Proteomic Analysis of the Soluble Fraction from Human Corneal Fibroblasts with Reference to Ocular Transparency*

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The transparent corneal stroma contains a population of corneal fibroblasts termed keratocytes, which are interspersed between the collagen lamellae. Under normal conditions, the keratocytes are quiescent and transparent. However, after corneal injury the keratocytes become activated and transform into backscattering wound-healing fibroblasts resulting in corneal opacification. At present, the most popular hypothesis suggests that particular abundant water-soluble proteins called enzyme-crystallins are involved in maintaining corneal cellular transparency. Specifically, corneal haze development is thought to be related to low levels of cytoplasmic enzyme-crystallins in reflective corneal fibroblasts. To further investigate this hypothesis, we have used a proteomic approach to identify the most abundant water-soluble proteins in serum-cultured human corneal fibroblasts that represent an in vitro model of the reflective wound-healing keratocyte phenotype. Densitometry of one-dimensional gels revealed that no single protein isoform exceeded 5% of the total water-soluble protein fraction, which is the qualifying property of a corneal enzyme-crystallin according to the current definition. This result indicates that wound-healing corneal fibroblasts do not contain enzyme-crystallins. A total of 254 protein identifications from two-dimensional gels were performed representing 118 distinct proteins. Proteins protecting against oxidative stress and protein misfolding were prominent, suggesting that these processes may participate in the generation of cytoplasmic light-scattering from corneal fibroblasts. Molecular & Cellular Proteomics 3:660–674, 2004.

The cornea, the only transparent connective tissue of the body, is responsible for ~70% of the total refractive power of visible light in the eye. The corneal stroma (thickness ~450 μm) mainly consists of collagen lamellae formed by uniform fibrils composed of type I, III, and V collagen (1, 2). The uniform diameter (~30 nm) and regular spacing of the collagen fibrils (~60 nm between the centers) lead to destructive interference of light except in the forward direction and thus optical transparency of the extracellular matrix of the cornea (3, 4). The stroma also contains multiple layers of quiescent corneal fibroblasts, termed keratocytes, which are interspersed between the collagen lamellae. Under normal conditions, the quiescent keratocytes are transparent except for the nuclei when studied in vivo using scanning slit specular microscopy (5), slit-lamp biomicroscopy, or in vivo confocal microscopy (6). This stealth-like invisibility indicates that the refractive index of the keratocyte cytoplasm and cellular processes is similar to that of the extracellular matrix of the corneal stroma (6).

The keratocytes have a dynamic potential for proliferation and transformation. Thus, after injury of the cornea such as excimer laser keratectomy for the treatment of myopia, the keratocytes transform into spindle-shaped fibroblastic cells (7, 8). The corneal fibroblasts repopulate the damaged region by migration and proliferation and deposit disordered and nontransparent matrix components (9–11). The damaged stroma is then remodelled through continuous degradation and synthesis of the extracellular matrix to recreate the highly ordered collagen structure (11, 12). In the latest phase of the wound-healing process, the corneal fibroblasts undergo a phenotype change into myofibroblasts, which are responsible for wound contraction (13–15). The wound-healing process reduces the optical clarity of the cornea due to an increase in the light-scattering from the cell bodies of the corneal fibroblasts compared with that of keratocytes (7, 8, 16, 17). These results suggest that development of corneal cellular haze during wound-healing is caused in part by an increase in the backscatter of light from the cytoplasm of the stromal cells.

The maintenance of ocular transparency and the development of opacities have been studied intensively in the ocular lens. The α- and β/γ-crystallins are the major water-soluble proteins of all vertebrate ocular lenses and are critical for the optical properties and transparency of this tissue. The α-crystallins are members of the small heat shock protein family and have chaperone-like activity preventing the aggregation of proteins (18, 19). The β/γ-crystallins are related to microbial...
oxidative stress proteins (20) and have been implicated in the development of cataracts (21). The high concentration and oligomeric complex formations of the α- and β/γ-crystallins are believed to give the ocular lens its refractive index and optical properties through short-range spatial orders (22).

Several studies of various vertebrates and invertebrates have shown taxon-specific accumulations of water-soluble proteins in the lens and epithelial cells of the cornea. The abundant taxon-specific proteins in the lens and cornea are often similar or identical to metabolic enzymes and, therefore, termed enzyme-crystallins. Most mammals accumulate aldehyde dehydrogenase 3 (ALDH3) in the corneal epithelium (23–26). Transketolase (TKT) is a major protein in the mouse and human corneal epithelium (27), and α-enolase is relatively abundant in human, mouse, and chicken corneas (26). Isocitrate dehydrogenase is overexpressed in the bovine corneal epithelium (28), while peptidyl-prolyl cis-trans isomerase and argininosuccinate lyase are found at high concentrations in the corneal epithelium of chicken (26). Glutathione S-transferase-related S-crystallin is abundant in squid cornea (26), while zebrafish accumulates gelsolin and actin in the corneal epithelium (29). It has been suggested that proteins present at a concentration exceeding 5% of the total water-soluble protein content of corneal cells should be defined as enzyme-crystallins (30). The function of the corneal enzyme-crystallins is not clear, but it has been suggested that they serve catalytic as well as structural roles reminiscent to the α- and β/γ-crystallins in the lens and thereby contribute to the transparency and optical properties of the corneal cells.

TKT and ALDH1 are abundant proteins in rabbit keratocytes (17). These two proteins comprise about 30% of the total water-soluble protein content in freshly isolated keratocytes from normal rabbit corneas, but are reduced to less than 15% in light-scattering corneal fibroblasts (17). These results indicate an apparent correlation between cellular transparency and the levels of enzyme-crystallins in stromal cells of the cornea. However, ALDH3A1-deficient mice (31) and TKT−/− (32) mice show no corneal phenotype, indicating that the enzyme-crystallins are not required for corneal transparency. Other studies have indicated that the abundant water-soluble enzyme-crystallins protect the cornea against ultraviolet (UV)-induced oxidative injury. Thus, isocitrate dehydrogenase prevents lipid peroxidation and oxidative DNA damage (33), while ALDH3A1 detoxifies the cellular environment by removing toxic aldehydes generated by lipid peroxidation (34, 35). In addition, ALDH3A1 may function as a UV-light filter in the cornea by directly absorbing UV radiation (36). However, despite the various suggestions concerning the roles of the abundant water-soluble proteins in the corneal, it is reasonable to believe that maintenance of corneal transparency and the development of cellular haze are dependent on the protein expression profile.

In the present study, the soluble proteome of cultured human corneal fibroblasts was analyzed by one-dimensional (1D) and two-dimensional (2D) PAGE, and 118 of the most abundant water-soluble proteins were identified by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) or MALDI quadrupole time-of-flight (TOF) tandem mass spectrometry (MS/MS). The identified proteins are involved in processes such as protein folding and degradation, cell proliferation, differentiation, and apoptosis, metabolism, cytoskeleton organization and cell motility, protection against oxidative stress, signal transduction, and secretion of proteins. Based on the identified proteins, we propose that oxidative stress may lead to protein unfolding that may participate in the enhanced backscatter of light from corneal fibroblasts. Aggregation of irreversible oxidized proteins is known to opacify the crystalline lens. Thus, it is possible that development of corneal cellular haze during wound-healing is somehow reminiscent to the formation of an oxidative stress-induced opacity in the crystalline lens.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Four human donor corneas (males of 39 and 51 years old and females of 66 and 73 years old) were obtained from the Danish Cornea Bank, Aarhus University Hospital, and dissected in Hanks’ buffered saline solution (HBSS; Life Technologies, Inc., Grand Island, NY). After removing the Descemet’s membrane-endothelial complex and the epithelium, the central stroma was punched from the endothelial side using a 7-mm trephine. The stromal buttons were cut into 1-mm³ pieces and washed three times in 1 ml of HBSS before being used for explants in 40-ml cell-culture flasks (Nunc, Roskilde, Denmark).

The corneal fibroblasts were cultured at 37 °C in a 5% CO₂-humidified incubator in Dulbecco’s modified Eagle’s medium (DMEM) (10% fetal bovine serum (Life Technologies, Inc.), 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Inc.), and 0.25 μg/ml fungizone (amphotericin B) (Life Technologies, Inc.). When the cultures had multiplied to confluence, the cells were trypsinized by 0.25% (w/v) trypsin/1 mM EDTA mixture (Life Technologies, Inc.) and transferred to a 250-ml cell-culture flask (first passage). Subsequently, confluent cultures were split in 1:4 and cells from the third passage were transferred to expanded cell-culture flasks (PBS) Dulbecco’s (Life Technologies, Inc.) before harvested by scraping using a cell scraper in PBS Dulbecco’s. Cells were spun down at 1,000 g for 10 min at 25 °C and washed thoroughly three times in the same buffer. After washing and centrifugation, the corneal fibroblasts were resuspended in 0.1 x PBS Dulbecco’s diluted in water and containing 2 mM 1,10-phenanthroline (Sigma, St. Louis, MO), 40 μM trans-epoxysuccinyl-L-leucyl-aminido(4-guanidino)-butane (E-64) (Sigma), and 2 mM pefabloc SC (Fluka, Buchs, Switzerland). Cells were lysed by sonication on ice, and the soluble fraction was isolated by centrifugation at 33,000 × g for 15 min at 4 °C and stored at −80 °C.

**1D and 2D PAGE**—For 1D SDS-PAGE, the soluble fraction of the corneal fibroblasts was mixed with SDS sample buffer containing 1
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mm dithiothreitol (DTT) and run in 5–15% gradient gels using the glycine, 2-amine-2-methyl-1,3-propanediol/HCl system described by Bury (37). Gels were Coomassie blue stained, and densitometry was performed on three samples.

For 2D PAGE, the soluble fraction of the corneal fibroblasts was mixed with sample buffer (5 μl urea, 2 μl thiourea, 2% (v/v) 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate (Chaps) (Sigma), 2% (v/v) N-decyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (SB3-10) (Sigma), 10 μM DTT, 2 μM EDTA, 2 μM 1,10-phe-panthroline, 40 μM E-64, 2 μM peflaciboc SC, and 0.5% (v/v) carrier ampholytes (Amersham Biosciences, Piscataway, NJ) of pH 4–7 or 6–11 in accordance with the immobilized pH gradients (38, 39) to a final protein concentration of 0.5–1 mg/ml and incubated for 1 h at 25 °C under rotation. Bromphenol blue was added to the samples to a final concentration of 10 μg/ml, and insoluble material was removed by centrifugation at 33,000 × g for 30 min at 25 °C. The solubilized sample (350 μl) was loaded onto 18-cm Immobiline DryStrips (Amer- sham Biosciences) with pH ranges of 4.5–5.5, 5.5–6.7, 4–7, or 6–9 and covered with DryStrip Cover Fluid (Amersham Biosciences). After incubation for 12–18 h at 25 °C, the isoelectric focusing was performed for 16–18 h using the IPGphor System I (Amer- sham Biosciences) and according to the protocol of Görg et al. (40). Proteins were focused in the first electrophoresis dimension for about 124 kVh.

The proteins were reduced and alkylated by incubating the immobilized pH gradient strips in 10 ml of reduction buffer (6 M urea, 50 mM Tris-HCl, pH 8.8, 30% (v/v) glycerol, 2% (v/v) SDS, and 6.5 μM DTT) at 25 °C for 15 min under rotation and in 10 ml of alkylation buffer (6 M urea, 50 mM Tris-HCl (pH 8.8), 30% (v/v) glycerol, 2% (v/v) SDS, and 10 mM iodoacetamide) at 25 °C for 15 min under rotation, respectively.

For the second-dimensional electrophoresis, the strips were transferred to 18 × 23.4-cm SDS-polyacrylamide gels (12.5%), covered with 0.5% agarose, and run in a Hoefer DALT™ tank (Amersham Biosciences) for 6 h at about 17,000 W per gel at 20 °C in running buffer containing 25 μM Tris, 192 mM glycerine, and 0.1% (w/v) SDS. The gels were fixed overnight, and proteins were visualized by silver-staining as described for MS analysis (41). Gels were scanned and stored in 5% (v/v) acetic acid at 6 °C.

Sample Preparation for MS Analysis—2D gel spots of interest were excised and washed in 0.5 ml of water and incubated two times in 0.1 ml of 50% (v/v) acetonitrile for 15 min. The gel plugs were dehydrated by incubation in 50 μl of acetonitrile for 15 min and equilibrated in 50 μl of 0.1 M NH₄HCO₃ for 5 min before 50 μl of acetonitrile was added, and the samples were incubated for 15 min. After removal of the supernatant, the gel plugs were lyophilized in a speed-vac for 10 min. The in-gel digestion was performed by adding 5 μl of 50 mM NH₄HCO₃ containing 25 μg/ml sequencing-grade modified trypsin from porcine (Promega, Madison, WI) followed by incubation at 20 °C for 5 min. NH₄HCO₃ (15 μl of 50 mM) was added to the samples followed by incubation at 37 °C for 16–18 h.

For MALDI-MS analysis, the resulting tryptic-digested peptides were isolated using ZipTip P10 pipette tips (Millipore, Bedford, MA) and spotted onto the MALDI sample target using 0.5 μl of matrix solution containing 70% (v/v) acetonitrile, 0.03% (v/v) trifluoroacetic acid (protein sequencer grade), and 0.4% (w/v) recrystallised α-cyano-4-hydroxy-cinnamic acid (Sigma).

RESULTS

1D PAGE and Quantification by Densitometry—When transparent keratocytes are exposed to fetal bovine serum, they transform into the reflective phenotype characterized by a spindle-shaped morphology and a fibroblastic cytoskeleton organization (44, 45). Thus, cultured human corneal fibroblasts of the third passage (Fig. 1A) were used as an in vitro model system for the study of the corneal fibroblast soluble proteome. For initial analysis, the soluble fraction was analyzed by 1D PAGE. Quantification by densitometry of 1D gels showed that no single protein isofrom exceeded 2.5% of the total amount of protein in the soluble fraction. Identification of the four most intensive bands revealed that the most abundant proteins were actin, two different vimentin isoforms, and annexin A2. Thus, actin (~47 kDa) makes up 2.1 ± 0.2% of the soluble protein content, while two vimentin isoforms of ~59 and ~51.3 kDa makes up 1.5 ± 0.1 and 1.1 ± 0.1%, respectively. Annexin A2 (~38 kDa) accounts for 1.0 ± 0.2% of the total protein in the soluble fraction (Fig. 1B).

Proteome Study Using 2D PAGE—The soluble proteome of the corneal fibroblasts was further analyzed by 2D PAGE using pH gradients 4–7 (Fig. 2A), 6–9 (Fig. 2B), 4.5–5.5 (Fig. 2C), and 5.5–6.7 (Fig. 2D). No obvious differences in the protein expression profiles among the donors were observed. All together, 254 gel spots were analyzed and identified by MALDI-MS or MS/MS. Thus, all spots indicated in Fig. 2 were identified by mass spectrometry. The identified proteins represented 118 distinct proteins, which were categorized according to function and cellular localization as reported in the current literature (Table I). In cases where it was obvious that the same spot had been analyzed on different gels, the spots were given the same spot ID (Fig. 2, A–D). Most of the identified proteins are involved in processes such as protein folding and degradation, cell proliferation, differentiation, and apo-
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Fig. 1. Cultured corneal fibroblasts and 1D SDS-PAGE. A, spindle-shaped human corneal fibroblasts cultured in 10% fetal bovine serum. B, 1D SDS gel of soluble fraction from human corneal fibroblasts. The gel is Coommasie blue stained, and the identities of the four most intense bands are indicated.

At present, the prevalent hypothesis of corneal cellular transparency and the development of cellular opacification during wound-healing involves enzyme-crystallins, which are highly expressed in corneal epithelium and keratocytes. A high level of enzyme-crystallins in corneal keratocytes are believed to contribute to the transparency and refractive properties of the cells by minimizing the refractive index fluctuations between the cytoplasm and the extracellular milieu (17, 47). Accordingly, corneal cellular haze development is thought to be related to low levels of water-soluble enzyme-crystallins in the cytoplasm of the reflective keratocyte phenotype. In the present study, 118 distinct proteins of the most abundant water-soluble proteins in human corneal fibroblasts were identified. Most of the identified proteins are housekeeping proteins common to most other cell types from nontransparent tissues. Thus, a significant number of the identified proteins are also expressed by other fibroblastic cells such as dermal fibroblasts (48, 49) and MRC5 fibroblasts (50) (visit proteomics.cancer.dk/jecelis/human_data_select.html). This finding may reflect a common mechanism of light scattering from reflective cells.

Corneal Enzyme-Crystallins and Various Isoforms—Densitometry of 1D gels revealed that no single band exceeds 5% of the total soluble protein fraction, which is the qualifying property of a corneal enzyme-crystallin according to the current definition. Because the definition of enzyme-crystallins is rather loose and does not consider the presence of various molecular mass isoforms (30), this result indicates that cul-
tured human corneal fibroblasts do not contain enzyme-crystal-
linns. In addition, neither ALDH3A1 nor TKT were found among
the most abundant water-soluble proteins, indicating that these enzyme-crystal-
linns are not present or only expressed at low levels, which is in accordance with previous results (51).

The proteome analysis identified five proteins that have previously been classified as enzyme-crystal-
linns in the corneal epithelium of various species (Table II). In a previous study (26), the full-length α-enolase (∼48 kDa) was shown to be highly expressed in human corneal epithelium. The finding of at least eight α-enolase isoforms (e.g. spots 110, 131, 140, 146, 175, 200, 244, and 246; Fig. 2, C and D) in human corneal fibroblasts indicates that this glycolytic enzyme/enzyme-cryst-
tallin is rather abundant also in corneal fibroblasts, but it remains unknown why it is present in several different isoforms. However, the 37-kDa isoforms can be explained by the finding that an alternative translation of the full-length α-enolase mRNA can produce a 37-kDa isoform, which is known to bind the c-myc promoter and function as a transcriptional repressor (52). In addition, the isoforms with similar molecular masses but different pI values may be explained by post-translational modifications such as phosphorylation. Thus, the isoforms of α-enolase (spots 175/246, and 140/200/131; Fig. 2D), peptidyl-prolyl cis-trans isomerase A (spots 181 and 180; Fig. 2B), actin (e.g. spots 14–17, and 152/153; Fig. 2C), and the isoforms of triosephosphate isomerase (e.g. spots 114, 211, and 228; Fig. 2D) may reflect post-translational modifications.
**TABLE I**

Proteins identified in the soluble fraction from human corneal fibroblasts

| Protein | Acc. no. | Function and localization | FL\textsubscript{obs}/M\textsubscript{obs}/pI\textsubscript{obs} | M\textsubscript{obs}/pI\textsubscript{obs} | Gel ID | Spot ID | Covered fragment |
|---------|----------|---------------------------|------------------|----------------------|-------|---------|------------------|
| Actin, β or γ | P02570 or P02571 | C/c | 375/41.7/5.3 | 44.6/5.2 | A/C | 14 | A19-K373 |
| | | | | 44.6/5.2 | A/C | 15 | A29-K373 |
| | | | | 44.6/5.3 | A/C | 16 | A19-K373 |
| | | | | 44.6/5.3 | A/C | 17 | A29-K373 |
| | | | | 35.5/5.4 | A/C | 83 | V66-R372 |
| | | | | 42.5/5.1 | C | 152 | A19-K336 |
| | | | | 42.5/5.1 | C | 153 | A19-K336 |
| | | | | 31.5/5.4 | C | 176 | G63-K373 |
| | | | | 35.5/5.5 | C | 186 | V66-R372 |
| | | | | 36.5/5.2 | C | 198 | I53-K336 |
| | | | | 37.5/5.2 | C | 199 | I53-K336 |
| Acyl-CoA-binding protein | P07108 | M/c/n | 87/9.9/6.1 | 9.5/5.6 | D | 216 | S2-K67 |
| Alcohol dehydrogenase [NADP\textsuperscript+] (Aldo-keto reductase 1A1) | P14550 | M/c | 326/35.6/6.3 | 40.9/6.2 | D | 145 | M14-K308 |
| α-Actinin 1 | P12814 | C/c | 892/103.5/5.2 | 29.1/4.7 | A/C | 66 | I53-R683 |
| Annexin A1 | P04083 | P/L/T/c/a/n | 346/38.8/6.6 | 28.7/5.1 | A/C | 34 | Q10-R228 |
| | | | | 28.3/5.0 | A/C | 48 | G30-R228 |
| | | | | 37.2/6.2 | B | 218 | Q10-R228 |
| Annexin A2 | P07355 | P/L/T/c/a | 339/38.7/7.6 | 17.5/5.6 | A | 53 | L11-R168 |
| | | | | 27.3/5.8 | A | 95 | A29-R205 |
| | | | | 33.8/5.2 | D | 121 | L11-R245 |
| | | | | 18.9/5.2 | C | 141 | L11-R168 |
| Annexin V | P08758 | P/L/T/c/a | 320/35.8/5.0 | 34.3/5.1 | A | 75 | G7-R285 |
| ATP synthase α chain, mitochondrial precursor | P25705 | M/m | 553/59.8/9.2 | 14.3/4.8 | C | 157 | T46-K194 |
| ATP synthase β chain, mitochondrial precursor | P06576 | M/m | 529/65.5/5.3 | 48.9/5.0 | A/C | 4 | L95-K480 |
| ATP synthase D chain, mitochondrial precursor | O75947 | M/m | 161/18.5/5.2 | 24.5/5.1 | C | 120 | T10-K121 |
| β\textsubscript{2}-microglobulin, precursor | P01984 | I/s | 119/13.8/6.1 | 10.8/6.1 | D | 230 | V102-K111 |
| Calcinin (S100A6) | P06703 | P/F/L/r/n | 90/10.2/5.3 | 9.1/5.1 | C | 133 | E41-R55 |
| | | | | 9.9/5.1 | C | 134 | E41-R55 |
| Calgizzarin (S100A14 or S100A11p) | NP_066369 | U/u | 102/11.4/7.8 | 8.9/6.4 | A | 61 | I4-K78 |
| Calmodulin | P02593 | L/S/P/c | 149/16.8/4.1 | 14.6/4.0 | A | 49 | E15-K149 |
| Calreticulin, precursor | P27797 | F/S/e | 417/48.3/4.3 | 56.8/4.3 | A | 74 | E25-K286 |
| Calumenin, precursor | O43852 | S/e/s | 315/37.1/4.5 | 47.0/4.5 | A | 43 | V28-K284 |
| Cathepsin B, precursor | P0758 | I/P/F/I | 396/38.8/5.9 | 27.6/5.1 | A | 22 | I20-R331 |
| | | | | 31.8/5.5 | A | 73 | L88-R331 |
| | | | | 26.7/5.7 | A | 94 | I20-R331 |
| Cathepsin D, precursor | P07339 | P/L/F/I/l | 412/45.0/6.1 | 31.9/5.2 | A/C | 72 | Y123-R411 |
| | | | | 31.0/5.9 | D | 109 | Q158-R411 |
| | | | | 32.3/5.4 | C | 187 | Y123-R411 |
| | | | | 33.2/5.1 | C | 196 | Q158-R411 |
| Cell division control protein 42 homolog (G25K GTP-binding protein) | P21181 | C/P/L/c | 191/21.7/5.8 | 23.6/5.6 | D | 31 | T108-R120 |
| Chloride intracellular channel protein 1 (CLIC1) | O00299 | O/T/n/c | 241/27.3/5.1 | 32.8/5.0 | C | 195 | I21-K238 |
| Chloride intracellular channel protein 4 (CLIC4) | Q9Y696 | C/P/c/m/n | 253/29.0/5.5 | 31.1/4.5 | C | 173 | A25-R227 |
| Clathrin light chain B | P09497 | M/l/c/a/l | 229/23.5/4.6 | 31.0/4.5 | A | 69 | L96-K204 |
| Protein                                                                                       | Acc. no.  | Function and localization | FL_{ex} / M_{in} / pI_{in} | M_{ins} / pI_{ins} | Gel ID | Spot ID | Covered fragment |
|-----------------------------------------------------------------------------------------------|-----------|---------------------------|-----------------------------|-------------------|--------|---------|------------------|
| Cofilin, non-muscle isoform                                                                  | P23528    | C/c/n                     | 166/18.7/8.2                | 13.8/5.4          | A/C    | 62      | K34-K92          |
| Destrin                                                                                       | P18282    | C/c/n                     | 165/19.0/8.1                | 18.8/8.3          | B      | 179     | A35-R146         |
| Dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex,     | P36957    | M/m                       | 453/49.0/9.0                | 22.0/5.0          | A/C    | 102     | D313-R325        |
| mitochondrial precursor (E2)                                                                  |           |                           |                             |                   |        |         |                  |
| Dynein light chain 2A or 2B, cytoplasmic                                                     | Q9NP97 or | C/P/c                     | 96/10.9/6.6–6.9             | 10.2/5.6          | D      | 217     | D59-R70          |
| Electron transfer flavoprotein β subunit (ETF-β)                                            | P38117    | M/m                       | 255/28.1/8.2                | 26.5/5.3          | C      | 142     | E60-R233         |
| Elongation factor 1-β (EF-1β)                                                                | P24534    | Y/c                       | 225/24.8/4.5                | 32.2/4.6          | A      | 97      | S8-K22           |
| Elongation factor 2 (EF-2)                                                                   | P13639    | Y/c                       | 858/96.1/6.4                | 11.9/6.0          | D      | 227     | A786-R801        |
| Endoplasmic reticulum protein 29, precursor (ERp29)                                          | P30040    | F/S/e/s                   | 261/29.0/6.8                | 29.8/6.5          | A      | 44      | G37-K253         |
| Endoplasmin, precursor (GRP94)                                                               | P14625    | F/S/e                     | 803/92.7/4.8                | 24.0/6.3          | D      | 165     | L494-R503        |
| α-Enolase                                                                                    | P06733    | M/T/c/n                   | 434/47.5/7.0                | 34.3/6.2          | A      | 1       | A33-K343         |
| Enoyl-CoA hydratase, mitochondrial precursor                                                 | P30084    | M/m                       | 290/31.8/8.3                | 29.1/6.4          | A      | 45      | G42-R283         |
| Ferritin light chain                                                                         | P02792    | O/T/c/l                   | 175/19.9/5.5                | 20.9/5.4          | C      | 189     | L155-R169        |
| Galectin-1                                                                                   | P09382    | P/C/s/n                   | 135/14.9/5.3                | 13.8/5.0          | A/C    | 39      | D38-K130         |
| Glutathione S-transferase, mitochondrial precursor                                           | Q9Y2Q3    | O/m                       | 226/25.5/8.5                | 14.3/6.3          | D      | 237     | H75-R225         |
| Glutathione S-transferase P (GSTP1–1)                                                        | P09211    | O/c                       | 210/23.5/5.4                | 26.9/5.4          | A/C    | 96      | P2-K141          |
| Glutathione transferase ω 1 (GSTO 1–1)                                                       | P78417    | O/c                       | 241/27.8/6.2                | 31.0/5.6          | D      | 101     | S2-K220          |
| Glyceraldehyde 3-phosphate dehydrogenase                                                     | P04406    | M/c                       | 335/35.9/8.6                | 10.6/5.9          | D      | 222     | L310-R323        |
| Glyoxalase I (lactoylglutathione lyase)                                                       | Q04760    | O/M/c                     | 184/20.8/5.3                | 24.2/5.0          | C      | 202     | D29-K179         |
| GTP-binding nuclear protein RAN (TC4)                                                         | P17080    | P/T/c/n                   | 216/24.6/7.0                | 27.2/6.8          | B      | 205     | Y39-R56          |
| Heat shock cognate 71-kDa protein (Hsc71)                                                     | P11142    | F/c                       | 646/71.1/5.4                | 35.5/4.7          | C      | 135     | N540-K550        |
| Heat shock protein 27-kDa                                                                    | P04792    | F/P/C/c                   | 205/22.8/6.0                | 28.4/6.4          | A      | 40      | R5-R188          |
|                                                                                             |           |                           |                             |                   |        |         |                  |
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| Molecular & Cellular Proteomics 3.7                                                           |           |                           |                             |                   |        |         |                  |
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#### Table I—continued

| Protein                                                                 | Acc. no. | Function and localization | FL_{aa}/M_{th}/pI_{th} | M_{ob}/pI_{ob} | Gel ID | Covered fragment |
|------------------------------------------------------------------------|----------|---------------------------|-------------------------|---------------|--------|-----------------|
| Heterogeneous nuclear ribonucleoprotein H (hnRNP H)                    | P31943   | T/n                       | 449/49.5/5.9            | 26.5/5.9      | D      | H99-R114        |
| Heterogeneous nuclear ribonucleoprotein K (hnRNP K)                    | Q07244   | T/c/h                     | 463/51.2/5.4            | 26.9/6.2      | D      | G17-R29        |
| Histidine triad nucleotide-binding protein 1                            | P49773   | T/c/n                     | 126/13.8/6.5            | 13.3/6.2      | D      | A8-R119        |
| 3-Hydroxyisobutyrate dehydrogenase, mitochondrial precursor             | P31937   | M/m                       | 336/35.7/8.4            | 29.9/5.1      | C      | M150-R167      |
| Hypothetical protein (fragment) (IL-25/SF20)                            | Q9BTK7   | U/u                       | 171/18.8/6.1            | 15.6/6.2      | D      | S99-K107       |
| Isocitrate dehydrogenase [NADP^+], cytoplasmic 78-kDa glucose-regulated protein, precursor (BiP) | P11021   | F/S/e                     | 654/72.4/5.1            | 32.6/6.7      | A      | V50-R214       |
| 60-kDa heat shock protein, mitochondrial precursor                      | P10809   | F/m                       | 573/61.2/5.7            | 54.9/5.2      | A/C    | A38-K516       |
| MIR-interacting saposin-like protein (MSAP)                             | NP_055070| C/P/c                     | 182/21.0/4.8            | 14.6/4.5      | A      | E14-R146       |
| Myosin alkali light chain isoform 1, smooth muscle and non-muscle       | NP_066299| C/P/L/c                   | 151/17.1/4.6            | 14.8/4.4      | A      | E14-K119       |
| Nuclear transport factor 2                                              | P13662   | T/c                        | 127/14.6/5.1            | 10.1/5.0      | A/C    | N107-R120      |
| Nucleophosmin (nucleolus phosphoprotein B23)                            | P06748   | T/P/F/n                   | 294/32.7/4.6            | 18.3/4.5      | A      | M81-R101       |
| Nucleoside diphosphate kinase A                                         | P15531   | M/c/h                     | 152/17.3/5.8            | 19.6/6.2      | A      | T7-R114        |
| Nucleoside diphosphate kinase B                                         | P22392   | M/c/n                     | 152/17.4/8.5            | 12.0/6.4      | A      | Q50-K143       |
| Peptidyl-prolyl cis-trans isomerase (FKBP65)                            | Q96AY3   | F/S/e                     | 582/64.8/5.4            | 60.6/6.3      | A      | E58-R577       |
| Peptidyl-prolyl cis-trans isomerase A (cyclophilin A)                   | P05092   | F/c                        | 165/18.1/7.8            | 17.6/7.8      | B      | V2-E165        |
| Peroxiredoxin 1                                                         | Q06830   | O/c                        | 199/22.3/8.3            | 26.8/8.5      | B      | V2-E165        |
| Peroxiredoxin 2                                                         | P32119   | O/c                        | 198/22.1/5.4            | 24.8/5.4      | A/C    | I8-R150        |
| Peroxiredoxin 3, mitochondrial precursor                                | P30048   | O/m                        | 256/28.0/7.7            | 25.6/6.5      | A      | T38-R214       |
| Peroxiredoxin 4                                                         | Q13162   | O/c                        | 271/30.8/5.9            | 28.4/6.1      | A/C    | T46-R223       |
| Peroxiredoxin 5, mitochondrial precursor                                | P30044   | O/m/p/c                   | 214/22.3/8.9            | 16.4/6.1      | A      | E160-R176      |
| Peroxiredoxin 6                                                         | P30041   | O/c/L/s                   | 224/25.0/6.0            | 28.4/6.8      | A      | F25-R174       |
| Phosphatidylethanolamine-binding protein                                | P30086   | L/B/c/s                   | 187/21.0/7.4            | 23.9/7.4      | B      | L63-R77        |
| Phosphoglycerate kinase 1                                               | P00558   | M/c                        | 418/44.7/7.5            | 27.6/5.3      | C      | A200-K382      |
| Phosphoglycerate mutase 1, isozyme B                                    | P18669   | M/c                        | 254/28.8/6.8            | 31.5/6.5      | D      | A107-R123      |
| Phosphophannomutase 2                                                   | Q15305   | S/c                        | 246/28.4/6.4            | 29.3/6.1      | D      | A2-S246        |
| Protein |
|---------|
| Polyadenylate and transcript release factor |
| Prefoldin subunit 1 |
| Proline I |
| Prohibitin |
| Prolyl 4-hydroxylase subunit |
| Prolyl 4-hydroxylase subunit (Protein disulfide isomerase), precursor |
| Protease inhibitor 6 |
| Proteasome activator 28-α |
| Proteasome activator 28-γ |
| Proteasome subunit α type 1 |
| Proteasome subunit α type 2 |
| Proteasome subunit β type 2 |
| Proteasome subunit β type 3 |
| Protein disulfide isomerase A3, precursor (ERp57) |
| Protein-L-isoaspartate (D-aspartate) O-methyltransferase |
| Pyruvate dehydrogenase E1 component β subunit, mitochondrial precursor |
| Pyruvate kinase, M1 or M2 isozyme |
| Retinoic acid-binding protein II, cellular (CRABP-II) |
| Rho GDP-dissociation inhibitor 1 |
| 60S acidic ribosomal protein P0 |
| snRNP core protein D3 |
| 40S ribosomal protein S12 |
| 40S ribosomal protein Sa (LBG/p40) |
| Stress-induced phosphoprotein 1 (HOP or Sti1) |
| Stress-70 protein, mitochondrial precursor (mortalin) |
| Succinyl-CoA:3-ketoacid-coenzyme A transferase, mitochondrial precursor |

**Proteomic Analysis of Human Corneal Fibroblasts**

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Metabolism — The transition of the quiescent keratocyte into the corneal fibroblast phenotype is characterized by development of extensive rough endoplasmic reticulum (ER), a prominent Golgi apparatus, and an increase in the number of vesicles and mitochondria (13, 45) reflecting an increase in the requirement of protein trafficking and respiration. This was confirmed by the identification of several mitochondrial metabolic enzymes in the soluble fraction from the corneal fibroblast. These proteins are involved in oxidative decarboxylation of pyruvate (E1 component /H9252 subunit of the pyruvate dehydrogenase complex), fatty acid oxidation (enoyl-CoA hydratase), the citric acid cycle (E2 component of the mitochondrial 2-oxoglutarate dehydrogenase complex), the respiratory chain (β subunit of electron transfer flavoprotein), and the ADP

| Protein                                                                 | Acc. no. | Function and localization | FL\textsubscript{prot}/M\textsubscript{prot}/pI\textsubscript{prot} | M\textsubscript{prot}/pI\textsubscript{prot} | Gel ID | Spot ID | Covered fragment |
|------------------------------------------------------------------------|----------|---------------------------|-----------------------------|---------------------------------|--------|---------|-----------------|
| Superoxide dismutase (Cu-Zn)                                           | P00441   | O/c                       | 154/16.0/5.7                | 18.6/5.6                        | D      | 215 G11-K24 |
| Superoxide dismutase (Mn), mitochondrial precursor                     | P04179   | O/m                       | 222/24.9/8.4                | 25.8/6.4                        | D      | 115 G76-R216|
| T-complex protein 1, β subunit (TCP-1-β)                               | P78371   | F/c                       | 535/57.5/6.0                | 18.1/5.1                        | A/C    | 51 L26-K170 |
| T-complex protein 1, ε subunit (TCP-1-ε)                               | P48643   | F/c                       | 541/60.1/5.4                | 55.9/5.4                        | D      | 171 I133-R525|
| Thioredoxin                                                            | P10599   | O/c                       | 105/12.0/4.8                | 13.4/4.9                        | A/C    | 60 T9-V105 |
| Transferring protein RhoA (H12)                                        | P06749   | L/c/a                     | 193/22.1/5.8                | 25.4/5.6                        | D      | 86 L8-R176 |
| Transgelin                                                             | Q01985   | C/P/c                     | 201/22.5/8.9                | 18.3/6.4                        | D      | 129 L30-K121|
| Transitional endoplasmic reticulum ATPase (TER ATPase)                 | P55072   | S/T/c/n                   | 806/90.0/5.1                | 36.0/5.4                        | C      | 185 M46-R53 |
| Translationaly controlled tumor protein (TCTP)                         | P13693   | P/Y/c                     | 172/19.7/4.8                | 25.2/4.8                        | A/C    | 99 J20-K34 |
| Triosephosphate isomerase                                              | P09398   | M/c                       | 249/26.8/6.5                | 27.3/6.5                        | A      | 23 K19-K188|
| Tropomyosin α-3 chain                                                  | P06753   | C/c                       | 247/29.1/4.7                | 32.7/4.9                        | A/C    | 35 K13-D247|
| Tropomyosin α-4 chain                                                  | P07226   | C/c                       | 248/28.6/4.7                | 32.8/4.8                        | A/C    | 41 K13-D247|
| Tropomyosin β chain                                                    | P07951   | C/c                       | 284/33.0/4.7                | 38.9/4.9                        | A      | 71 K77-L284 |
| Tubulin α-1 chain                                                      | P05209   | C/c                       | 451/50.8/4.9                | 40.0/5.8                        | A      | 87 T41-K280 |
| Tubulin α-6 chain                                                      | Q9BQ7E3  | C/c                       | 449/49.9/5.0                | 38.6/5.4                        | C      | 174 T41-K280|
| Tubulin β-4 chain                                                      | Q13509   | C/c                       | 450/50.9/4.8                | 36.5/5.4                        | C      | 184 I47-K58 |
| Tubulin β-5 chain                                                      | P05218   | C/c                       | 444/50.1/4.8                | 37.0/5.3                        | A/C    | 52 F20-K297 |
| Ubiquitin                                                             | P02248   | F/c/n                     | 76/8.6/6.6                  | 9.3/6.5                         | B      | 188 E64-R72 |
| Ubiquitin-conjugating enzyme E2 N (Ubc13)                              | Q16781   | D/F/c/n                   | 152/17.2/6.1                | 15.3/5.6                        | D      | 224 L15-R141|
| Ubiquitin C-terminal hydrolase-L1                                     | P09936   | F/c                       | 223/25.2/5.3                | 28.3/5.3                        | A/C    | 26 M1-K195 |
| Vimentin                                                              | P08670   | C/c                       | 466/53.8/5.0                | 48.1/5.0                        | A/C    | 7 L97-K439 |
|                                                                      |          |                           |                             |                                 |        | 44.5/4.8   | 8 T101-R424 |
|                                                                      |          |                           |                             |                                 |        | 43.3/4.7   | 9 L97-R378 |
|                                                                      |          |                           |                             |                                 |        | 43.3/4.8   | 10 L97-R378 |
|                                                                      |          |                           |                             |                                 |        | 43.3/4.9   | 11 T101-R424|
|                                                                      |          |                           |                             |                                 |        | 27.6/5.1   | 91 D271-E466|
|                                                                      |          |                           |                             |                                 |        | 45.7/4.6   | 98 T101-R424|
|                                                                      |          |                           |                             |                                 |        | 27.2/4.9   | 147 F114-R440|
|                                                                      |          |                           |                             |                                 |        | 40.8/4.7   | 159 L97-K402|
|                                                                      |          |                           |                             |                                 |        | 45.9/4.9   | 162 L97-R440|
|                                                                      |          |                           |                             |                                 |        | 43.9/5.0   | 164 L97-R424|
phosphorylation (ATP synthase chains). In addition, succinyl-CoA-3-ketoacid-coenzyme A transferase, which is a key enzyme for ketone body catabolism, was found.

Also several enzymes from the glycolytic pathway were identified. Thus, in addition to α-enolase, these included triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase 1, phosphoglycerate mutase 1B, and pyruvate kinase. In addition, 6-phosphogluconolactonase from the oxidative branch of the pentose phosphate pathway was found. Other metabolic enzymes included nucleoside diphosphate kinases, isocitrate dehydrogenase, and an alcohol dehydrogenase, isozyme 1A1. Thus, the present protein identifications are in accordance with a high metabolic activity in corneal fibroblasts. Interestingly, all six isoforms of glyceraldehyde 3-phosphate dehydrogenase had molecular masses and pI values (M_{ob} ~10–22 kDa, pI_{ob} ~5.9–6.5) much smaller than the theoretical values (M_{th} ~36 kDa, pI_{th} ~8.6), which indicate profound fragmentation of this enzyme (Table I; Fig. 2D).

The secretory pathway—The secretory pathway involves ER luminal proteins (Table I) assisting the folding and processing of secreted proteins. In the present study, protein disulfide isomerases, peptidyl-prolyl cis-trans isomerases, and molecular chaperones within the ER lumen were found. Protein disulfide isomerase A3 stimulates the formation and rearrangement of disulfide bonds in proteins, while peptidyl-prolyl cis-trans isomerase (FKBP65) catalyzes the cis-trans isomerization of the peptidyl-prolyl residue and facilitates the folding of secretory proteins during protein synthesis. In addition, the ER molecular chaperones, endoplasm, ER protein 29, and 78-kDa glucose-regulated protein (BiP) are known to prevent aggregation of the secretory proteins.

Proteins involved in modification of secretory proteins are also present in the soluble fraction from corneal fibroblasts. Prolyl 4-hydroxylation (α-1 and β subunits) catalyzes the post-translational hydroxylation of proline in collagen and other proteins, and the β subunit is also a protein disulfide isomerase. Some secretory proteins are γ-carboxylated in the ER lumen by a system converting glutamate into γ-carboxyglutamate. The function of calumenin is not clear, but it has been shown to inhibit the vitamin-K-dependent γ-carboxylation of proteins in the ER lumen (53). Phosphomannomutase 2 is a cytosolic enzymes converting mannose 6-phosphate (man-6-P) to manrose-1-phosphate (man-1-P), which is required for the initial steps of protein glycosylation performed in the ER lumen and Golgi apparatus.

The finding of key enzymes involved in protein folding, proline hydroxylation, and glycosylation of secretory proteins is in accordance with the wound-healing process accomplished by corneal fibroblasts.

Identification of Hypothetical Protein/IL-25—The hypothetical human protein (accession number Q9BTK7, TrEMBL entry) revealed high homology to the novel mouse IL-25/SF20, which is a bone marrow stroma-derived growth factor. According to the previous findings, IL-25/SF20 is a secretory protein that stimulates cell proliferation (54, 55). Interestingly, recent reports have shown that both the corneal epithelium and stroma contain a significant number of resident bone marrow-derived cells such as macrophages and dendritic cells (56, 57). The bone marrow-derived cells are highly efficient in antigen presentation and initiate immune responses that are critical in corneal inflammation and allograft rejection. The identified human homologue of the mouse bone marrow stroma-derived growth factor IL-25/SF20 in corneal fibroblasts may play a regulatory role in the wound-healing process, which could include activation and transition of the resident bone marrow-derived cells in the corneal stroma.

Oxidative Stress Defense—Reactive oxygen species (ROS) are cytotoxic because they react with lipids and carbohydrates to generate highly reactive agents, damage DNA, and irreversible oxidize proteins that can lead to protein-protein cross-linking, fragmentation, unfolding and degradation, or formation of protein aggregates (58, 59). UV radiation is an exogenous source of ROS, while metabolic enzyme systems such as the respiratory chain are important endogenous sources of ROS and reactive cytotoxic agents (60). Thus, the many mitochondria in proliferating corneal fibroblasts compared with quiescent keratocytes presumably result in a higher level of oxidative stress in fibroblasts than in keratocytes. In accordance, several mitochondrial and cytosolic proteins involved in the redox regulation and protection against oxidative stress were identified in corneal fibroblasts. These proteins included mitochondrial and cytoplasmic glutathione S-transferases, thioredoxin, and several cytoplasmic and mitochondrial peroxiredoxins that play important roles in eliminating various peroxides formed during metabolism. In addition, mitochondrial superoxide dismutase (Mn) and superoxide dismutase (Cu-Zn) catalyzing the conversion of O_{2}^{-} radicals into hydrogen peroxide were found. Glyoxalase I is part of the glyoxalase system, which catalyzes the conversion of reactive aldehydes into acids and thereby detoxifies the cytosol. The fact that several of the identified proteins are involved in defense against oxidative stress indicates that the corneal fibroblasts senses oxidative stress, and it is reasonable to believe that the level of ROS generated endogenous is higher in proliferating corneal fibroblasts than in quiescent keratocytes.

TABLE II

| Enzyme-crystallin | Species | Ref. |
|-------------------|---------|-----|
| Actin, non-filamentous (in complex with gelsolin) | Zebrafish | 29 |
| α-Enolase | Human, mouse, chicken | 26 |
| Isocitrate dehydrogenase | Bovine | 28 |
| Glutathione S-transferase | Squid | 26 |
| Peptidyl prolyl cis-trans isomerase A | Chicken | 26 |
Proteomic Analysis of Human Corneal Fibroblasts

Protein Folding and Degradation—Molecular chaperones and the protein degradation machinery are cellular protection systems that assist protein folding and prevent the accumulation of unfolded and damaged proteins. In the present study, both cytosolic and mitochondrial proteins involved in proteins folding and degradation were identified.

Prefoldin is a cytosolic chaperone that delivers unfolded protein, principally actins and tubulins, to the cytosolic chaperonin T-complex protein 1, which is essential for the folding of these cytoskeleton proteins (61). Hsp27 is an ubiquitously expressed member of the small heat shock proteins and has been implicated in various functions including cellular resistance during heat shock, oxidative stress, cytokine treatment, and is involved in cytoskeleton organization. Stress-induced phosphoprotein 1 is similar to the cytoplasmic yeast heat shock protein STI1 that is known to interact with hsp90 and hsp70 and regulate their ATPase activities. In addition, the cytosolic heat shock cognate 71-kDa protein was found. Mitochondrial 60-kDa heat shock protein is implicated in mitochondrial protein import and prevents unfolding under mitochondrial stress conditions, while mitochondrial stress-70 protein may be a chaperone.

The ubiquitin (Ub)-proteasome system catalyzes the destruction of unfolded or impaired proteins generated in cells. It has previously been shown that the Ub-proteasome system is induced during the phenotypic transition from keratocyte to fibroblast and that the higher levels of free Ub, Ub-protein conjugates, and the 26S proteasome in corneal fibroblasts were maintained during subculturing (51). Thus, the corneal fibroblast has a persisting pool of Ub conjugates targeted to the 26S proteasome for degradation. These results are consistent with the present findings of several proteins from the Ub-proteasome system including proteasome subunits, free Ub, Ub-conjugating enzyme, and Ub C-terminal hydrolase. The abundance of free Ub and proteasome subunits in subcultured corneal fibroblasts could indicate that the Ub-proteasome system in corneal fibroblasts is required for degradation of damaged or unfolded proteins rather than for protein degradations associated with cell transformation and differentiation as suggested for TKT (61). Thus, the present results in combination with the previous findings might indicate that the requirement of protein degradation, which could include the degradation of oxidized proteins, is higher in corneal fibroblasts than in keratocytes. However, certain oxidized proteins and severe oxidized proteins are poor substrates of the proteasome, which can lead to accumulation of oxidized protein aggregates in intracytoplasmic inclusions as observed in some diseases (62, 63).

Perspectives—Increased knowledge on the various keratocyte phenotypes is important for understanding corneal physiology and pathophysiology. The biochemical mechanisms regulating cellular transparency of the cornea are not fully understood. This study has applied a proteomic approach to identify soluble cellular components in primary corneal fibroblasts that are responsible for the cellular haze observed following corneal injury. Most of the identified proteins are common to other nontransparent fibroblastic cells from other tissues. The rather prominent finding of multiple proteins involved in oxidative stress defense, protein folding, and degradation in corneal fibroblasts provides the basis to propose that protein unfolding induced by oxidative stress may participate in the backscattering of light from this keratocyte phenotype. In that respect, it should be noted that oxidative stress is known to cause cataract owing to oxidation of lens proteins and formation of light-scattering, high-molecular-mass protein aggregates (64). Thus, it is possible that development of corneal cellular haze during wound-healing could somehow be reminiscent to the development of oxidative stress-induced opacity in the crystalline lens. Further studies are needed to explore this hypothesis.

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