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A Unique TGFBIP Protein in Granular Corneal Dystrophy Types 1 and 2

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Abstract

PURPOSE—Types 1 and 2 granular corneal dystrophies (GCD) are primarily associated with accumulation of the R555W and R124H mutant transforming growth factor- β inducible proteins (TGFBIP) in corneal stroma, respectively. However, specific components of TGFBIP responsible for granular deposits have not been delineated. This study was undertaken to identify the mutant TGFBIP components potentially responsible for GCD.

METHODS—Recombinant TGFBIP of wild-type (WT) and three mutants, R124C, R124H and R555W, were generated in HEK293FT cells. WT and TGFBIP mutants were collected from cell lysates. Immunoblot analyses were performed with five different antibodies directed against various regions of WT TGFBIP.

RESULTS—WT and TGFBIP mutants showed differential reactivities with these antibodies. In contrast to our prior observation in purified WT and TGFBIP mutants, TGFBIP from cell lysates were less prone to polymerize. A unique 35kD fragment was detected in cell lysates of R555W and R124H, but not in those of WT or R124C, by a commercial antibody raised against amino acids 199–406 of TGFBIP.

CONCLUSIONS—Monomeric and polymeric WT and TGFBIP mutants were observed in vitro. The 35kD fragment found only in R555W and R124H, but not in WT and R124C cell lysates, is likely a degraded TGFBIP derived from the central domain of these mutants and this fragment may be contributory to the non-amyloid granular deposits observed in GCD 1 and 2.

Hereditary corneal dystrophies related to transforming growth factor β -inducible protein (TGFBIP) are a group of heterogeneous conditions characterized by accumulation of untoward protein deposits within the corneal tissue and progressive corneal opacities with vision loss. TGFBIP (the protein product of the *TGFBIP* gene, also referred to as keratoepithelin [KE], BIGH3, beta-igh3, β igh3, or RGD-CAP) is a 683-a.a. secretory matrix protein containing a secretory signal peptide sequence at the N-terminus, four “Fas 1-like” domains (~140 a.a. in each domain) homologous to *Drosophila* fasciclin-1 (Fas-1), and an

arginine-glycine-aspartate (RGD) motif located at the C-terminus, which can mediate cell adhesion, migration, proliferation and differentiation via integrins.¹

Based mainly on the affected corneal layer, the International Committee for Classification of Corneal Dystrophies (IC3D) anatomically organized corneal dystrophies as epithelial and subepithelial, Bowman layer, stromal and those affecting Descemet membrane and corneal endothelium.² Granular corneal dystrophy (GCD) is an autosomal dominant condition characterized by progressive deposition of granular materials in the corneal stroma leading to visual impairment. These granular deposits were thought to be microfibrillar protein by relatively primitive histochemical studies in the 1960s.^{3, 4} In the 1990s, Eiberg, et al. and Stone, et al. mapped GCD to mutations in human chromosome 5(5q31).^{5, 6} Subsequently, *TGFBI* became a strong candidate gene for those inherited corneal dystrophies that had been mapped to the same locus (5q31).⁷ Klintworth et al. further isolated the predominant protein from corneas with GCD and identified it as BIGH3 protein (now known as TGFBIp) by amino acid (a.a.) sequencing.⁸

To date, many mutations of *TGFBI* have been identified and linked to a spectrum of corneal dystrophies, characterized by the presence of abnormal amyloid fibril and/or non-amyloid deposits in subepithelial and stromal layers in the cornea.⁹ Deposits of differentially degraded TGFBIp fragments were noted in the dystrophic corneas and they likely contribute to the pathogenesis of TGFBIp-related corneal dystrophies.¹⁰ Conformational change and misfolding of TGFBIp are proposed to be the prevailing mechanisms for amyloid fibril formation.¹¹ Among all known mutations, two mutation hot spots are located at codon Arg124 and codon Arg555, respectively. The R555W mutation results in the GCD1 with primarily non-amyloid, granular stromal deposits. By contrast, the R124H mutation results in GCD2 with a more complex stromal aggregation of both granular (non-amyloid) and lattice (amyloid) deposits, whereas a R124C mutation is usually associated with lattice corneal dystrophy (LCD1) with only amyloid deposits. Therefore, the study of molecular properties of dystrophy-causing TGFBIp mutants under amyloid-conducive conditions should shed light on the TGFBIp aggregation and related pathogenic pathways for this group of corneal dystrophies.

As transgenic animal models have failed to manifest corneal dystrophic phenotypes, studies with recombinant proteins are currently the only avenue for understanding the amyloid fibril formation of TGFBIp.¹² We have previously demonstrated that purified recombinant TGFBIp can successfully form amyloid fibrils in vitro¹³ and two shorter TGFBIp are unique in recombinant mutants related to LCD.¹⁴ To further understand the pathogenesis of TGFBIp in GCD, we used a battery of antibodies against various regions of TGFBIp in this report to identify the potential components responsible for GCD in mutant TGFBIp.

MATERIALS AND METHODS

Production of recombinant wild-type and mutant TGFBI protein

To study the role of TGFBIp in corneal dystrophies, we generated recombinant wild-type and several mutant TGFBI proteins (R124C, R124H and R555W) for comparison as those in our previous reports.^{14, 15}

To obtain the cell lysates, transfected HEK293FT cells were cultured in DMEM with reduced puromycin (10% FBS, 1 μ g/mL puromycin) in 100 mm petri dishes with media as described above. When the cells grew up to 75%–80% confluency, culture medium was removed from petri dishes and each plate was rinsed thoroughly with cold PBS buffer. A radioimmunoprecipitation assay (RIPA) buffer (1XPBS; 1% Igepal; 0.1% SDS; 0.5% Deoxycholic acid disodium salt) containing fresh protease inhibitor was added to each plate (0.6ml per 100mm plate) to lyse the cells. The lysed cells were scraped off the plate with a cell scraper and lysates were collected using a syringe with 21-gauge needle and transferred to microcentrifuge tubes. Cell lysates were incubated with 10 μ L of 10mg/ml phenylmethylsulfonyl fluoride (PMSF) for 30–60 minutes at 4°C. Crude cell lysates were centrifuged at 15000 $\times$ G for 20 min at 4°C to remove nuclear fragments and tissue debris. The remaining supernatants were stored at –20°C until used for protein analyses. The supernatants were not dialyzed against phosphate buffer and not further concentrated by filtration. Total protein contents in the cell lysates were measured by Bradford assay¹⁶ on aliquots of each supernatant thus collected.

Gel electrophoresis and immunoblotting

Lysate proteins (20 μ g/lane) from transfected cells were separated by electrophoresis on 7.5% SDS-PAGE gels (Bio-Rad, Hercules, CA). A commercial full-length recombinant TGFBIp, BIGH3 (amino acids 1–683, R&D systems, MN, 50ng/lane) was used as a positive control. The protein solution was mixed with a concentrated sample buffer and gel electrophoresis was conducted as previously reported.¹⁴ The separated proteins were transferred to 0.2 μ m PVDF membranes (Bio-Rad, Hercules, CA) in transfer buffer (10% 10XTGS from Bio-Rad, CA; 70% MilliQ H₂O; 20% Methanol) using 0.35A power for 75 minutes using a plastic cassette. After transfer, PVDF membranes were blocked with 0.2% nonfat dry milk in tris-buffered saline-Tween (TBST) solution (containing 20 mM Tris, pH 7.5; 500 mM NaCl; 0.1% Tween-20) for 1 h at room temperature and then incubated overnight at 4°C with a battery of 5 anti-TGFBIp antibodies (as summarized in Table 1) including a mouse monoclonal TGFBI antibody raised against a.a. 24–683 (R&D Systems, Minneapolis, MN) at 1:5000 dilution; a rabbit polyclonal TGFBI antibody, A01, raised against a.a. 481–581 (Abnova corporation, Taipei, Taiwan) at 1:2000 dilution; a custom made rabbit polyclonal TGFBI antibody, KE50, against a.a. 125–683 (University of Minnesota, MN) at 1:5000 dilution; a rabbit polyclonal TGFBI antibody against a.a. 199–406 (Proteintech Group, Chicago, IL) at 1:5000 dilution; a rabbit polyclonal TGFBI antibody, AV44268, raised against a.a. 24–649 (Sigma, Saint Louis, MO) at 1:5000 dilution.

After blocking, blots were washed 3–4 times every 15 minutes with TBST and then incubated for 1 h at room temperature with corresponding secondary antibodies such as goat anti-mouse or anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:20000 dilution and Precision Strep-Tactin-HRP conjugate (Bio-Rad, Hercules, CA) at 1:30000 dilution for Western C molecular weight marker. Blots were washed again and the protein bands were visualized on a film with an enhanced chemiluminescence (ImmobilonTM Western, Millipore Corporation, Billerica, MA) reagent.

Immunoblottings were performed at least 3 times with different batches of recombinant proteins for each antibody. Consistent results were observed among different batches of recombinant proteins and representative immunoblots were included in this report. To compare if the protein bands identified by different antibodies were similar, sequential immunoblotting was performed. After marking the bands identified by the first anti-TGFBIP antibody, the antibody was stripped off the blot by a Western Re-Probe solution (G-Biosciences, St. Louis, MO) according to the manufacturer's instructions. The blot was then restained with a different anti-TGFBIP antibody to compare if the proteins were found on the same location.

To further study the effects of salt ionic strength on protein aggregation, we treated the control BIGH3 (50 ng/lane) and cell lysates (20 µg/lane) of recombinant TGFBIP WT and mutants (R124C, R124H) with various concentrations (from 500mM to 2M) of NaCl or LiCl for comparison. After adding the salt, the samples were incubated in room temperature for 16 to 18 hours. Gel electrophoresis and immunoblotting were then performed as described above.

RESULTS

As noted in our previous study,¹⁷ the anti-TGFBI antibody from R&D (against a.a. 24–683) showed a strong reactivity to WT TGFBI proteins. Herein, we further confirmed that this antibody could detect a prominent band of 68kD (BIGH3 monomer) and to a lesser extent a 140kD (BIGH3 dimer) in the unfolded BIGH3. The antibody also detected two corresponding bands, a distinct 62kD band (TGFBIP monomer) and a less noticeable 125kD band (TGFBIP dimer) in the cell lysates of WT, R124C, and R124H (Figure 1A). This antibody did not react with the R555W cell lysates.

We further analyzed the blots with four other polyclonal antibodies raised against different regions of TGFBIP for comparison. Similar to the anti-TGFBI from R&D, the A01 anti-TGFBI (raised against a.a. 481–581) from Abnova reacted predominantly with the corresponding monomer bands in BIGH3 and WT cell lysates (68kD and 62kD, respectively, Figure 1B). In addition, this antibody reacted predominantly with the monomeric TGFBIP (62kD) in R124C and R124H cell lysates (Figure 1B). A less noticeable 125kD band was also detected in the R124H cell lysates. Similar to the R&D antibody, A01 did not react with R555W from either purified proteins or cell lysates (cf. Figure 1A and 1B).

By contrast, our custom-made antibody, KE 50 (raised against a.a. 125–683) detected the 62kD bands in all recombinant proteins from all cell lysates including R555W, in addition to the corresponding 68kD band in BIGH3 (Figure 2A). A less prominent band of 140kD was noted in BIGH3 along with corresponding bands of 125kD in cell lysates of WT, R124C and R124H, respectively. Of note, while also reacting with monomeric R555W, KE50 did not react with dimeric TGFBIP in the R555W cell lysates (Figure 2A).

We further investigated if other antibodies that would react favorably with both R555W (GCD1) and R124H (GCD2), as they share similar granular stromal deposits. The

polyclonal anti-TGFBI from Proteintech Group (PTG) was raised against a.a. 199–406, a region containing neither mutation hotspots of R124 or R555. As expected, this antibody detected primarily the 62kD in WT and all tested TGFBIp mutants in cell lysates including R555W as well as a monomeric 68kD band in BIGH3 (Figure 2B). It also reacted with R124H dimers to a lesser extent, but not with the dimers of WT, R124C and R555W. Of note, this PTG antibody also detected a unique 35kD band only in cell lysates of R124H and R555W. No such band was noted in BIGH3, WT or R124C (Figure 2B).

Another antibody, AV44268 (raised against a.a. 24–649) from Sigma readily detected monomeric TGFBIp (62kD) in all cell lysates (Figure 2C). It reacted only modestly with the related dimers in BIGH3 and in all cell lysates except R555W. Of note, a 40kD band was detected by this antibody in all cell lysates (Figure 2C).

To further confirm that the 40kD protein in the cell lysates detected by AV44268 is not the same as the 35kD band detected by the PTG antibody, we performed a sequential immunoblotting. The blot was first stained with AV44268 to confirm that the 40kD bands were present in the cell lysates of all recombinant TGFBIp. After the location of the 40kD band in each lane was marked, AV44268 was then stripped off the blot as described in Methods. The stripped blot was restained with the PTG antibody. The second immunoblotting with PTG showed that a 35kD band (not at the 40kD location) was detected only in R124H and R555W, but not in WT and R124C (data not show, it was essentially identical to result shown in Figure 2B). The preferential expression of 35kD only in R124H and R555W also speaks against that the 35kD band is similar to the 40kD, which was detected in all recombinant proteins by AV44268.

As we and other investigators have previously reported that TGFBIp aggregation is ionic strength-dependent,¹³ we further investigated the effects of salt on protein aggregation. When NaCl from 0.5 to 2M was added to the cell lysates, a progressive increase of polymeric TGFBIp was noted in each blot (Figure 3A–C), indicating the protein polymerization was dose-dependent on the NaCl concentrations. Of note, when 2M NaCl was added to the cell lysates, there was a significant protein polymerization which was nearly indiffusible through the gel during electrophoresis (the uppermost band in each gel, \$ in Figure 3A–C). A corresponding reduction of monomeric and dimeric recombinant TGFBIp by 2M NaCl was also noted in Figures 3A–C. In contrast, LiCl from 0.5 to 2 M did not affect the TGFBIp polymerization. Of note, 2M LiCl neither induced significant TGFBIp polymerization nor reduced the related monomeric or dimeric TGFBIp (Figure 3A–C). It is also noteworthy that the unique 35kD fragment was detected in all R124H cell lysates independent of the salt concentration (Figure 3C), which is consistent with the findings in Figure 2B.

DISCUSSION

Although molecular genetics have unequivocally established the role of mutations of *TGFBI* gene in the human chromosome 5q31-related hereditary corneal dystrophies, the pathogenic mechanisms responsible for abnormal protein aggregations in human corneas remain to be elucidated. In this study, we undertook an *in vitro* investigation of the potential components

of TGFBIp responsible for causing the untoward corneal deposits using a battery of anti-TGFBIp antibodies and three recombinant mutant proteins (R124C, R124H and R555W) representing the most common genotypes for TGFBIp-related corneal dystrophies.

From our results, all 5 antibodies tested reacted with monomeric forms of BIGH3, WT, R124C and R124H. In addition, these antibodies can detect dimeric and polymeric TGFBIp when present. However, neither the antibody from R&D or Abnova reacted with R555W. As the R&D and Abnova A01 anti-TGFBIp were raised against a.a. 24–683 and 481–581 of TGFBIp, respectively, we speculate that these two antibodies do not react with the mutated region of R555W at the C-terminus of TGFBIp, but they can react with other TGFBIp without mutation in that region.

In this study, we identified several previously unreported TGFBIp-related fragments in the cell lysates. These fragments may contribute to TGFBIp aggregation in corneal dystrophies, as other degraded TGFBI proteins isolated from corneal deposits in clinical specimens have been previously implicated in the pathogenesis of TGFBIp-related corneal dystrophies.¹⁰ For example, a 40kD band was detected by AV44268 in cell lysates of WT and all three TGFBIp mutants (Figure 2C). Most importantly, we also found a unique 35kD fragment in cell lysates of R124H and R555W by the PTG antibody (Figure 2C). As this fragment is only present in R555W (GCD1) and R124H (GCD2) and not in WT or R124C (LCD1), we further surmise that this fragment might contribute to the granular (non-amyloid) deposits commonly observed in corneas with GCD 1 and 2 and it potentially could be used as a molecular marker of GCD 1 and 2. Reis-Bucklers corneal dystrophy (RBCD) with superficial granular corneal deposits in the Bowman layer is associated with R124L and sometimes referred to as GCD3.^{18, 19} As we have not generated R124L recombinant proteins, we can not comment on the presence of 35kD in GCD 3.

Previous investigations have studied the mutant TGFBIp with either anti-TGFBIp raised against the N- or C-terminus.²⁰ However, the 35kD fragment was only recognized by the PTG anti-TGFBIp (raised against a.a. 199–406) and not detected by other antibodies, this finding suggests that this fragment is not likely to be associated with either terminus of TGFBIp and is likely cleaved from TGFBIp with the central domain of TGFBIp relatively preserved. Furthermore, our study indicates the differential degradation exists between WT and three TGFBIp mutants, given the unique presence of 35kD in R124H and R555W. This is in sharp contrast to the earlier notion that WT and all mutant TGFBI proteins displayed similar degradation patterns when expressed in transfected corneal epithelial cells and CHO cells.^{21, 22}

The detection of shorter TGFBIp fragments such as 35kD or 40kD bands only in cell lysates but not in purified proteins as described in our recent report¹⁴ is not surprising. Such a finding is related to the purification process of recombinant TGFBIp. To concentrate the recombinant proteins during protein production, a gel filtration with M.W. cutoff of 50kD was used in that report¹⁴. Consequently, these smaller fragments were removed from the purified recombinant proteins. Thus, the functional significance of these smaller TGFBIp fragments may have been overlooked in previous investigations using only purified recombinant proteins.

Zinn et al., previously suggested that the four repeat Fas 1 domains of TGFBIp are folded into a potential bivalent tetrameric structure.²³ This notion can be further substantiated by the recent report of secondary amyloidosis in non-dystrophic corneas with aggregations of WT TGFBIp.²⁴ However, we did not find significant TGFBIp polymerization in the cell lysates in this study. We speculate that the presence of other non-TGFBIp proteins in the cell lysates may act as a protein chaperone to prevent TGFBIp aggregation.

In general, the severity of corneal dystrophies correlates well with the extents of mutant TGFBIp aggregations. GCD1 associated with the R555W mutation is characterized by granular opacities made of non-amyloid protein aggregates in the subepithelial to anterior stroma layer.^{6, 25–27} In contrast, GCD2 (also known as Avellino corneal dystrophy) associated with the R124H mutation shows discrete granular opacities in the anterior stromal layer and fusiform opacities (lattice lines) in the deeper stroma.^{28, 29} Many proteomic studies have suggested that the disease-causing proteins form abnormal degradations under physiological conditions.^{30–33} Thus, mutations may alter the protein degradation pathways and have differential effects on the structures and stability of the deposited proteins.

To further understand the impact of solute and ionic strength on protein aggregation, we compared the effect of NaCl and LiCl on TGFBIp. As shown in Figure 3A–C, we noted TGFBIp polymerized as insoluble aggregates under high [NaCl]. This finding corroborates our recent findings regarding the ionic strength on WT TGFBIp aggregation.¹³ In contrast, recombinant TGFBIp remained in their monomeric and dimeric forms without significant polymerization even under high [LiCl]. The finding suggests that LiCl may impede the aggregations of TGFBIp *in vitro*. A recent report also indicates that lithium can down-regulate the expression and aggregation of TGFBIp in corneal fibroblast cultures from corneas with GCD2.³⁴ Our results may also imply that LiCl at low concentration may prevent the aggregation of mutant TGFBIp *in vitro*.

While our findings of a 35kD fragment in R124H and R555W and TGFBIp aggregation under high [NaCl] are relatively novel, discrepancies may exist when correlating experimental results with the clinical observations. The pathogenic role of 35kD protein in GCD 1 and 2 awaits further confirmation. Ultimately, better understanding of the pathogenic role of TGFBIp in corneal dystrophies may lead to the development of novel therapeutic strategies for these sight-threatening corneal dystrophies.

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FIGURE 1A

FIGURE 1B

FIGURE 1.

FIGURE 1A. a representative immunoblot of 2-tag (His)₆-TGFBIP-Strep recombinant TGFBIP in cell lysates stained with a mouse monoclonal anti-TGFBIP antibody from R&D Co. The antibody detected a 62kD band (a.a. 30–641, the lower band) and a less prominent 125kD band (*) in WT, R124C and R124H, but none in R555W. A commercial BIGH3 protein (a.a. 1–683, M.W. of 68kD, Sigma Co.) also reacted with this antibody in a similar pattern with a 68kD monomeric band and a 140kD dimeric band.

FIGURE 1B. a representative immunoblot of 2-tag recombinant proteins in cell lysates stained with a mouse polyclonal anti-TGFBIP antibody from Abnova Co. (AO1). This antibody detected a 62kD band in WT, R124C and R124H (the lower band) and a 125kD band (*) in R124H, but none in R555W. The antibody also detected a prominent 68kD monomeric band in BIGH3.

FIGURE 2A

FIGURE 2B

FIGURE 2C

FIGURE 2.

FIGURE 2A. a representative immunoblot of 2-tag recombinant proteins in cell lysates stained with a custom-made rabbit polyclonal anti-TGFBI antibody, KE50. This antibody detected a 62kD band (the lower band) and a less prominent 125kD band (*) in WT, R124C and R124H. Only a 62kD band was detected in R555W. KE50 also reacted with BIGH3 in a similar pattern with a prominent 68kD monomeric band and a faint 140kD dimeric band. FIGURE 2B. a representative immunoblot of 2-tag recombinant proteins in cell lysates stained with a rabbit polyclonal anti-TGFBI antibody from Proteintech Group (PTG) Co. The PTG antibody detected a 62kD band in WT, R124C, R124H and R555W and a 125kD

band (*) in R124H. Of note, this antibody also detected a 35kD band (>) only in R124H and R555W, but not in WT and R124C. This antibody also reacted with primarily monomeric BIGH3.

FIGURE 2C. a representative immunoblot of 2-tag recombinant proteins in cell lysates stained with AV44268, a rabbit polyclonal anti-TGFBIP antibody from Sigma Co. This antibody detected a 62kD band in WT, R124C, R124H and R555W and a 125kD band (*) in WT, R124C and R124H. Of note, this antibody detected a 40kD band (@) in WT, R124C, R124H and R555W. This antibody also reacted with BIGH3 but no 40kD band was detected in BIGH3.

FIGURE 3A

FIGURE 3B

FIGURE 3C

FIGURE 3. Immunoblots of cell lysates of WT (A), R124C (B) and R124H (C) under different concentrations (500mM-2M) of NaCl and LiCl stained with a rabbit polyclonal anti-TGFBI antibody from Proteintech Group (PTG) Co

A. a representative immunoblot of WT cell lysates with the antibody detected a major monomeric 62kD band and a dimeric 125kD band (*) in all preparations. A prominent band of highest molecular weight (\$) was detected in NaCl-containing preparations in a dose-dependent manner from 500mM to 2M. This band was barely present in the preparations containing 2M LiCl. Of note, the intensity of the monomeric and dimeric bands decreased with the increase of salt concentrations (c.f. 2M vs. 500mM).

B. a representative immunoblot of R124C cell lysates with the antibody detected a major monomeric 62kD band and a minor dimeric 125kD band (*) in all preparations. A prominent band of highest molecular weight (\$) was detected in the preparation containing 2M NaCl. This band was less noticeable in the preparations containing 500mM or 1M NaCl. A similar band was barely detected in the preparation containing 2M LiCl (\$). Such a band was not readily noted in the preparations containing 500mM or 1M LiCl. Of note, the intensity of the monomeric and dimeric bands decreased inversely with the increase of salt concentrations from 500mM to 2M.

C. a representative immunoblot of R124H cell lysates with the antibody detected a major monomeric 62kD band and a dimeric 125kD band (*) in all preparations. A prominent band of highest molecular weight (\$) was detected in preparations containing 2M NaCl and to a lesser extent in 2M LiCl. This band was not evident in the preparations containing lower salt concentrations. The intensity of the dimeric band decreased inversely in the presence of 2M NaCl. Of note, the 35kD fragment of TGFBIp (>) was present and not affected by the salt concentrations in all R124H preparations.

TABLE 1

List of Antibodies

Name	Antigens from TGFBIp	Dilution Titer
R&D Systems	a.a. 24–683	1:5000
Abnova A01	a.a. 481–581	1:2000
KE50	a.a. 125–683	1:5000
Proteintech Group	a.a. 199–406	1:5000
Sigma AV44268	a.a. 24–649	1:5000