Splice-Site Mutation of Exon 3 Deletion in the Gamma-Glutamyl Carboxylase Gene Causes Inactivation of the Enzyme

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TO THE EDITOR

Gamma-glutamyl carboxylase (GGCX) is an integral membrane protein that catalyzes the post-translational modification of certain glutamates to gamma-carboxyglutamates in vitamin K-dependent (VKD) proteins. Carboxylation is required for the biological activity of numerous VKD proteins involved in a broad range of physiological functions. Mutations in GGCX have been mainly associated with bleeding disorders because these mutations cause undercarboxylation of VKD coagulation factors and of the anticoagulant proteins (Napolitano et al., 2010). GGCX mutations have also been linked to pseudoxanthoma elasticum (PXE)-like syndrome, a non-bleeding disorder caused by functional defects in the matrix Gla protein (MGP) (Vanakker et al., 2007). Patients with PXE-like syndrome have been reported to have comorbid bleeding disorder of vitamin K-dependent coagulation factors deficiency (VKCFD), which is characterized by the simultaneous functional defects of multiple VKD coagulation factors (Li et al., 2009; Rongioletti et al., 1989; Vanakker et al., 2007).

Recently, Kariminejad et al. (2014) reported 13 patients with phenotypes typical of PXE-like syndrome, but with no coagulation abnormalities (Kariminejad et al., 2014). Genetic analysis of these patients' ATP-binding cassette subfamily C member 6 gene (the causative gene for the classical PXE syndrome) excluded the ATP-binding cassette subfamily C member 6 mutations. However, all affected members were found to be homozygous for a splice-site mutation (c.373+3G>T) in the GGCX gene, which causes the deletion of exon 3 in the GGCX mRNA (GGCX-Δex3). It has been suggested that the phenotypes displayed by the affected patients were associated with the GGCX-Δex3 mutation (Kariminejad et al., 2014). However, the reason for the absence of bleeding diathesis in these patients remained unclear, and no functional study on the GGCX-Δex3 mutation was available.

To examine the functional consequences of the GGCX-Δex3 mutation, we determined the carboxylation activity of this mutant using our recently established GGCX-deficient cell-based assay with two reporter proteins (a chimeric coagulation factor, prothrombin with its Gla domain replaced by that of FIX and MGP) (Supplementary Methods online). This approach, unlike the traditional in vitro GGCX activity assay, allows us to assess the functionality of GGCX using its natural protein substrates in a cellular milieu that requires the enzyme to interact with its physiologic components for VKD carboxylation (Tie et al., 2016). Our result shows that the GGCX-Δex3 mutant *abolished* carboxylation activity for *both* reporter proteins (Figure 1a). These results agree with the findings of Kariminejad et al. (2014), who observed uncarboxylated MGP in the affected patients. However, the inability of the GGCX-Δex3 mutant to carboxylate the coagulation factor reporter protein disagrees with their observation that all the affected patients had normal coagulation factor activities.

To further clarify the effect of GGCX-Δex3 mutation on the carboxylation of coagulation factors, we characterized GGCX mutants of a patient with VKCFD with compound heterozygous mutations of GGCX-Δex3 and Arg485-Pro (Rost et al., 2004). If the GGCX-Δex3 mutation located on one allele expresses an inactive enzyme, as we observed (Figure 1a), the Arg485Pro mutation on the other allele should play a major role in the clinical phenotype of this patient with VKCFD, whose coagulation disorder has been partially corrected by vitamin K administration (Rost et al., 2004). To test this hypothesis, we titrated the carboxylation activity of the Arg485Pro mutant in response to increasing concentrations of vitamin K. Compared with the wild-type enzyme, the Arg485Pro mutant requires an approximately 5-fold higher vitamin K concentration to reach half-maximal carboxylation of prothrombin with its Gla domain replaced by that of FIX (Figure 1b). At the optimum vitamin K concentration, the Arg485Pro mutant has ameliorated coagulation factor carboxylation up to approximately 50%. This agrees with the patient's clinical result, in which vitamin K administration partially restored coagulation factor activities to approximately 60% (Rost et al., 2004). These results support our hypothesis that, when the GGCX-Δex3 mutation on one allele encodes an inactive enzyme, the Arg485Pro mutation on the other allele plays a key role in the clinical phenotype of this patient with VKCFD.

Deletion of exon 3 results in an in-frame deletion of 53 amino acid residues (between Phe73 and Gly125) in the GGCX molecule, which includes part of the first and second transmembrane domains (TMDs) and the lumenal loop between TMD1 and TMD2 (Figure 1c) (Tie et al., 2000). Therefore, exon 3 deletion could disrupt the proper integration of TMD1 and TMD2 into the endoplasmic reticulum (ER) membrane, which would

Abbreviations: ER, endoplasmic reticulum; GGCX, gamma-glutamyl carboxylase; MGP, matrix Gla protein; PXE, pseudoxanthoma elasticum; TMD, transmembrane domain; VKCFD, vitamin K-dependent coagulation factors deficiency; VKD, vitamin K-dependent

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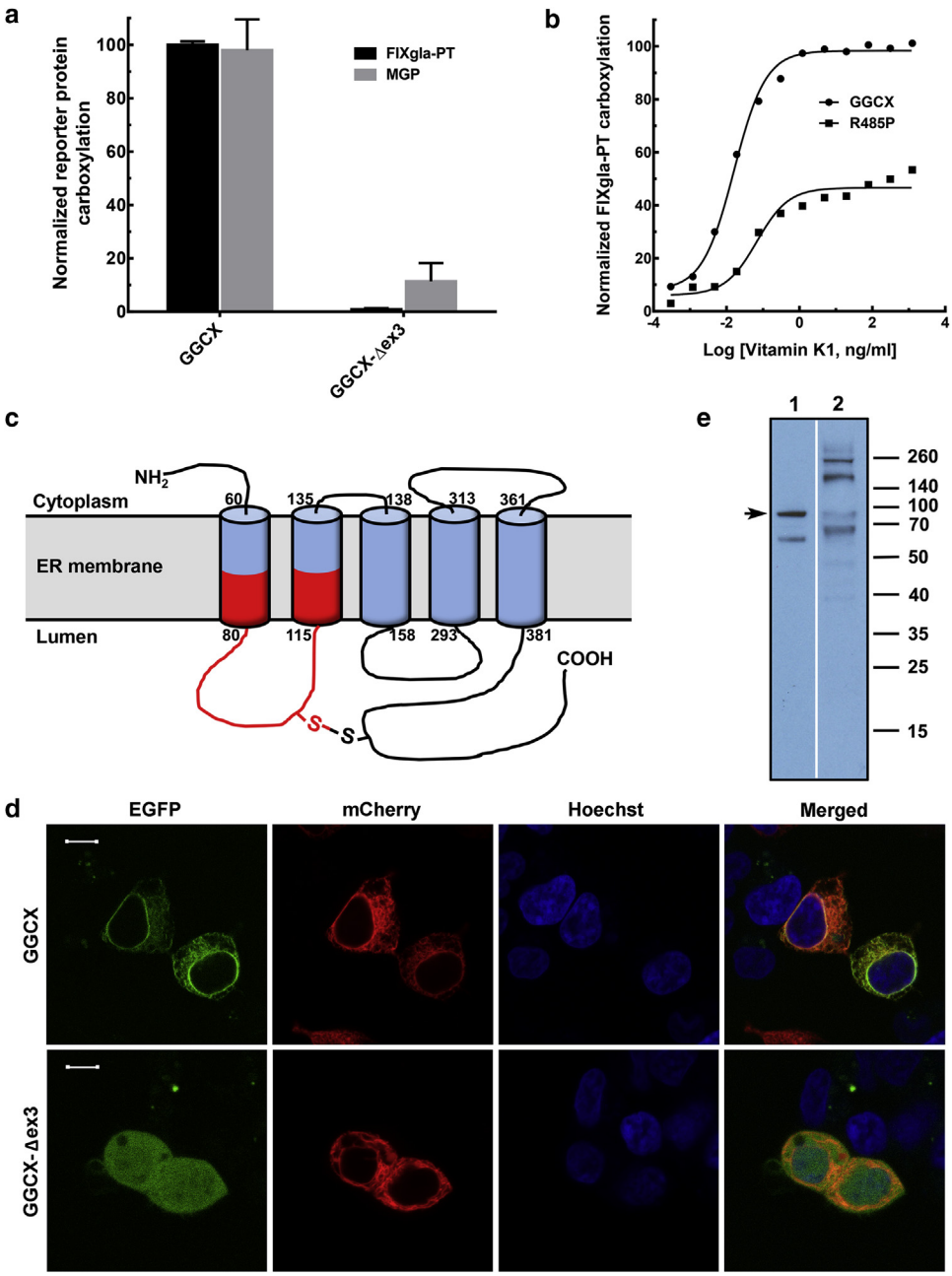

Figure 1. Characterization of GGCX mutations identified in patients with PXE-like syndrome and VKCFD. (**a**) Cell-based activity assay of GGCX and its mutant identified from patients with PXE-like syndrome. Wild-type GGCX and the GGCX-Δex3 mutant were transiently expressed in GGCX-deficient HEK293 reporter cells. Transfected cells were cultured in complete medium containing 5 μg/ml vitamin K. The concentrations of the carboxylated reporter proteins (FIXgla-PT, black bars; MGP, gray bars) in the cell culture medium were measured by ELISA. Wild-type GGCX activity was normalized to 100%. Data are presented as mean ± SD (n = 3). (**b**) Carboxylation activity of GGCX and the Arg485Pro mutant in response to increasing concentrations of vitamin K. Wild-type GGCX (filled circles) and the Arg485Pro mutant (filled squares) were transiently expressed in GGCX-deficient HEK293 reporter cells. The enzymatic activity for FIXgla-PT carboxylation was determined as described above. (**c**) Schematic representation of the proposed membrane topology of GGCX. The exon 3 encoded region is shown in red. (**d**) Subcellular localization of GGCX and the GGCX-Δex3 mutant in HEK293 cells. EGFP-tagged GGCX or GGCX-Δex3 was transiently coexpressed with the ER marker mCherry-ER-3 in HEK293 cells. Forty-eight hours after transfection, the cell nucleus was stained by Hoechst 33342 and directly used for fluorescence confocal image collection. GGCX fusions were visualized by the green fluorescence of EGFP, ER marker was visualized by the red fluorescence of mCherry, and cell nuclei were visualized by the blue fluorescence of Hoechst. Scale bar = 10 μm. (**e**) Immunoblotting analysis of GGCX (lane 1) and the GGCX-Δex3 mutant (lane 2) proteins. Full-Length GGCX band is indicated by an arrow. ER, endoplasmic reticulum; EGFP, enhanced green fluorescence protein; FIXgla-PT, prothrombin with its Gla domain replaced by that of FIX; GGCX, gamma-glutamyl carboxylase; HEK, human embryonic kidney; MGP, matrix Gla protein; PXE, pseudoxanthoma elasticum; SD, standard deviation; VKCFD, vitamin K-dependent coagulation factors deficiency.

result in the misfolding of the GGCX protein. Exon 3 also encodes residue Cys99, which forms the only disulfide bond with Cys450 in the GGCX molecule that is essential for GGCX folding and maturation (Tie et al., 2003). To test the effect of exon 3 deletion on GGCX maturation, we fused an enhanced green fluorescence protein at the