Functional consequence of fibulin-4 missense mutations associated with vascular and skeletal abnormalities and cutis laxa

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Abstract

Fibulin-4 is a 60 kDa calcium binding glycoprotein that has an important role in development and integrity of extracellular matrices. It interacts with elastin, fibrillin-1 and collagen IV as well as with lysyl oxidases and is involved in elastogenesis and cross-link formation. To date, several mutations in the fibulin-4 gene (FBLN4/EFEMP2) are known in patients whose major symptoms are vascular deformities, aneurysm, cutis laxa, joint laxity, or arachnodactyly. The pathogenetic mechanisms how these mutations translate into the clinical phenotype are, however, poorly understood. In order to elucidate these mechanisms, we expressed fibulin-4 mutants recombinantly in HEK293 cells, purified the proteins in native forms and analyzed alterations in protein synthesis, secretion, matrix assembly, and interaction with other proteins in relation to wild type fibulin-4. Our studies show that different mutations affect these properties in multiple ways, resulting in fibulin-4 deficiency and/or impaired ability to form elastic fibers. The substitutions E126K and C267Y impaired secretion of the protein, but not mRNA synthesis. Furthermore, the E126K mutant showed less resistance to proteases, reduced binding to collagen IV and fibrillin-1, as well as to LTBP1s and LTBP4s. The A397T mutation introduced an extra O-glycosylation site and deleted binding to LTBP1s. We show that fibulin-4 binds stronger than fibulin-3 and -5 to LTBP1s, 3, and 4s, and to the lysyl oxidases LOX and LOXL1; the binding of fibulin-4 to the LOX propeptide was strongly reduced by the mutation E57K. These findings show that different mutations in the fibulin-4 gene result in different molecular defects affecting secretion rates, protein stability, LOX-induced cross-linking, or binding to other ECM components and molecules of the TGF-β pathway, and thus illustrate the complex role of fibulin-4 in connective tissue assembly.

Introduction

Fibulin-4 is a member of the fibulin family of extracellular matrix proteins and found in elastic tissues, tendon and ligaments, periosteum and bone. The structural characteristics of this protein family are characterized by a tandem array of calcium-binding (cb) epidermal growth factor (EGF)-like modules and a fibulin-type C-terminal domain [1] (Fig. 1A, B, C). Fibulin-4 was identified by a homology search for new secreted proteins [2] and also named using different acronyms, MBP1 (mutant p53-binding protein 1) [3] and EFEMP2 (EGF-containing fibulin-like extracellular matrix protein 2 [4]. A high level of fibulin-4 mRNA expression was found in heart, and prominent staining with a specific antibody was detected in large and small blood vessels [2,5], smooth and skeletal muscle, tendon, bone, periosteum and endomysium [2,6]. The importance of fibulin-4 in development and maintenance of vascular tissues was demonstrated in Fbln4-null mice [7] and Fbln4-hypomorphic mice (fibulin-$4^{R/R}$) [8], which showed aneurysm and arterial tortuosity; however abnormalities in lung were only found in Fbln4-null mice. Fbln4-null mice die perinatally from rupture of the aorta and other cardiovascular complications; in the aorta of those mice, disorganized elastic fibers and perturbation of TGF-β signaling were observed. Furthermore, newborn Fbln4 null mice show forelimb contractures, reduced collagen crosslinking and highly irregular fibril sizes of bone collagen fibrils due to impaired activation of pro-lysyl oxidase in the absence of fibulin-4 [6,9].

Recently, a number of mutations in the human fibulin-4 gene have been identified in patients affected with vascular abnormalities including aneurysm, arterial tortuosity, cardiovascular diseases, cutis laxa, arachnodactyly, enhanced bone fragility and joint laxity [10–18]. Some mutations such as E126K [13,17] or D203A [18] theoretically affect Ca^{2+} binding of cbEGF-like modules (Fig. 1C), but the molecular mechanisms of the genotype–phenotype relationships of the various mutants remain unclear.

Fibulin-4 is involved in multiple ways in the formation and maintenance of elastic tissue. Experimental evidence indicates that fibulin-4 regulates elastic fiber assembly, cross-linking and TGF-β activity in the extracellular matrix [6,7,19]. Fibulin-4 has been shown to bind to elastin and fibrillin-1 [7,20] and thus may participate in the fibrillin network which includes latent TGF-β binding protein (LTBP). Disruption of the fibrillin network results in aberrant activation of TGF-β, as observed in a mouse model for Marfan syndrome [21].

Furthermore, fibulin-4 is involved in the regulation of cross-linking of collagen and elastic fibers; it binds to lysyl oxidase (LOX) and lysyl oxidase like-1 (LOXL1), respectively [6,19,22,23] which are the key enzymes involved in cross-linking of collagens and elastin. Interaction between fibulin-4 and LOX may target LOX to microfibrils and facilitate cross-linking of elastin [19,22]. The desmosine content in fibulin-4 null mouse tissues is reduced to about 10% of that in wild type mice, although *Eln* (elastin) and *Lox* expression are not reduced [7]. Furthermore, Fbln4-null mice have phenotypes similar to *Lox*-null mice, suggesting that fibulin-4 may be required for the activation or the activity of LOX [24–26]. Recently we have reported that fibulin-4 null and knock-in mice showed abnormal collagen fibril assembly in bone, tendon and skin due to impaired LOX processing [6,9,27].

Thus, mutations in the fibulin-4 gene may lead to conformational changes and structural deformities, resulting in complete or partial loss of protein synthesis or secretion, in reduced protein stability against proteases, or in impaired assembly into elastic fibers due to reduced binding to other matrix molecules. All these effects will result in deficient elastic fiber formation. Other mutations may affect connective tissue stability due to reduced binding to and activation of pro-LOX, resulting in impaired elastin and collagen cross-linking and uncontrolled collagen fibrillogenesis. Third, some mutations may affect interactions with components of the fibrillin/TGFβ / LTBP complex and thus interfere with anabolic and catabolic mechanisms of elastic tissue homeostasis.

Only sparse information, however, exists on how single amino acid mutations affect these functions. Analysis of the molecular consequences of fibulin-4 mutations should provide insight not only into the specific role of distinct amino acids and structural modules in controlling the different functions of fibulin-4, but also help us to understand how the various mutations translate into distinct, but partially overlapping clinical phenotypes. Therefore, in this study we analyzed at molecular level the consequences of missense mutations using recombinantly expressed fibulin-4 mutant proteins bearing single amino acid substitutions E57K [10,16], E126K [13,17], C267Y [12], R297C [11], and A397T [13], and P47S (The substitution of proline 47 by serine was identified as a polymorphism, Dr. Loeys, personal communication); it was therefore used here as control mutant. We compared their synthesis and secretion rates, their proteolytic stability, and their ability to assemble into extracellular fibers, as well as their interaction with extracellular matrix components and enzymes involved in cross-link formation and factors of the TGFβ/LTBP pathway with respect to wild type fibulin-4. Our studies show that each missense mutation affects these functions at different extent, which may result at the end either in pathologic deficiency of fibulin-4 in patient tissues, or in non-functional fibulin-4 molecules with impaired abilities to interact with other elastic matrix components, with lysyl oxidases, or with components of the TGFβ pathway.