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Mutation in transforming growth factor beta induced protein associated with granular corneal dystrophy type 1 reduces the proteolytic susceptibility through local structural stabilization

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ABSTRACT

Hereditary mutations in the transforming growth factor beta induced (*TGFBI*) gene cause phenotypically distinct corneal dystrophies characterized by protein deposition in cornea. We show here that the Arg555Trp mutant of the fourth fasciclin 1 (FAS1-4) domain of the protein (TGFBIp/keratoepithelin/βig-h3), associated with granular corneal dystrophy type 1, is significantly less susceptible to proteolysis by thermolysin and trypsin than the WT domain. High-resolution liquid-state NMR of the WT and Arg555Trp mutant FAS1-4 domains revealed very similar structures except for the region around position 555. The Arg555Trp substitution causes Trp555 to be buried in an otherwise empty hydrophobic cavity of the FAS1-4 domain. The first thermolysin cleavage in the core of the FAS1-4 domain occurs on the N-terminal side of Leu558 adjacent to the Arg555 mutation. MD simulations indicated that the C-terminal end of helix α3′ containing this cleavage site is less flexible in the mutant domain, explaining the observed proteolytic resistance. This structural change also alters the electrostatic properties, which may explain increased propensity of the mutant to aggregate *in vitro* with 2,2,2-trifluoroethanol. Based on our results we propose that the Arg555Trp mutation disrupts the normal degradation/turnover of corneal TGFBIp, leading to accumulation and increased propensity to aggregate through electrostatic interactions.

1. Introduction

Transforming growth factor beta induced protein (TGFBIp; also called keratoepithelin or βig-h3), is found in the extracellular matrix of several tissues in the human body, but is especially abundant in the cornea [1–4]. TGFBIp is a 68 kDa protein consisting of an N-terminal Cys-rich EMILIN-1 (EMI) domain [5] and four homologous fasciclin 1 (FAS1) domains of approximately 140 residues [6]. Mutations in the transforming growth factor beta induced (*TGFBI*) gene, encoding TGFBIp, are associated with protein aggregation in the cornea [7,8]. To date, at least 30 mutations in *TGFBI* are known to cause corneal dystrophies. A unique property of TGFBIp compared to other proteins linked to hereditary protein misfolding diseases is that specific single-point mutations in *TGFBI* consistently cause phenotypically different corneal dystrophies related to the nature of the protein aggregates. The different phenotypes include lattice corneal dystrophy (LCD) type 1 and its variants, which are characterized by formation of TGFBIp

Abbreviations: TGFBIp, transforming growth factor beta induced protein; *TGFBI*, transforming growth factor beta induced gene; EMI, EMILIN-1 domain; LCD, lattice corneal dystrophy; GCD, granular corneal dystrophy; TBCD, Thiel–Behnke corneal dystrophy; WT, wild-type; FAS1, fasciclin 1 domain; FAS1-4, fourth FAS1 domain of TGFBIp; NMR, nuclear magnetic resonance; MD, molecular dynamics; SUMO, small ubiquitin-like modifier; LB, Lysogeny broth; OD, optical density; IPTG, isopropyl β-D-1-thiogalactopyranoside; DSS, 2,2-Dimethyl-2-silapentane-5-sulfonate; RDC, residual dipolar couplings; PDB, Protein Data Bank; RMSD, root-mean-square deviation; RMSF, root-mean-square fluctuation

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amyloid, different types of granular corneal dystrophy (GCD) typified by accumulation and deposition of TGFBIp in non-amyloid granular opacities in the cornea [9], and Thiel–Behnke corneal dystrophy (TBCD) characterized by deposition of curly fibers in the superficial cornea [7].

The structural basis for the different types of TGFBIp depositions remains unknown. However, recent studies of normal and mutant TGFBIp variants from human corneas suggest that proteolytic degradation of TGFBIp plays a significant role in the pathobiology of the *TGFBI*-linked cornea dystrophies. In the normal human cornea, TGFBIp is proteolytically processed in a highly orchestrated manner and the observed fragments strongly suggest that proteolysis of the FAS1-4 domain (residues Val505–Leu632) plays a key role in the turnover of TGFBIp in the cornea [10]. However, in *TGFBI*-linked corneal dystrophies the processing of TGFBIp is altered and different mutations are associated with specific changes in the degradation pattern [11] and proteolytic cleavage sites [12]. In a previous *in vitro* study, we showed that the FAS1-4 domain is the most proteolytically susceptible of the four FAS1 domains in full-length WT TGFBIp and phenotypically distinct mutations in the FAS1-4 domain alter the thermodynamic stability of the domain [13].

In the present study, we have investigated the altered proteolytic susceptibility of the FAS1-4 domain with reference to the structural changes caused by mutation Arg555Trp in TGFBIp. This mutation causes GCD type 1, which is one of the most common *TGFBI*-linked cornea dystrophies [14–16]. We show by limited proteolysis that the most susceptible thermolysin as well as tryptic cleavage site in the WT fourth FAS1 domain is between residues Arg557 and Leu558 positioned right next to the mutation site Arg555 while the Arg555Trp mutant FAS1-4 domain is resistant to proteolysis. To explore these observations in detail, we have determined the high-resolution structures of the isolated WT and Arg555Trp mutant FAS1-4 domains by liquid-state NMR spectroscopy. Our data reveal very similar overall structures of the WT and the Arg555Trp mutant domains. However, while residue Arg555 is solvent exposed in the WT structure, Trp555 of the mutant domain is buried in the hydrophobic cavity formed by helices α1, α3, α3′, and α4 thereby stabilizing the overall structure.

The dynamical behaviors of the WT and Arg555Trp variants of the FAS1-4 domain were further addressed through long time-scale molecular dynamics (MD) simulations and NMR relaxation studies. The MD simulations support the finding from the NMR structure that the hydrophobic Trp555 side chain of the Arg555Trp mutant stabilizes the structure. Significantly, the C-terminal end of helix α3′ containing the protease-accessible residues Arg557 and Leu558, and the subsequent loop are more rigid in the Arg555Trp mutant domain compared to the WT FAS1-4 domain, which explains the increased proteolytic resistance of the mutant domain. Furthermore, calculations of the electrostatic potential of the two NMR structures reveal differences in the electrostatic surface potential, which are consistent with the increased propensity of the Arg555Trp mutant domain to aggregate through electrostatic interactions *in vitro*.

Based on our results, we propose that the Arg555Trp mutation in TGFBIp both disrupts the degradation/turnover and increases the aggregation propensity of TGFBIp, which concomitantly lead to its accumulation and deposition in the GCD type 1 corneas.

2. Material and methods

See the Supplementary Experimental Procedures for additional details.

2.1. Expression and purification

The FAS1-4 constructs were generated as previously described [13] with the WT and Arg555Trp constructs consisting of residues 502–634. An additional full-length WT FAS1-4 domain, residues 502–657, was also made. For all constructs, an Ala–Gly dipeptide was included at the N-terminus to facilitate cleavage by the SUMO protease (see details in Supplementary Experimental Procedures). ^{13}C,^{15}N-labeled proteins were prepared by transferring one colony of transformed cells to a 50 mL solution of Lysogeny broth (LB) media with antibiotic. The solution was incubated overnight at 37 °C with shaking. Thereafter, the LB medium was stepwise diluted into 4 L M9 minimal medium containing ^{13}C-glucose and $^{15}NH_4Cl$ to obtain uniformly ^{13}C,^{15}N-labeled protein. When the optical density at 600 nm (OD_{600}) reached approximately 0.7, the protein expression was induced by adding 200 mg isopropyl β-D-1-thiogalactopyranoside (IPTG) per liter medium. When OD_{600} had reached a stable value the cells were harvested by spinning at 6000 rpm for 15 min.

The proteins were purified as previously described [13]. Following purification, the WT and Arg555Trp mutant proteins were dialyzed against a 50 mM phosphate buffer containing 100 mM NaCl, 50 mM arginine, and 50 mM glutamate in order to prevent protein aggregation [17]. The protein solutions were concentrated with Centricons (Amicon, Inc., Beverly, MA, USA) with a molecular weight cut-off of 10 kDa to yield protein concentrations of approximately 4 mg/mL. 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was added for chemical shift reference, NaN_3 to avoid bacterial growth, and D_2O to provide field lock. The final concentrations were 0.5 mM, 0.5%, and 10% for DSS, NaN_3, and D_2O, respectively. The samples were transferred to a Wilmad 541-pp NMR tube. The sample volumes were 550 μL.

2.2. Limited proteolysis, polyacrylamide gel analyses, and Edman degradation

Proteolytic fragments were separated by sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) in 10–15% polyacrylamide gels [18]. Samples were reduced using 30 mM dithiothreitol (DTT) and boiled in 1% SDS prior to electrophoresis. The proteolytic susceptibilities of the domains were investigated using thermolysin proteolysis (preference for cleavage at the N-termini of residues L, I, F, V, A, M). The FAS1-4 domain variants were incubated for 1 h with increasing amounts of thermolysin, ranging from thermolysin:FAS1-4 domain ratio 1:1000 to 1:1 (w/w). Subsequently, proteolysis was quenched by inhibiting thermolysin with 5 mM EDTA for 15 min at room temperature. For N-terminal sequencing the proteolytic fragments of the FAS1-4 domains were separated by SDS-PAGE as described above. For these analyses, the stacking gel was allowed to polymerize overnight prior to electrophoresis and samples were heated for 3 min at 80 °C only. Following electrophoresis proteins were transferred to a polyvinylidene diflouride membrane (Immobilon-P, Millipore) in 10 mM CAPS, 10% (v/v) methanol, pH 11 as described previously [19]. Samples were then analyzed by automated Edman degradation using an Applied Biosystems PROCISETM 494 HT sequencer with online phenylthiohydantoin analysis by HPLC (Applied Biosystems Model 120A).

2.3. NMR spectroscopy

All the NMR experiments for assignment and structure determination were performed on a Bruker Avance-II 700 MHz wide-bore NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) using a standard 5 mm triple resonance TXI probe equipped with a *z*-gradient. The experiments were performed at 300 K. Relaxation data were recorded on a Bruker Avance-III 800 MHz NMR spectrometer equipped with a standard cryoprobe. Data collection was done with TopSpin 1.3 (Bruker BioSpin, Rheinstetten, Germany), while the processing was performed with NMRPipe [20,21].

HNCA, HNCO, HN(CA)CO, HN(CO)CA, CBCANH, and CBCA(CO)NH triple-resonance experiments were performed to assign the backbone of the protein [22]. The side-chain assignment was based on 3D HCCH-TOCSY and N-TOCSY-HSQC. The distance restraints were