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Differential expression and processing of transforming growth factor beta induced protein (TGFB β Ip) in the normal human cornea during postnatal development and aging

Henrik Karring^a, Kasper Runager^b, Zuzana Valnickova^b, Ida B. Thøgersen^b, Torben Møller-Pedersen^b, Gordon K. Klintworth^c, and Jan J. Enghild^{b,*}

^aInstitute of Chemical Engineering, Biotechnology and Environmental Technology, Faculty of Engineering, University of Southern Denmark, Niels Bohrs Allé 1, DK-5230 Odense M, Denmark

^bCenter for Insoluble Protein Structures (inSPIN) and Interdisciplinary Nanoscience Center (iNANO), Department of Molecular Biology, Science Park, University of Aarhus, Gustav Wieds Vej 10c, DK-8000 Aarhus C, Denmark

^cDepartments of Pathology and Ophthalmology, Duke University Medical Center, Durham, NC 27710, USA

Abstract

Transforming growth factor beta induced protein (TGFB β Ip, also named keratoepithelin) is an extracellular matrix protein abundant in the cornea. The purpose of this study was to determine the expression and processing of TGFB β Ip in the normal human cornea during postnatal development and aging. TGFB β Ip in corneas from individuals ranging from six months to 86 years of age was detected and quantified by immunoblotting. The level of TGFB β Ip in the cornea increases about 30% between 6 and 14 years of age, and adult corneas contain 0.7–0.8 μ g TGFB β Ip per mg wet tissue. Two-dimensional (2-D) immunoblots of the corneal extracts showed a characteristic “zig-zag” pattern formed by different lower-molecular mass TGFB β Ip isoforms (30–60 kDa). However, the relative abundance of the different isoforms was different between infant corneas (< 1 year) and the child/adult corneas (> 6 years). Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) data of TGFB β Ip isoforms separated on large 2-D gels show that TGFB β Ip is proteolytically processed from the N-terminus. This observation was supported by in silico 2-D gel electrophoresis showing that sequential proteolytical trimming events from the N-terminus of mature TGFB β Ip generate TGFB β Ip isoforms which form a similar “zig-zag” pattern when separated by 2-D polyacrylamide gel electrophoresis (PAGE). This study shows that in humans TGFB β Ip is more abundant in mature corneas than in the developing cornea and that the processing of TGFB β Ip changes during postnatal development of the cornea. In addition, TGFB β Ip appears to be degraded in a highly orchestrated manner in the normal human cornea with the resulting C-terminal fragments being retained in the cornea. The age-related changes in the expression and processing of corneal TGFB β Ip suggests that TGFB β Ip may play a role in the postnatal development and maturation of the cornea. Furthermore, these observations may be relevant to the age at which mutant TGFB β Ip deposits in the cornea in those dystrophies caused by mutations in the transforming growth factor beta induced gene (*TGFB β*) as well as the mechanisms of corneal protein deposition.

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*Corresponding author. Tel.: +45 8942 5062; fax: +45 8942 5063. jje@mb.au.dk (J. J. Enghild).

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Keywords

TGFB1; TGFB1p; postnatal development; cornea maturation; age-related changes; protein degradation; corneal dystrophy

1. Introduction

The transforming growth factor beta induced protein (TGFB1p, also named keratoepithelin) is an extracellular matrix protein encoded by the transforming growth factor beta induced gene (*TGFB1*), which was discovered in a lung adenocarcinoma cell line exposed to transforming growth factor beta (TGF- β) (Skonier et al., 1992). TGFB1p is composed of 683 residues and contains an N-terminal secretory signal peptide (Met₁-Ala₂₃) and a C-terminal RGD sequence (Arg₆₄₂-Gly₆₄₃-Asp₆₄₄). According to sequence analyses, the N-terminal part of secreted TGFB1p contains a cysteine-rich EMI domain (residues Gly₄₅-Ala₉₉) originally recognized in EMILINs (Doliana et al., 2000), and residues Ala₁₀₀-Pro₆₃₅ contains four 140-amino acid residue repeats with sequence identity with the fasciclin-1 (FAS1) domain from insects (Zinn et al., 1988). TGFB1p interacts with fibronectin (Billings et al., 2002), different integrins (Bae et al., 2002; Kim, J. E. et al., 2000; Kim, J. E. et al., 2002; Kim, M. O. et al., 2003; Ohno et al., 1999), and collagens (Hanssen et al., 2003; Hashimoto et al., 1997), and plays an important role in adhesion of cells to the collagenous network of connective tissues but its specific biochemical role remains unclear (Runager et al., 2008).

TGFB1p is a major protein in the human cornea (Escribano et al., 1994; Klintworth et al., 1994). Most of the human corneal TGFB1p lacks C-terminal residues Ser₆₅₈-His₆₈₃, which is likely to leave the integrin-binding RGD sequence exposed for interactions (Andersen et al., 2004). In addition to the C-terminally processed and mature corneal TGFB1p isoform, the human cornea contains small amounts of full-length TGFB1p (residues Gly₂₄-His₆₈₃) (Andersen et al., 2004) and numerous isoforms of TGFB1p with distinct molecular masses and isoelectric points (Karring et al., 2005). About 60% of TGFB1p in the human cornea is associated with water-insoluble components of the collagenous extracellular matrix through a disulfide bridge (Andersen et al., 2004).

Mutations in *TGFB1* cause several phenotypically different inherited corneal diseases with different clinical and histopathological characteristics. These different phenotypes include variants of lattice corneal dystrophy (LCD) type I, and variants of granular corneal dystrophy (GCD), as well as Thiel-Behnke corneal dystrophy. The *TGFB1* related corneal dystrophies are characterized by an extracellular accumulation of TGFB1p within the cornea. Particularly noteworthy is the fact that specific mutations in *TGFB1* consistently cause specific types of corneal deposits. Thus, the deposits in the GCDs contain rod-shaped or trapezoid bodies of TGFB1p-containing material, LCD type I and its variants are characterized by amyloid deposition, and Thiel-Behnke corneal dystrophy involve the formation of subepithelial “curly fibers” in the cornea (Kannabiran and Klintworth, 2006; Klintworth, 2003, 2009).

TGFB1p is expressed in several fibrous connective tissues and organs, but mutations in *TGFB1* only lead to an accumulation of TGFB1p deposits in the cornea (El Kochairi et al., 2006). The clinical age of onset of the *TGFB1* corneal dystrophies depends on the specific mutation in *TGFB1*. Thus, while some mutations lead to an onset in the first or second decade of life (e.g. R555W and R124C) (Kannabiran et al., 2005; Okada et al., 1998; Romero et al., 2008), others become manifest later in life (e.g. L527R and A546T) (Dighiero et al., 2000; Fujiki et al., 1998). The molecular basis for the different types of deposits, the ages of onset, and the cornea-specific accumulation of TGFB1p caused by mutations in *TGFB1* remains unknown. Previous studies have shown that the turnover of TGFB1p in the cornea is affected

by the mutations (Korvatska et al., 2000). Thus, the degradation patterns of TGFBIp in diseased corneas are different from that in the normal cornea and depend on the mutation in *TGFBI*.

In the present study, we have investigated the expression and processing of TGFBIp in the normal human cornea at different ages. In addition, we present mass spectrometry data showing that all the lower-molecular mass isoforms of TGFBIp identified in the healthy human cornea are N-terminally truncated isoforms of the protein. The observed age-related expression and orchestrated processing of corneal TGFBIp are likely to provide a better understanding of the biology of TGFBIp and the molecular pathobiology of corneal diseases associated with TGFBIp.

2. Materials and methods

2.1. Human corneas

Corneas from three infants (male of 6 months, post mortem time (pmt) < 48 h; female of 9 months, pmt 51 h; male of 12 months, pmt < 48 h) and five children/adults (male of 6 years, pmt 43 h; male of 14 years, pmt 44 h; male of 19 years, pmt 59 h; female of 51 years, pmt 25 h; male of 86 years, pmt 95 h) were obtained post mortem from individuals intended for autopsy at the Department of Forensic Medicine, Aarhus University Hospital, Denmark. The bodies were kept refrigerated before the cornea samples were removed. None of the individuals had any known corneal disorders or previous eye surgery. The collection of human tissue was approved by the Regional Committee for Scientific Medical Ethics in Aarhus, Denmark. The central area of the corneas was excised using a 7-mm diameter trephine. Pieces of the corneas were lyophilized and homogenized in liquid nitrogen, and the resulting fine powder was used for two-dimensional (2-D) polyacrylamide gel electrophoresis (PAGE) analyses.

2.2. One-dimensional (1-D) SDS-PAGE

Pieces of cornea were weighted and added 1 × sodium dodecyl sulfate (SDS) sample buffer with 50 mM dithiothreitol (DTT) to a final concentration of 50 mg wet tissue per ml sample buffer. The samples were boiled for 10 minutes and extracted overnight under rotation before 20 µl (equalling 1.0 mg wet cornea) were analysed on 10% SDS-polyacrylamide gels (10 × 10 × 0.1 cm) using the 2-amino-2-methyl-1,3 propanediol (ammidiol)/glycine/hydrogen chloride (HCl) buffer system (Bury, 1981). As reference for the quantifications, 0.5 µg of purified recombinant human TGFBIp was analyzed on the same gel (Andersen et al., 2004; Runager et al., 2009). The samples for quantification were analyzed in triplicates.

2.3. 2-D SDS-PAGE

Proteins from the corneal powders were extracted for 2-D gel electrophoresis as previously described (Karring et al., 2005). Briefly, corneal powder (0.5–1.0 mg) was dissolved in lysis buffer (5 M urea, 2 M thiourea, 2% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (Chaps) (Sigma), 2% (w/v) N-decyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (SB3-10) (Sigma), 10 mM DTT, 2 mM EDTA, 2 mM 1,10-phenanthroline, 40 µM E-64 (Sigma), 2 mM pefabloc SC (Fluka), and 0.5% (v/v) IPG Buffer of pH 4–7) to a final concentration of 1 mg dry tissue per ml and incubated for 1 hour at 25 °C under rotation. Subsequently, the samples were sonicated and centrifuged before 125 µl of the solubilized sample was loaded onto 7-cm Immobiline DryStrips (GE Healthcare) with pH range 4–7. The strips were rehydrated over night and the isoelectric focusing was performed using the IPGphor System II (GE Healthcare) for 7.5 kVh. After the isoelectric focusing, the proteins were reduced in 6 M urea, 50 mM Tris-HCl (pH 8.8), 30% (v/v) glycerol, 2% (v/v) SDS containing 6.5 mM DTT and then alkylated in the same buffer containing 10 mM iodoacetamide. The strips were placed on top of 10 × 10 × 0.1 cm 10% SDS-polyacrylamide gels and the second-dimension

electrophoreses were run at 15 mA per gel in running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS).

2.4 Detection and quantification of TGFBIp by immunoblotting

Proteins separated by 1-D or 2-D SDS-PAGE were electroblotted to a polyvinylidene difluoride (PVDF) membrane (Millipore Immobilon transfer membranes, Millipore, Bedford, MA) (Matsudaira, 1987) for immunoblotting. The membranes were blocked in 30 ml 5% dry milk solution in 20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.1% Tween (TBS-T) for one hour at room temperature and an antiserum from rabbit against the central domains (residues Gly₁₃₄-Pro₅₀₀ in precursor TGFBIp) of human TGFBIp (Andersen et al., 2004) was added to the blocking solution. Dilutions of 1 : 5 000 of the primary antiserum in 5% milk were used for the 1-D and 2-D immunoblots, respectively. The membranes were incubated with the primary antiserum over night at 4 °C, washed thoroughly with TBS-T, before incubated for two hours at room temperature in TBS-T containing goat anti-rabbit IgG peroxidase conjugate (Sigma Chemical Co., St. Louis, MO). The secondary antibody was used in ratios of 1 : 10 000 and 1 : 5 000 in 5% milk for the 1-D and 2-D immunoblots, respectively. Finally, the membranes were washed thoroughly with TBS-T and developed for 0.5–2.0 minutes using the enhanced chemiluminescence (ECL) Western blotting detection system and reagents (GE Healthcare) as previously described (Karring et al., 2007). The levels of TGFBIp were determined through densitometry on the developed films from 1-D immunoblots using the Biorad Quantity One software. The absolute concentrations of mature TGFBIp in the human corneas were estimated using the signal of reactivity from a known amount of purified recombinant TGFBIp (Andersen et al., 2004; Runager et al., 2009) analysed on the same gel.

2.5 Identification of TGFBIp fragments by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS)

In a previous study (Karring et al., 2005, 2006), the adult human cornea proteome was analyzed by 2-D SDS-PAGE using large Immobiline DryStrips (18 cm, GE Healthcare) and 12.5% SDS-polyacrylamide gels (18 × 23.4 × 0.1 cm). The raw MALDI-TOF MS data from that work were used to determine the TGFBIp regions covering the identified tryptic TGFBIp peptides for each of the recognized TGFBIp-containing gel spots. Thus, the sequence of the actual TGFBIp fragment in the gel spot may not be fully covered by the identified peptides from the MALDI-TOF MS data.

2.6. Simulation of 2-D PAGE patterns of N- and C-terminal truncation events of human TGFBIp

The theoretical 2-D gel patterns of N- and C-terminal truncated isoforms of human TGFBIp were obtained using the simulated 2-D gel-function of the GPMW 8.10 program (Peri et al., 2001). Thus, the sequence of mature human corneal TGFBIp (residues Gly₂₄-Ala₆₅₇) was saved and the theoretical isoelectric point (pI) and molecular mass values of N- and C-terminal-trimmed isoforms of TGFBIp were calculated and displayed. The generation of the simulated 2-D gel was based on the assumption that human corneal TGFBIp does not contain any post-translational modifications.

3. Results

3.1. The expression of corneal TGFBIp increases during postnatal development

The 1-D immunoblots (Fig. 1A) show that the 65 kDa C-terminally truncated mature TGFBIp (residues Gly₂₄-Ala₆₅₇ of precursor TGFBIp) is the major isoform of the protein in the human cornea at all ages (0.5–86 years of age). In addition, some bands migrating at lower molecular masses (30–60 kDa) are detected predominantly in the adult corneas when the 1-D

immunoblots were developed for longer time (Fig. 2). Densitometry measurements reveal that the level of mature TGFBIp in the analyzed corneas increases about 30% between 6 and 14 years of age. Thus, while the concentration of TGFBIp is 0.45–0.55 μg per mg wet tissue in infant and child corneas (< 6 years of age), the teenage and adult corneas (> 14 years of age) contain 0.7–0.8 μg per mg wet tissue (Fig. 1B). There is no correlation between the level of corneal TGFBIp and the post mortem times of the autopsies.

3.2. Age-related changes in the processing of corneal TGFBIp

To investigate the processing of TGFBIp in the cornea during postnatal development and aging, corneal protein extracts from the donor corneas were separated by 2-D gel electrophoresis and TGFBIp was detected by immunoblotting. The resulting 2-D immunoblots revealed a number of TGFBIp isoforms forming a characteristic “zig-zag” pattern (MW: 30–60 kDa; pI: 5.5–6.2) (Fig. 2). From the 2-D immunoblots it is evident that TGFBIp is processed in a highly orchestrated manner. The overall “zig-zag” pattern generated by TGFBIp isoforms is the same for all the individuals (data not shown), but the relative abundance of the different TGFBIp isoforms changes during infancy (0–1 years of age, Figs. 2A, B) through childhood (1–6 years of age, Figs. 2B, C) and is then maintained through the rest of life. Thus, several of the lower-molecular mass isoforms of TGFBIp (30–45 kDa) in the “zig-zag” pattern on the immunoblots are relatively more abundant in the child/adult corneas (Fig. 2C) than in the infant corneas (Fig. 2A,B), when compared to the higher molecular mass species of TGFBIp (~60 kDa) in the “zig-zag” pattern. There are no indications that the relative abundances of the different TGFBIp isoforms in the “zig-zag” pattern are related to the post mortem times of the autopsies.

3.3. Corneal TGFBIp is proteolytically processed from the N-terminus in a highly orchestrated manner

Proteins extracted from normal adult human corneas were analyzed by 2-D PAGE using a large gel-strip with pH gradient 4–7. Except from two isoforms migrating at about 70 kDa and two lower-molecular mass isoforms (~14 kDa) of TGFBIp, all the 21 identified TGFBIp isoforms have pI values between 5.7 and 6.5, and migrate as proteins with molecular masses of 35–45 kDa (Fig. 3A). The most intense silver-stained TGFBIp spots on the 2-D gel are likely to be identical to the most intense spots on the 2-D immunoblots (Fig. 2C). In accordance with this, the most intense spots in the “zig-zag” pattern on the 2-D immunoblots have pI values between 5.5–6.2 and molecular masses of about 40 kDa.

From the MALDI-TOF MS data, the TGFBIp regions covering the identified tryptic TGFBIp peptides were determined for the detected TGFBIp-containing protein spots (Fig. 3A, spots 1–21). As expected, the estimated molecular masses of the TGFBIp regions based on the peptide-covered sequence are lower than the actual masses of the isoforms according to their migration on the 2-D gels. This is due to the fact that the peptide coverage is never complete and that the C-terminal peptide of proteins digested with trypsin are rarely detected by mass spectrometry as they lack terminal lysine or arginine. The mass spectrometry-based analyses reveal that the lower-molecular mass isoforms of TGFBIp (35–45 kDa) (Fig. 2 and 3) are all N-terminal truncated versions of the mature protein. According to the mass spectrometry data, most of the TGFBIp isoforms (spot numbers 3–20) are lacking 210–375 residues (about 23–41 kDa reduction in mass) from the N-terminus of the mature protein. Thus, these results suggest that mature corneal TGFBIp in the normal human cornea is proteolytically processed from the N-terminus in a highly orchestrated manner, which gives rise to multiple C-terminal fragments with slightly different molecular masses and isoelectric points.

3.4. N-terminal trimming events of TGFBIp generate a characteristic “zig-zag” pattern on simulated 2-D gels

The observations from 2-D immunoblots and mass spectrometry-based identification of different TGFBIp fragments separated by 2-D gel electrophoresis suggest that the characteristic “zig-zag” pattern on 2-D gels is generated by N-terminal truncated isoforms of TGFBIp. This is supported by theoretical 2-D PAGE patterns of human TGFBIp sequentially trimmed by one residue from either the N-terminus or the C-terminus (Fig. 4). Thus, the characteristic “zig-zag” pattern of TGFBIp isoforms observed on 2-D gels is recognized in the simulated 2-D PAGE pattern of N-terminal trimming events indicating that heterogeneous N-terminal truncations of mature TGFBIp are responsible for the characteristic “zig-zag” fragmentation pattern. Thus, the result from this *in silico* experiment is consistent with the data showing that most of the TGFBIp isoforms are lacking 210–375 residues from the N-terminus (Fig. 2 and Fig. 3).

4. Discussion

Despite the fact that TGFBIp is induced by the important growth factor TGF- β and is a major protein in the cornea, the biochemical properties and function of the protein are poorly understood. In this study, we have investigated the expression and processing profiles of TGFBIp in the human cornea during postnatal development and aging. In addition, we have presented data showing that all identified fragments of TGFBIp from healthy human corneas are N-terminally truncated isoforms.

The protein quantifications showed that the level of TGFBIp in the cornea increases by 30% between 6 and 14 years of age. Thus, while corneas from infants and young children contain 0.45–0.55 μg per mg wet tissue, teenage and adult corneas contain 0.7–0.8 μg per mg wet tissue. In addition, our data suggest that the level of TGFBIp in the mature cornea is maintained throughout life (Fig. 1). The ages at which the expression and processing of corneal TGFBIp changes are in good agreement with other observations on postnatal corneal changes and maturation. Thus, studies by other investigators suggest that both central and paracentral corneal thicknesses reach mature thicknesses at 3–5 years of age (Ehlers et al., 1976; Hussein et al., 2004). Likewise, after the age of 2–3 years the growth behavior and composition of Descemet membrane change (Kabosova et al., 2007; Murphy et al., 1984). Thus, major changes in the composition and distribution of different basement membrane proteins have been observed between infant/child corneas (< 3 years) and teenage/adult corneas (aged 13–75 years) (Kabosova et al., 2007). The differences in the expression and distribution of specific proteins between infant and adult human corneas may relate to the structural changes in the cornea associated with its postnatal maturation. Therefore, the present results could indicate that TGFBIp also plays a role in the postnatal development and maturation of the cornea. Furthermore, the 30% increase in the level of TGFBIp in the human cornea between the age of 6 and 14 years could explain why clinical symptoms in the corneal dystrophies caused by mutations in *TGFBI* rarely occur in early infancy. For example, homozygous R555W and R124H mutations in *TGFBI* become clinically evident at 2–10 years of age at an earlier age than heterozygotes (Kannabiran et al., 2005; Mashima et al., 1998; Okada et al., 1998). A dramatic increase in the concentration of TGFBIp in the cornea is likely to enhance the risk of aggregation of misfolded and structural labile TGFBIp mutants. Thus, the formation of crystalloid and amyloid deposits in the corneas of patients with TGFBIp-related GCDs and LCDs could be stimulated by the increase in TGFBIp concentration.

The observed age-dependent changes in the processing of corneal TGFBIp could also be important for the onset of the *TGFBI* related corneal dystrophies. It is noteworthy, that age-dependent proteolytic processing from the N-terminus of TGFBIp has also been observed in the human endothelial cell-Descemet's membrane complex (Jurkunas et al., 2009). Our

MALDI-TOF MS data for the identified 2-D gel spots indicate that all the TGFBIp fragments are generated by N-terminal proteolytic processing events of the mature C-terminally truncated isoform. Thus, we have for the first time identified several C-terminal TGFBIp fragments using a direct protein identification method. The identified fragments of TGFBIp are lacking up to about 375 residues from the N-terminus which will include the cysteine-rich EMI domain and the FAS1-1 and FAS1-2 domains. These results are in accordance with the results reported by Korvatska et al. (Korvatska et al., 2000) showing that some of TGFBIp in normal and diseased corneas are proteolytically processed. The very small differences in pI's and molecular masses of some of the TGFBIp isoforms resolved on the large 2-D gel (Fig. 3A) together with the simulated 2-D gel pattern obtained by in silico trimming of TGFBIp from the N-terminus indicate, that the protein is processed by an aminopeptidase. Thus, mature corneal TGFBIp may be cleaved first by an endoprotease followed by sequential trimming of the resulting new N-terminus by an aminopeptidase. In addition, it can be concluded that the N-terminal parts of TGFBIp are either completely degraded or somehow cleared from the cornea, while the C-terminal regions are maintained in the cornea. According to a previous study, larger fragments of TGFBIp such as the N-terminal part is not in circulation (Karring et al., 2007), suggesting that corneal TGFBIp is not processed through a single endo-protease cleavage.

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Figure 1. Quantification of TGFBIp in the normal human cornea during postnatal development and aging

A; TGFBIp is detected by immunoblotting in reduced extracts from normal human corneas from individuals ranging from 0.5 to 86 years of age. The age in years of the individual is indicated above each lane. **B;** The plot shows the level of TGFBIp at different ages in μg per mg wet cornea. The concentration of mature corneal TGFBIp increases about 30% between 6 and 14 years of age before reaching a plateau maintained throughout life. The quantifications were performed in triplicates using 0.5 μg of purified recombinant human TGFBIp as reference (Control).

Figure 2. Processing of corneal TGFBIp during postnatal development and aging

A; 2-D immunoblot of reduced corneal TGFBIp from a 0.5 year old individual. A similar 2-D immunoblot pattern was obtained from a 0.75 year old individual. **B**; 2-D immunoblot of reduced corneal TGFBIp from a 1 year old individual. **C**; 2-D immunoblot of reduced corneal TGFBIp from a 6 years old individual. The 2-D immunoblot is representative for individuals with age 6–86 years. All the immunoblots show a characteristic “zig-zag” pattern formed by the different TGFBIp isoforms, but the relative abundance of the different TGFBIp isoforms changes between the age-groups. The 1-D immunoblot inserted left to panel A shows the detection of lower-molecular mass isoforms of TGFBIp from the cornea of a 14 years old individual when the blot is developed for longer time.

Figure 3. Identification of N-terminally processed isoforms of TGFBIp in the adult human cornea
A; 2-D PAGE of extract from adult human corneas with pH gradient 4.0–7.0. The indicated protein spots are different TGFBIp isoforms that have been identified by MALDI-TOF mass spectrometry. **B**; Schematic presentation of the TGFBIp regions covering the tryptic TGFBIp peptides identified by mass spectrometry. For each TGFBIp-containing spot in panel A, the region covering the identified peptides is indicated with residue numbers and a schematic presentation. All the identified isoforms of TGFBIp are proteolytically processed from the N-terminus. The region of TGFBIp that the antiserum is directed against is indicated.

Figure 4. N-terminal trimming-events of mature human corneal TGFBIp generate a characteristic “zig-zag” pattern on a simulated 2-D gel

The mature human TGFBIp (residues Gly₂₄-Ala₆₅₇, marked with a red star) is sequentially trimmed by one residue from the N-terminus (green line) or C-terminus (pink line) resulting in reduced molecular mass and altered absolute charge. The theoretical molecular masses and isoelectric points of the truncated protein species are then plotted. Every 20 amino acid-trimming events from the N-terminus (green circles) and C-terminus (pink squares) are numbered. A characteristic “zig-zag” pattern reminiscent to those presented in Fig. 2 is observed for the N-terminal trimming. In addition, the theoretical masses and isoelectric points of TGFBIp fragments lacking 200–340 residues from the N-terminus are reminiscent to those observed for the identified isoforms of TGFBIp (Fig. 3).