Intracellular Evaluation of ER Targeting Elucidates a Mild Form of Inherited Coagulation Deficiency

Lara Rizzotto, Mirko Pinotti, Paolo Pinton, Rosario Rizzuto, and Francesco Bernardi

INTRODUCTION

Blood coagulation involves numerous serine-proteases with procoagulant and anticoagulant function (1,2), whose plasma levels are regulated to maintain the hemostatic balance and prevent hemorrhagic or thrombotic events.

Partial deficiency of procoagulant factors is associated with variable hemorrhagic phenotypes (3,4), ranging from asymptomatic to very severe. These observations, together with the notion that levels above 10% of normal plasma are sufficient to prevent major hemorrhagic symptoms, make the characterization of residual levels of great importance to understand and classify coagulation deficiencies.

The extensive genetic investigations in patients revealed the mutational pattern for a number of inherited hemorrhagic coagulation factor disorders (5-10), which is mainly characterized by missense mutations reducing circulating protein levels in plasma. So far, the molecular mechanisms through which these mutations impair the intracellular biosynthetic pathways, and their relationship with plasma and clinical phenotype in patients, have been poorly investigated (11-16). Missense mutations affecting the prepeptide region of coagulation factors (5-10), crucial for targeting of nascent molecules to the endoplasmic reticulum (ER) (17), represent peculiar models to address this issue. The causative role of substitutions affecting this region, and their effects on protein levels, are difficult to predict because of the extreme variability of prepeptide sequences (18,19) even among members of the coagulation serine protease family.

In the present study, through in vitro expression of native as well as of chimeric proteins with green fluorescent protein (GFP) (20), we elucidated the effect within living cells of the prepeptide mutations in factor VII (FVII), the serine protease triggering the clotting cascade (21,22).

MATERIALS AND METHODS

FVII Mutations

The mutations in the FVII prepeptide have been selected from the FVII mutation database (8) and the International Registry of FVII deficiency (9). The homozygous L-48P mutation was associated with FVII activity (FVIIc) and antigen (FVIIag) levels of 11% (23). The L-42P substitution, in doubly heterozygous condition with the V252M change, was associated with FVIIc levels of 12% (24).

Expression Vectors, Cell Culture, and Transfection

To make the pWTFVII-GFP vector, the FVII coding sequence cloned in the
ALTERED ER TARGETING IN FVII DEFICIENCY

pCDNA3-FVII (25) was amplified with PfuTurbo DNA polymerase (Stratagene, La Jolla, CA, USA) using the forward primer in the CMV promoter (5′-GGC CTTACGGTGCCGGAGGTC-3′) and the reverse primer designed to abrogate the GTGTACGGTGGGAGGTC-3′ primer in the CMV promoter (5′-La Jolla, CA, USA) using the forward primer in the pCDNA3 vector (26).

Mutations were introduced into the pCDNA3-FVII and pWtFVII-GFP vectors using the Quick-Change Mutagenesis Kit (Stratagene) with the following oligonucleotides: 5′-CTCTGCTTCTGCTGGGC CTCAGGGC-3′ (L-48P), 5′-GGCCTTCAG GGCCTCCCGG TCGAGTCTCGTA ACCC-3′ (L-42P) and 5′-CGTGTACC TGACGGTCATCGTCAGCT GG-3′ (G360V). The amplified fragment was digested with BamH1 and cloned in frame with the coding sequence of the enhanced GFP, inserted in the pCDNA3 vector (26).

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Cells were observed 48 h posttransfection using a Zeiss-Axiovert 200M fluorescence microscope (470 nm excitation, 505 nm emission). After digitonin treatment, fluorescence was monitored at 5-s intervals for 5 min.

Fluorescence Microscopy
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Images were analyzed using the Meta-morph/Metafluor 4.5 software.

Digitonin Treatment
Transfected cells were treated with 200 μM digitonin in medium mimicking intracellular ion composition (140 mM KCl, 10 mM NaCl, 1 mM K2PO4, 5.5 mM glucose, 2 mM MgSO4, 1 mM ATP, 2 mM sodium succinate, 20 mM Heps, pH 7.05). Digitonin complexes specifically with unesterified 3-β-hydroxy steroids, forming in situ precipitates and selectively perforating the plasma membrane, leaving ER and Golgi apparatus membranes sealed.

Subcellular fractionation
Cells were washed twice with 1× ice-cold PBS and harvested via scraping into 1 mL CLB (10 mM HEPES, 10 mM NaCl, 1 mM KH2PO4, 5 mM NaHCO3, 1 mM CaCl2, 0.5 mM MgCl2)/5 mM EDTA/1 mM PMSF. The homogenate was centrifuged at 110,000 g for 30 min. The resulting supernatant, containing the soluble plasma (cytosolic) proteins, was conserved for further assays. The pellet, containing nuclei, debris, and membrane proteins, was resuspended in 1 mL lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF), incubated on ice for 30 min, and centrifuged at 10,000 g at 4 °C for 10 min to clear cellular debris from the lysate. The supernatant was removed and conserved for further assays.

Putative cytosolic and organelle fractions were run on SDS-PAGE (NuPAGE 4-12% bis-tris gel; Invitrogen, Carlsbad, CA, USA) under reducing conditions. Membrane was probed with monoclonal antibodies directed against the β-actin (Cell signaling Technology, Danvers, MA, USA) or the protein disulfide isomerase (Calbiochem, San Diego, CA, USA). After incubation with the primary antibody, filters were incubated with the secondary anti-rabbit antibody (Pierce, Rockford, IL, USA).

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Expression of Mutations in the Native FVII
The causative role of the prepeptide mutations was approached through the expression of FVII variants in eukaryotic cells and investigation at both the extracellular and intracellular level. As control, besides the WtFVII, we also expressed the naturally occurring 360VFVII variant that possesses a correct prepeptide sequence and a Gly-to-Val substitution in the C-terminal domain.

Expression Levels in Conditioned Medium
Expression levels of FVII variants in medium were evaluated in 5 independent transient transfection experiments. Mean FVII protein levels of WtFVII in

RESULTS

Computational Analysis
Secondary structure analysis (SOPMA method) predicted that the introduction of proline for leucine at positions 48 and 42 in the FVII prepeptide alters and reduces the α-helix content of the hydrophobic core (Figure 1A), crucial for proper interaction with the signal recognition particle (17).

When a panel of computational programs devoted to the signal peptide prediction was used, no major differences in the score among prepeptide sequences were observed (Figure 1B).

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Expression Levels in Conditioned Medium
Expression levels of FVII variants in medium were evaluated in 5 independent transient transfection experiments. Mean FVII protein levels of WtFVII in
medium at the steady state (24-48 h post-transfection) were 125 ± 32 ng/mL, whereas levels of the 360V-FVII were undistinguishable from those of the negative control. Protein levels of the –42PFVII and –48PFVII variants were 15.4 ± 5.7 ng/mL (12% of WtFVII) and 69.1 ± 20.1 ng/mL (55% of WtFVII), respectively (Figure 2B).

The activity of FVII was investigated in these media through fluorogenic FXa generation assays, enabling us to detect even very low levels. FVII activity of the 360V-FVII was undetectable, whereas that of the –42PFVII and –48PFVII variants was 11% ± 5% and 66% ± 3% of WtFVII, respectively (Figure 2A).

Expression Levels of the Prepeptide Variants

Cells stably expressing WtFVII and prepeptide variants were also selected and investigated. The secreted protein levels of –42PFVII (30 ± 10 ng/mL) and –48PFVII (117 ± 39 ng/mL) from the 3 best clones were significantly lower than those of Wt-FVII (1314 ± 490 ng/mL) (Figure 2C). These concentrations permitted us to evaluate the specific activity of the –42PFVII (2092 ± 117 Rfu/sec/nMFVII) and –48PFVII (2125 ± 493 Rfu/sec/nMFVII) molecules, which was comparable to that of WtFVII (2100 ± 553 Rfu/sec/nMFVII).

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Intracellular FVII Expression Levels

To evaluate FVII proteins at the intracellular level, lysates of either transiently or stably transfected cells were processed to separate cytosol from organelles. The successful fractionation of cell content was indicated by Western blotting analysis, which identified β-actin almost exclusively in the cytosolic fraction and the protein disulphide isomerase (PDI) almost exclusively in the organelle fraction (Figure 2B-C, right panels).

FVII protein levels inside cell organelles of transiently transfected cells were detectable only for the WtFVII (10.3 ± 3.7 ng/mL) and the 360V-FVII (22.8 ± 4.4 ng/mL) but not for the –42PFVII and –48PFVII variants (Figure 2B, left panel). In contrast, investigations in stable clones revealed the presence of very reduced protein levels in organelles for the –48PFVII (10.5 ± 4.9 ng/mL) compared with those of Wt-FVII (130 ± 43.4 ng/mL) (Figure 2C). The –42PFVII levels in this compartment ranged around the sensitivity limit of the ELISA (~4 ng/mL), and reached values of 4.1 and 6.1 ng/mL in 2 clones.

FVII protein levels in the cytosolic fractions from transiently or stably transfected cells were not measurable by ELISA. This is likely explained by the inability of the anti-human FVII antibody to recognize the improperly folded FVII molecules in the cytosol environment, which does not permit the required extensive posttranslational modifications of FVII to occur (29).

Expression of Mutations in the FVII-GFP Chimera

To investigate at the intracellular level in living cells the effect of these substitutions on the first biosynthetic steps, FVII was expressed as a fusion protein with the GFP (Figure 3A). Fluorescence of the FVII-GFP variants was evaluated by microscopy of transfected cells.

Cells expressing the –42PFVII-GFP and the –48PFVII-GFP showed a diffuse fluorescence, also present in the nuclear region (Figure 3D and E, time 0). Transfected cells were subsequently treated with digitonin to permeabilize the plasma membrane. In these conditions, the cytosolic cell content leaks out of the permeabilized membranes, leaving ER and Golgi apparatus membranes sealed. Upon digitonin treatment, fluorescence of the –42PFVII-GFP and –48PFVII-GFP rapidly decreased over time (see Figure 3D and E). The residual fluorescence observed for the
–48PFVII-GFP was approximately 10%, while that of the –42PFVII-GFP was approximately 20% (Figure 3D and E). Similarly, a diffuse fluorescence was observed by expressing the protein kinase Cβ (PKCβ) isoform-GFP chimera, which has been demonstrated to be largely cytosolic (26). After digitonin treatment, the residual fluorescence levels of PKCβ-GFP were indistinguishable from the background (27). Detection by Western blotting of the β-actin (~42 kDa) and PDI (~55 kDa) proteins as markers of the cytosolic and organelle fractions. Molecular weights are given in kDa.

Expression levels of the WtFVII-GFP were also investigated in conditioned medium 48 to 72 h posttransfection. The ELISA assay, exploiting a capture monoclonal antibody recognizing the aminoterminal portion of FVII and a detecting polyclonal anti-FVII antibody, revealed that levels of extracellular WtFVII-GFP chimera were very low (2 ± 1 ng/mL) compared with those of the native FVII (125 ± 32 ng/mL). Moreover, the FXa generation activity of the WtFVII-GFP in medium was virtually undetectable. These findings are likely explained by the presence of the GFP attached to the carboxy terminus of the catalytic domain of FVII that is important for secretion (30).

DISCUSSION

The natural L-48P and L-42P mutations in FVII were chosen as models to elucidate mechanisms of altered biosynthesis and reduction of protein levels and their correlation with plasma and clinical phenotype in patients. Because of their localization at crucial positions of the prepeptide (17), the L-48P (hydrophobic core) and L-42P (position –3 preceding the signal-peptidase cleavage site) changes are candidates to affect targeting of nascent FVII to ER. However, computational analyses failed to predict significant alterations of the prepeptide properties.

Prepeptide variants of the native FVII were initially expressed in transient transfection experiments to evaluate consequences of mutations on secreted and intracellular FVII levels. The steady-state expression levels in medium of the –42PFVII variant were significantly reduced whereas those of the –48PFVII, though showing an altered early secretion profile, were slightly reduced compared with those of WtFVII. The G360V substitution in the FVII carboxyl-terminal domain, associated to undetectable FVII protein levels in plasma, was investigated as an example of mutation affecting a highly conserved residue in all serine proteases (31) and therefore expected to alter FVII folding/biosynthesis but not targeting to ER. Not surprisingly, the 360VFVII variant was not secreted in conditioned medium.

Studies at the intracellular level revealed appreciable protein amount of the WtFVII and 360VFVII in organelles, thus indicating access of FVII molecules to the secretory pathway. The significantly altered targeting of FVII to ER is likely to play a major role in the plasma deficiency in patients with FVII mutations.
higher protein levels of the 360VFVII compared with those of WtFVII indicated retention and accumulation of this variant into the organelle lumen. Conversely, the –42PFVII and –48PFVII into organelles were undetectable, which prompted us to select and investigate stably expressing cells.

Comparison among the best expressing clones revealed markedly reduced secretion levels for the –42PFVII and –48PFVII variants, thus clearly indicating the causative role of the L-42P and L-48P changes. On the other hand, both variants possessed a specific activity comparable to WtFVII, which supported correct processing of the N-terminus of the secreted FVII molecules. Investigation of stable clones also unraveled markedly reduced FVII levels of both variants into cell organelles, a finding compatible with impaired targeting of nascent FVII to ER, a first step of the secretory pathway.

The FVII-GFP chimera, which permits direct investigation of protein trafficking in living cells, was exploited to overcome the inability of the ELISA to reveal protein levels into the cytosol. Some limitations of this approach, i.e. very low levels of the secreted chimeric proteins, do not preclude an informative analysis of ER targeting. The diffuse fluorescence of the –42PFVII-GFP and –48PFVII-GFP rapidly decreased over time upon treatment of cells with digitonin, thus convincingly demonstrating that these variants were mainly cytosolic and thus excluded from cell organelles. A well-characterized cytosolic protein, used as control, did not produce appreciable fluorescence after plasma membrane permeabilization, thus further indicating that the residual values of chimeric FVII prepeptide variants were attributable to molecules entering the ER lumen. In particular, the residual fluorescence observed in organelles for the –48PFVII-GFP and –42PFVII-GFP indicated that 10% to 20% of mutant proteins entered the ER, a finding compatible with plasma FVII levels of patients (11% to 12%).

Taken together, these data suggest the FVII-GFP strategy as an informative tool to evaluate the effect of FVII prepeptide mutations on ER targeting in living cells. The expression studies with the native and chimeric FVII molecules indicated that both prepeptide mutations were associated to residual FVII levels, which are sufficient to prevent major hemorrhagic symptoms and to explain the presence in patients of a mild form of FVII deficiency. This approach, extendable to other coagulation serine proteases, clearly contributed to elucidate the relationship of genotype with plasma and clinical phenotype.
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