, it is not clear how LMAN1 and MCFD2 bind to each other to form a complex, and how the complex recognizes FV/FVIII.

In mammals, the LMAN1-MCFD2 complex is so far the only known example of a specific receptor for soluble cargo proteins. The requirement of both a transmembrane component (LMAN1) and a soluble cofactor (MCFD2) is a unique feature of this secretory pathway. Understanding how the receptor complex is organized and how it recognizes the FV/FVIII cargo proteins is important in understanding the mechanism of this receptor-mediated secretory pathway. In this study, we present evidence that the EF hands of MCFD2 contain separate binding sites required for...
metabolically labeled with [35S] methionine/cysteine (250 Ci/mL) in methionine/cysteine-free Dulbecco modified Eagle medium (MP Biomedicals) followed by a 30-minute incubation in complete medium.

**Crosslinking and immunoprecipitation**

Crosslinking using dithio-bis(succinimidyl propionate) (DSP; Thermo Electron) and immunoprecipitation were described previously. Briefly, cells were washed 3 times in ice-cold phosphate-buffered saline, pH 7.4, containing 0.9mM CaCl₂ and 0.5mM MgCl₂ and then incubated with 1mM DSP in crosslinking buffer (phosphate-buffered saline, pH 7.4, with 1mM CaCl₂ and 100mM glucose) on ice for 1 hour with gentle shaking. The crosslinking reaction was quenched in 20mM Tris-HCl, pH 7.5, for 5 minutes. Cells were lysed in Nonidet P-40 buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 1% Nonidet P-40, 0.05% sodium dodecyl sulfate, 2mM CaCl₂) plus protease inhibitors and subjected to immunoprecipitation using various antibodies. Immunoprecipitates were washed 3 times using NP-40 buffer with 0.5M NaCl. Proteins were separated on 4% to 12% Criterion Bis-Tris gels (Bio-Rad) using MES running buffer and visualized by exposing to a Kodak Bio-Max film using a Kodak Biomax Transcreen LE. Each experiment was performed at least twice.

**Immunofluorescence**

Immunofluorescence staining of HeLa cells transfected with different MCFD2 and LMAN1 expression vectors was previously described. Images were viewed on a Leica DMRXE confocal microscope (Wetzlar) using a 40× oil-immersion objective with a 1.25 numeric aperture. Confocal images were acquired using the Leica Confocal Software, Version 2.61.

**Purification of MCFD2 proteins**

Cloning of MCFD2 into pET15b was described previously. The D89A and D122V point mutations were introduced into the DNA construct using the Stratagene mutagenesis II XL kit (Stratagene) according to manufacturer’s instruction. All mutation constructs were confirmed by DNA sequencing. Mutagenesis primer sequences are available on request. A human ceruloplasmin expression construct (in pcDNA3 vector) is a gift from P. Fox (Cleveland Clinic Foundation).

**COS-1** cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 IU/mL streptomycin. Cells were transfected using FuGENE 6 (Roche Diagnostics) according to the manufacturer’s instructions. Twenty hours after transfection, cells were metabolically labeled with [35S] methionine/cysteine (250 µCi/mL in methionine/
Circular dichroism spectroscopy
CD spectra were determined on a Jasco J-815 CD spectropolarimeter using a cuvette of 2-mm path length. Before measurement, all protein concentrations were adjusted to 0.1 mg/mL in 5mM Bis-Tris buffer, pH 7.0, with 10mM CaCl2. To remove Ca2+ from MCFD2, protein was incubated with 5mM Bis-Tris buffer, pH 7.0, with 10mM ethylenediaminetetraacetic acid for 2 hours and dialyzed against 5mM Bis-Tris buffer, pH 7.0, at 4°C overnight. The spectra were measured from 200 to 250 nm at room temperature. Each sample was scanned 11 times. The scans were averaged after correction for the buffer spectrum. Secondary structure proportions were calculated using programs SELCON3, CDSSTR, and CONTINLL (CDPro package; Colorado State University).

Results
MCFD2 missense mutations differentially affect the protein structure
To date, 6 MCFD2 missense mutations have been identified in F5F8D patients,3 all of which are localized to the EF-hand domains. The EF-hand domains are characteristic structures consisting of a Ca2+-binding loop flanked by 2 α-helices. The 4 α-helices are hereby designated helix 1 through 4 (Figure 1A). Most mutations (D81Y, D89A, D129E, Y135N, and I136T) are localized to the Ca2+-binding loops of both EF-hand domains (Figure 1A). D122V, the only mutation located outside the Ca2+-binding loops, replaces a charged amino acid with a hydrophobic one on helix 3 in the second EF-hand domain (Figure 1A; supplemental Figure 1, available on the Blood website; see the Supplemental Materials link at the top of the online article).

To investigate the consequences of different missense mutations on MCFD2 secondary structure, we performed CD spectroscopy measurements on the wild-type MCFD2, as well as MCFD2 with the D89A, D122V, or I136T mutations in the presence of Ca2+ (Figure 1B). The spectrum of the wild-type MCFD2 shows minima at approximately 208 nm and 222 nm. The spectra of D89A and I136T mutants show a shallow 222-nm band and a minimum shifted from 208 nm to approximately 205 nm, consistent with previous reports.19 Interestingly, MCFD2 with the D122V mutation displays a completely different CD spectrum (Figure 1B). The 208-nm band is much smaller than the 222-nm band, so that it largely overlaps with the 222-nm band to form a single broad minimum at 222 nm. We calculated portions of secondary structures in MCFD2 proteins using 3 different computer programs SELCON3, CDSSTR, and CONTINLL.20 All 3 programs predict that the D122V mutant contains a smaller portion of α-helix than the wild-type MCFD2, whereas the D89A and I136T mutations have no significant effect on the amount of α-helical content (data not shown). In the absence of Ca2+, CD spectrum of the wild-type MCFD2 changed to a pattern similar to those of D89A and I136T mutants (Figure 1B), consistent with the previous report.19 On the other hand, spectra of all 3 mutant proteins showed only slight changes in the absence of Ca2+ (Figure 1B), indicating that the folding status of these mutants is not significantly affected by Ca2+ in the buffer. Taken together, our results suggest that, although all 3 mutations likely abolish calcium binding, there are independent and separable effects of mutations in calcium binding loops (D89A, D129E, and I136T) and mutations in helical regions (D122V) on MCFD2 conformation.

The EF-hand domains of MCFD2 are sufficient and necessary for LMAN1 binding
The N-terminal sequence of mature MCFD2 contains 42 amino acid residues that are conserved among vertebrates.19 To investigate whether this N-terminal domain is required for LMAN1 binding, we constructed a series of deletion mutants with a myc tag at the C terminus (Figure 2A). COS-1 cells were transfected with these constructs and metabolically labeled with 35S-methionine/cysteine. Lysates prepared from labeled cells were subjected to immunoprecipitation with anti-myc, anti-MCFD2, and anti-LMAN1 antibodies. Anti-myc detects the myc-tagged mutant MCFD2, whereas anti-MCFD2 detects both the endogenous and myc-tagged MCFD2. MCFD2 with deletion of the entire N-terminal sequence plus 3 amino acids in the first EF-hand domain (Δ45) still efficiently coimmunoprecipitate with LMAN1 (Figure 2C). Immunofluorescence staining of cells transfected with the Δ40 deletion mutant demonstrated colocalization with endogenous LMAN1, similar to the wild-type MCFD2 (Figure 2B), indicating that MCFD2 mutants are retained in the ER/GIC via their interactions with LMAN1. These results suggest that the N-terminal sequence of MCFD2 is not essential for LMAN1 binding and that the EF-hand domains are sufficient for LMAN1 binding.

MCFD2 with deletions of up to 47 amino acids, or 5 amino acids into helix 1 can still significantly coimmunoprecipitate with LMAN1 (Figure 2C). Further deletions into this helix markedly decreased the coimmunoprecipitation (co-IP). Consequently, immunofluorescence staining of the Δ50 mutant (Figure 2B) showed a diffused pattern that resembles that of the D129E missense mutant, which is unable to bind LMAN1.12 Similarly, deletion of the first 5 amino acids of helix 3 (ΔEF2-N) abolishes the LMAN1 interaction, indicating that the structure of this helix is important for LMAN1 binding. Consistent with our findings, a previous report showed that a patient mutation that truncates the last 3 amino acids of helix 4 markedly decreased MCFD2 binding to LMAN1.21 This mutation significantly altered MCFD2 3-dimensional structure.21 Similarly, our partial helix 1 and helix 3 deletion mutants probably also alter the structure of the EF-hand domains. Taken together, these results suggest that the integrity of the core structures of both EF-hand domains of MCFD2 is necessary for LMAN1 interaction.

We next asked whether the linker region between the 2 EF-hand domains is critical for the interaction with LMAN1. The linker region of MCFD2 is defined as the sequence between the 2 EF-hand domains and is also well conserved in vertebrate species.19 In the NMR structure, this region is 10 amino acids long and largely unstructured.19 We deleted 5 amino acids at a time and assessed the LMAN1 interaction. Results showed that partial deletions of the linker region had minimal effect on the interaction with LMAN1 (Figure 2D), suggesting that the length or the amino acid sequence of the linker region is not critical for LMAN1 binding.

MCFD2 missense mutations found in F5F8D patients can interact with FV and FVIII
The interaction between the LMAN1-MCFD2 complex with FV/FVIII is detected with a crosslinking/co-IP assay. In this assay, cells are treated with a membrane-permeable, thiol-cleavable chemical crosslinker, DSP, before lysis and immunoprecipitation. We previously demonstrated specific crosslinking of FVIII to the LMAN1-MCFD2 complex.12 We now further demonstrate the specificity of this interaction by cotransfecting MCFD2 with ceruloplasmin (Cp), a protein with homology to the FVIII A domains. Cells were treated with DSP and analyzed by immunoprecipitation. The co-IP of LMAN1 and MCFD2 with FVIII
was readily detected with anti-myc and anti-LMAN1 antibodies. In contrast, no co-IP was detected between LMAN1 or MCFD2 and Cp with anti-myc, anti-LMAN1, or anti-Cp antibodies (supplemental Figure 2).

Our CD spectroscopy experiments suggest that different MCFD2 missense mutations exert different effects on the MCFD2 structure, although all previously reported missense mutations abolish LMAN1 binding.12,13 The D129E mutant has previously been shown to maintain interaction with FVIII, and M, myc tag. The N-terminal domain is defined as the first 42 amino acids after the signal sequence. (B) Immunofluorescence staining of the wild-type (WT), Δ40, and the Δ50 N-terminal deletion mutants, as well as the D129E missense mutant MCFD2. (C) co-IP of various N-terminal deletion mutants with LMAN1. (D) co-IP of MCFD2 deletion constructs used in the study. SS indicates signal sequence; L, linker; and M, myc tag. The N-terminal domain is defined as the first 42 amino acids after the signal sequence. (B) Immunofluorescence staining of the wild-type (WT), Δ40, and the Δ50 N-terminal deletion mutants, as well as the D129E missense mutant MCFD2. (C) co-IP of various N-terminal deletion mutants with LMAN1. (D) co-IP of MCFD2 deletion constructs used in the study. SS indicates signal sequence; L, linker; and M, myc tag. The N-terminal domain is defined as the first 42 amino acids after the signal sequence. (B) Immunofluorescence staining of the wild-type (WT), Δ40, and the Δ50 N-terminal deletion mutants, as well as the D129E missense mutant MCFD2. (C) co-IP of various N-terminal deletion mutants with LMAN1. (D) co-IP of MCFD2 deletion constructs used in the study. SS indicates signal sequence; L, linker; and M, myc tag. The N-terminal domain is defined as the first 42 amino acids after the signal sequence.

To study the structural requirements for the interactions between MCFD2 and FV/FVIII, we introduced a missense mutation, D129E, into 2 N-terminal MCFD2 deletion mutant constructs (Δ35 and Δ40) depicted in Figure 2A. The presence of the D129E mutation abolishes LMAN1 binding (Figure 3).5 Thus, the observed co-IP between these MCFD2 mutants and FV/FVIII indicate direct binding, not an indirect interaction mediated through LMAN1. We next asked the question whether the N-terminus of MCFD2 is involved in FV/FVIII binding by monitoring co-IP of the 2 N-terminal deletion mutants of MCFD2 with FV and FVIII. As expected, introducing the D129E mutation into either MCFD2 truncation mutant abolished the co-IP with FV or FVIII (Figure 4). Little FV (Figure 4A) or FVIII (Figure 4B) was pulled down by anti-myc (to detect transfected MCFD2) or anti-LMAN1 (to detect endogenous LMAN1) in the absence of DSP (lanes 2, 3, 6, 7, 10, and 11). However, DSP treatment significantly increased the amount of FV/FVIII that was pulled down by both antibodies (lanes 14, 15, 19, 22, and 23). The co-IP of LMAN1 with FV/FVIII was the result of the presence of endogenous MCFD2. The amount of FV or FVIII pulled down by the anti-myc antibody was indistinguishable from the wild-type MCFD2 for both N-terminal deletion mutants (lanes 14, 18, and 22), suggesting that the N-terminal domain of MCFD2 is not critical for binding FV and FVIII. As a negative control, no co-IP of
**Discussion**

The LMAN1-MCFD2 cargo receptor is unique in that both a transmembrane component (LMAN1) and a soluble cofactor (MCFD2) are absolutely required for its function.\(^3\) Mutations in either LMAN1 or MCFD2 result in nearly identical manifestations.\(^2,3,22\) However, a systematic study of all available cases of F5F8D with known mutations uncovered a subtle difference between patients with LMAN1 mutations and patients with MCFD2 mutations.\(^13\) The mean FV and FVIII levels in the MCFD2 group are significantly lower than those of the LMAN1 group, suggesting that MCFD2 plays a more direct role in the cargo receptor function. In the current study, we demonstrated a central role for the EF-hand domains of MCFD2 in the formation of the LMAN1-MCFD2 complex, as well as in interactions with both FV and FVIII. These 2 interactions are distinct and probably involve separate binding sites of the protein.

The core structure of MCFD2 EF-hand domains consists of 2 helix-loop-helix units connected by a flexible linker.\(^19\) The 2 Ca\(^{2+}\)-binding loops are localized in close proximity (supplemental Figure 1). Although all missense mutations previously reported in F5F8D patients abolish LMAN1 binding,\(^12,13\) their effects on MCFD2 structure vary. Most of these mutations directly change the Ca\(^{2+}\)-chelating amino acids and are expected to abolish Ca\(^{2+}\) binding of the mutated EF hand. The I136T mutation is localized to the second EF-hand domain, but it does not directly change a Ca\(^{2+}\) chelating residue (Figure 1). Rather, side chains of Y135-I136 are thought to form a very short antiparallel \(\beta\)-sheet with the first Ca\(^{2+}\) binding loop.\(^23\) Because the CD spectra of D89A, D129E, and I136T are very similar (Figure 1),\(^19,21\) we speculate that mutations
in either Ca\(^{2+}\)-binding loops lead to the same structural consequence of disrupting interaction of the 2 loops. The conformational changes to the D89A, D129E, and I136T mutants are probably caused by their inability to bind Ca\(^{2+}\) because of the central role of Ca\(^{2+}\) in maintaining the structure of EF-hand proteins.\(^2\)\(^3\) Although removal of Ca\(^{2+}\) significantly altered the CD spectrum of the wild-type MCFD2, it had little impact on the spectra of all 3 mutant proteins (Figure 1B), supporting the notion that all 3 mutations abolish the Ca\(^{2+}\) binding. The unique CD spectrum of the D122V missense mutant along with either FV (A) or FVIII (B). Cells were incubated with or without DSP before lysis. Cell lysates were immunoprecipitated with anti-myc (myc), anti-LMAN1 (L), and either anti-FV (F5 in panel A) or anti-FVIII (F8 in panel B) antibodies.

The interaction of MCFD2 with FV/FVIII is clearly distinct from the interaction of MCFD2 with LMAN1, although both interactions involve the EF-hand domains. The missense mutations and the ∆EF2-N mutation that fail to bind LMAN1 can still interact with FV and FVIII (Figures 3,5). As discussed in the second paragraph above, mutations in the Ca\(^{2+}\)-binding loop probably disrupt the 3-dimensional structure of EF hands without significantly affecting the α-helical secondary structure. Thus, Ca\(^{2+}\) binding-induced 3-dimensional conformation of MCFD2 is not essential for this interaction. We speculate that secondary structure...
elements are sufficient to form the binding site for FV and FVIII. The D122V and JEF-2N mutations that damage helix 3 can also interact with FV/FVIII, suggesting that this helix is not directly involved in this interaction. We propose that MCFD2 is the protein that initiates the cargo capture in the ER and brings FV/FVIII to the proximity of LMAN1 for additional interactions. The less rigid structural requirement for FV/FVIII binding is consistent with this function.

It is somewhat surprising that the N-term of MCFD2 appears to be dispensable for interactions with either LMAN1 or FV/FVIII. This sequence is conserved in vertebrate species. Although a defined structure cannot be assigned to the N-term of MCFD2, Guy et al pointed out that this sequence is not completely without order and it could still assume a folded state in a multitudesubunit protein complex.25 Kawasaki et al showed that MCFD2 can interact with the CRD of LMAN1 in vitro and that binding of MCFD2 enhanced the binding of CRD to the surface glycans of HeLa cells.25 Because our crosslinking assay was performed in living cells, it can potentially pick up weak and transient interactions. Therefore, we cannot rule out a role of the N-terminal sequence in further enhancing the interaction with LMAN1 or FV/FVIII. In addition to FV and FVIII, other potential LMAN1 cargo proteins have been identified.26-31 Some of these cargo proteins, such as CatC and CatZ, do not seem to require MCFD2.24 Thus, MCFD2 may be a specific cargo selection protein for the recruitment of FV and FVIII, but not other cargo proteins, such as CatC and CatZ. Detailed structure-function analysis of MCFD2 may lead to the development of a selective inhibitor of LMAN1-MCFD2 interaction as a new anticoagulant. Such an inhibitor would reduce FV/FVIII secretion without affecting other LMAN1-dependent cargo proteins.

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Authorship

Contribution: C.Z. and H.L. performed the experiments; C.Z., H.L., J.Z., and B.Z. designed the research, analyzed the results, made the figures, and wrote the manuscript.

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References

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