Transduction of TAT Fusion Proteins into the Human and Bovine Trabecular Meshwork

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PURPOSE. To examine the applicability of TAT (the protein transduction domain of transactivating transcription polypeptide)-mediated protein-transduction technology in introducing proteins of interest into trabecular meshwork (TM) cells in various culture systems.

METHODS. Normal human TM cell cultures, human tissues in organ cultures, and bovine eyes in perfusion organ cultures were incubated or perfused for various lengths of time with TAT- and hemagglutinin (HA)-tagged fusion proteins, TAT-HA-β-galactosidase (TAT-HA-β-gal), TAT-HA-myocilin, and TAT-HA-myocilin-EGFP. Transduction of TAT-HA-β-gal was detected by X-gal staining. Transduction of myocilin or myocilin-EGFP was evaluated by immunostaining or fluorescence. β-Gal and EGFP proteins were used as the negative control.

RESULTS. Blue X-gal staining, signifying β-gal activity resulting from transduction, was observed in cultured TM cells in a concentration- and time-dependent manner. TAT-HA-β-gal was also transduced into cells in all regions of TM tissues in organ cultures. TM cell cultures, after TAT-HA-myocilin incubation, showed an enhanced myocilin staining compared with the control cultures. Stronger myocilin or HA staining was also noted in TM tissues of TAT-HA-myocilin-incubated or –perfused eyes. Myocilin transduction resulted in a loss of actin stress fibers and focal adhesions in TM cells in culture. The level of phosphorylated myosin light chain was reduced. Human and bovine TM tissues after TAT-myocilin transduction also exhibited a diminished actin and paxillin–vinculin staining.

CONCLUSIONS. TAT fusion proteins can be efficiently transduced into TM cells and tissues. The TAT-mediated protein transduction technology may be valuable in studies of proteins such as myocilin in the TM (Invest Ophthalmol Vis Sci. 2006;47: 4427–4434) DOI:10.1167/iovs.06-0047

A TAT-mediated protein transduction methodology has been developed recently to introduce proteins of interest into a variety of cultured cells. TAT is a transactivator gene in the human immunodeficiency virus type 1 (HIV-1) that plays key roles in controlling productive and processive viral gene transcription. In 1988, it was discovered that the full-length 86-amino-acid HIV-1-tat polypeptide crossed biological membranes to be taken up by HeLa cells. A region at the N terminus (amino acid residues, 37–47) of HIV-1-Tat was identified to be the protein transduction domain crucial for the efficient and rapid transport of HIV-1-Tat through cell membranes. Nagai et al. subsequently generated a vector that contains an N-terminal 6-histidine leader sequence followed by the 11-amino-acid-acid protein transduction domain (YGRKKRRQRRR), termed TAT; a hemagglutinin (HA) tag; and a polynucleotide. This methodology has since been used to transduce full-length proteins up to 120 kDa into various cultured cells to inhibit apoptosis, suppress cancer growth, block G-protein signaling, or control inflammation. TAT fusion proteins can also be transduced on injection into mice to trigger biological activity or effects in vivo.

The protein transduction technology has been applied to eye research. For instance, TAT-HA-β-galactosidase (TAT-HA-β-gal) has been transduced into cultured human corneal epithelial cells and the corneas in ex vivo and organ-cultured rat eyes. Inhibition of proliferation of corneal epithelial cells has been achieved using TAT-fused p15INK4a. In the retina, transduction of TAT-BclXl in vivo into mouse retinal ganglion cells by intraocular injection effectively prevents retrograde neuronal apoptosis after optic nerve transection.

The trabecular meshwork (TM) at the chamber angle is an important site for regulation of the aqueous outflow. TM cells cover layers of trabecular beams made up of extracellular matrices, maintaining the normal structure of TM tissues. Both the cells and matrices in the TM are believed to contribute to the outflow resistance. High resistance to the aqueous humor drainage may lead to elevated intraocular pressure (IOP), a major risk factor for the development of glaucoma.

Myocilin is the product of the GLC1A gene, which has been linked directly to both juvenile- and adult-onset primary open-angle glaucoma (POAG), the most common form. Multiple mutations of myocilin have been identified in POAG families. Myocilin was initially identified as a 57-/55-kDa protein secreted into the medium of TM cultures after induction with glucocorticoids such as dexamethasone. Analyses of the genomic sequence of myocilin have identified an N-terminal myosin-like domain and a C-terminal ofactomedin-like domain. The myocilin mRNA and protein are present in many ocular and nonocular tissues including the TM and retina. That myocilin expression can be induced dramatically by dexamethasone has been shown to be a distinct feature of TM cells. Myocilin has been localized to both intracellular and extracellular sites. When overexpressed, it causes a loss of actin stress fibers and focal contacts in cultured human TM cells. Despite these efforts, however, the exact functions of myocilin in the TM remain obscure.

The present study was conducted to examine systematically the potential of using the TAT fusion protein technology to facilitate studies of novel proteins such as myocilin in relation to the TM physiology and/or diseases. Transduction of TAT-HA-β-gal into TM cells and tissues was tested. The possibility of transducing TAT-HA-myocilin into the TM was subsequently evaluated.
MATERIALS AND METHODS

TAT Fusion Vectors and Protein Purification

BL-21 bacteria containing the pTAT-HA-β-gal vector and BL-21 bacteria containing pHA-β-gal were the generous gift of James Zieskea (Scheinens Eye Research Institute, Boston, MA). The pTAT-HA vector, which contains an N-terminal 6x-histidine purification tag, the 11-amino-acid sequence of the TAT protein transduction domain of HIV, and the HA tag, was prepared from pTAT-HA-β-gal by digestion with NcoI and EcoRI. pTAT-HA-myocilin and pTAT-HA-myocilin-EFGP were then generated. Briefly, the open reading frame of myocilin was amplified by polymerase chain reaction (PCR) from plasmid pRSET/myocilin. The 1.5-kb PCR products were digested with NcoI and EcoRI and then subcloned in frame into the pTAT-HA vector at the same restriction sites, yielding pTAT-HA-myocilin. We created pTAT-HA-myocilin-EFGP by PCR amplification of the coding regions for myocilin without stop codon (nucleotide 61-1572, gene accession number BC029261) and EFGP (720 bp from stop to stop codon) from the plasmids pRSSET-myocilin and pEFGP-N1 (BD Biosciences/Clontech, Mountain View, CA), respectively. NcoI-SphI-digested myocilin PCR products and SphI-EcoRI digested EFGP PCR products were coligated in frame into the pTAT-HA vector. Proper orientations and sequences were confirmed by a DNA analysis system (CEQ 2000 XL; Beckman Coulter, Fullerton, CA).

TAT-HA-β-gal was purified under native conditions using nickel nitrilotriacetate affinity columns (Qiagen, Valencia, CA).3 pTAT-HA-myocilin and pTAT-HA-myocilin-EFGP were transformed into Rosetta2(DE3)pLys (Novagen, Madison, WI), and the proteins were purified in denaturing conditions. The denaturation of 8 M urea in the purified proteins was removed by PD-10 column chromatography (GE Healthcare, Piscataway, NJ).20 Electrophoresis was performed on 10% SDS gels. Correct protein expressions and purifications were confirmed by Coomassie blue staining and Western blot with anti-HA constant flow rate (16.7 μL/min) for 3 hours, and then with DMEM alone (n = 4). 0.25 (n = 4), or 0.5 (n = 4) μM TAT-HA-β-gal: 0.5 μM TAT-HA-myocilin (n = 8); or 0.5 μM β-gal (n = 4) for 6 hours. The IOP ranged from 12 to 18 mm Hg. A further perfusion with SFCM continued for 16 hours to 3 days. The perfused tissues were fixed with buffered formalin and were either processed and embedded in paraffin blocks or frozen in OCT compound (Sakura FineTek, Torrance, CA).

Actin and Immunofluorescence Staining in TM Cell Cultures and Tissues

Cultured TM cells were fixed in paraformaldehyde-lysine-phosphate buffer24 and permeabilized in 0.2% Triton X-100. F-actin was stained with Alexa Fluor 488 phalloidin (1:40 dilution, 20-minute incubation; Invitrogen-Molecular Probes, Eugene, OR).24 To detect the unpolymerized G-actin, Alexa Fluor 594–conjugated DNsase (1:550 dilution, 20-minute incubation; Invitrogen-Molecular Probes) was used. For immunofluorescence, cells were stained for 90 minutes with primary antibodies, including rabbit anti-human myocilin (1:500; the kind gift of Daniel Stamer, University of Arizona, Tucson, AZ),29 rabbit anti-β-actin (1:100), rabbit anti-fibrillin-1 (1:200; Elastin Products Company, Owensville, MO), mouse anti-paxillin (1:50; Upstate Biotechnology, Lake Placid, NY), rabbit anti-phosphorylated (Thr18/Ser19) myosin light chain 2 (phospho-MLC, 1:500; Cell Signaling, Beverly, MA), or mouse anti-β-tubulin (1:200; Sigma-Aldrich, St. Louis, MO). The cells were subsequently incubated for 60 minutes with FITC- or Cy3-conjugated goat anti-rabbit IgG and/or Cy3-5-antigoat mouse IgG. For β-tubulin staining, the secondary antibody was biotin-labeled goat anti-mouse IgG. The cells were incubated in addition with FITC-labeled streptavidin for 45 minutes. Some cell specimens were double stained for actin and paxillin.

Human TM tissues in organ cultures were fixed in 4% paraformaldehyde for 16 hours and embedded in paraffin blocks. Deparaffinized sections (5 μm) were unmasked with 10 mM citrate buffer solution (for heat-induced epitope retrieval; Laboratory Vision, Fremont, CA) with microwave irradiation for 10 minutes. Sections were blocked with normal goat serum and incubated with anti-myocilin, anti-HA, or anti-fibrillin-1 (used as a control). After incubation with biotin-goat anti-rabbit IgG, the sections were treated for 45 minutes, either with FITC-labeled streptavidin or horseradish peroxidase-avidin-biotin complex (for fibrillin-1). For actin and paxillin double staining, 8-μm frozen sections were washed to remove OCT compound and incubated with mouse anti-paxillin (1:50) for 90 minutes, Cy3-goat anti-mouse IgG for 60 minutes, and Alexa Fluor 488 (1:100) phalloidin for 20 minutes. For

Transduction of TAT Fusion Proteins into Human Tissues in Conventional Organ Cultures

Corneoscleral rims of normal human donors that included TM tissues were obtained after penetrating keratoplasty from the Cornea Service at the University of Illinois at Chicago. The rims, originally from the Illinois Eye Bank, were from donors 33, 36, 49, and 62 years of age. After overnight incubation in sterile conditions with SFCM, the tissues were gently cut into 5-mm-wide strips with the aid of a dissecting microscope. The strips were cultured for 2 or 4 hours at 37°C in 500 μL of DMEM alone, 0.5 μM β-gal or EFGP (negative control), or DMEM containing 0.25, 0.5 or 1 μM TAT-HA-β-gal or 0.5 μM TAT-HA-myocilin.

Transduction of TAT Fusion Protein into Bovine Anterior Segments in Perfusion Organ Cultures

A total of 24 freshly enucleated bovine eyes were obtained from a local abattoir. The eyes were washed and bisected at the equator. The iris, lens, and vitreous were carefully removed, and the anterior segments were placed in an incubation assembly containing three chamber units28 built on the prototype developed by Johnson and Tschumper.22 The tissues were first perfused at 37°C with DMEM at a constant flow rate (16.7 μL/min) for 3 hours, and then with DMEM alone (n = 4), 0.25 (n = 4), or 0.5 (n = 4) μM TAT-HA-β-gal; 0.5 μM TAT-HA-myocilin (n = 8); or 0.5 μM β-gal (n = 4) for 6 hours. The IOP ranged from 12 to 18 mm Hg. A further perfusion with SFCM continued for 16 hours to 3 days. The perfused tissues were fixed with buffered formalin and were either processed and embedded in paraffin blocks or frozen in OCT compound (Sakura FineTek, Torrance, CA).
bovine tissues in perfusion organ cultures, similar staining procedures were followed. Mouse anti-vinculin (1:200; Chemicon, Temecula, CA) was used instead of anti-paxillin, because the bovine tissues were not immunoreactive to the mouse anti-human paxillin antibody.

Staining in the cells and tissue sections was examined by light (Axioskop2 Plus; Carl Zeiss Meditec), fluorescence (Axiovert 100M; Carl Zeiss Meditec), or confocal laser scanning (SP2 AOBS; Leica, Deerfield, IL) microscopy. Photographs were taken either with one of three digital systems (AxioCam, Carl Zeiss Meditec; SensiCam, Cooke Camera, Romulus, MI; or the SP2 AOBS camera system, Leica). In certain experiments, images were analyzed with deconvolution software (AutoDeblur and AutoVisualize Software 9.3; AutoQuant-Media Cybernetics, Troy, NY).

**RESULTS**

**Transduction of Active β-Gal into TM Cells in Tissue Culture**

TAT-HA-β-gal in concentrations ranging from 0.05 to 1 μM was introduced into normal human TM cells in culture. Figure 1 shows the blue X-gal staining resulting from β-gal enzyme activity in various tissues. The blue staining intensifies with the increase of the TAT-HA-β-gal concentration (plateau at 0.5 μM). (Images captured with AxioCam; Carl Zeiss Meditec, Oberkochen, Germany). Bar, 50 μm.

**Figure 1.** TAT-HA-β-gal transduction into normal human TM cells in tissue culture. Cells were transduced for 60 minutes with either 0.5 μM β-galactosidase (β-gal) without the TAT domain (negative control, A) or 0.05 (B), 0.1 (C), 0.25 (D), 0.5 (E), or 1 (F) μM TAT-HA-β-gal; fixed; and stained with X-gal. Diffuse blue staining signifying β-gal activity was observed in the cytoplasm and nuclei of nearly all cells in the culture. The blue staining intensified with the increase of the TAT-HA-β-gal concentration (plateau at 0.5 μM). (Images captured with AxioCam; Carl Zeiss Meditec, Oberkochen, Germany). Bar, 50 μm.

**Figure 2.** TAT-HA-β-gal and TAT-HA-myocilin transduction into organ-cultured tissues. Human corneoscleral rims in conventional organ cultures were transduced for 4 hours with either 0.5 μM β-gal lacking the TAT domain (A, negative control; NC) or 0.5 μM TAT-HA-β-gal (B). Bovine anterior segments in perfusion organ cultures were transduced for 6 hours with either 0.5 μM β-gal (C) or 0.5 μM TAT-HA-β-gal (D). X-gal staining was performed. The resultant blue staining signifies the activity of β-gal in the various tissues. (A, B, insets) Higher magnification of X-gal staining in cells of the human TM. (Images were captured by AxioCam; Carl Zeiss Meditec; Oberkochen, Germany). In a separate experiment, human corneoscleral tissues were transduced with DMEM alone (E, NC) or with 0.5 μM TAT-HA-myocilin (F). Immunofluorescence staining for HA was performed. The green HA staining was evident in TAT-HA-myocilin (F)-transduced eyes, but was barely observed in DMEM control eyes (E). Blue: DAPI staining. (Images were captured by SensiCam and were analyzed using deconvolution software AutoQuant-Media Cybernetics, Silver Spring, MD) (★) Schlemm’s canal. TM, trabecular meshwork; CB, ciliary body; Endo, corneal endothelium. Bar, 50 μm.
activity in the cytoplasm and nucleus of nearly all cells in the culture after incubation with TAT-HA-β-gal for 60 minutes, indicative of successful transduction of functionally active β-gal into the cells. The staining was diffuse and the intensity increased with increasing concentrations (plateau at 0.5 μM) of TAT-HA-β-gal (Figs. 1B–F). When TM cells were incubated with 0.5 μM β-gal without the TAT protein transduction domain as a negative control, little or no staining was seen (Fig. 1A). Experiments performed with various incubation periods (15, 30, 45, 60, and 90 minutes) revealed that the X-gal staining intensity also increased (plateau at 60 minutes) in a time-dependent manner (data not shown).

Transduction of TAT-HA-β-Gal into Human and Bovine TM Tissues in Conventional and Perfused Organ Cultures

To test whether TAT-HA-β-gal can be transduced into TM tissues, experiments were performed using organ-culture systems. First, human corneoscleral rims (n = 4) in conventional organ cultures were incubated with TAT-HA-β-gal ranging from 0.25 to 1 μM for 2 to 4 hours. As depicted in Figure 2, the transduction was efficient in 4 hours with 0.5 μM of TAT-HA-β-gal. Blue X-gal staining was prominent in almost all cells of all regions of the TM, Schlemm’s canal, the ciliary body, and corneal endothelium (Fig. 2B). The staining was associated mostly with cells (Fig. 2B, inset), and the staining intensity was concentration and time dependent (data not shown). For the second series of experiments, perfusion organ cultures that mimic the aqueous humor dynamics across the TM29 were used. Bovine anterior segments in these cultures were infused continuously for 6 hours with 0.25 or 0.5 μM TAT-HA-β-gal.

Again, β-gal activity was seen in TM areas, Schlemm’s canal, and the ciliary body and corneal endothelium (Fig. 2D) of these tissues in a dose-dependent fashion (data not shown) but not in the β-gal control (Fig. 2C). This finding is consistent with data reported very recently, that a 90-minute perfusion of a much higher concentration (2 μM) of β-gal fusion protein into anterior segment cultures resulted in X-gal staining in the TM and the corneal endothelium.31

Transduction of TAT-HA-Myocilin or TAT-HA-Myocilin-EGFP into TM Cells

Protein transduction of two TAT-HA-myocilin proteins (with and without additional EGFP domain at the C-terminus of myocilin) into tissue-cultured human TM cells was performed. Immunostaining experiments showed that cells treated for 60 minutes with either SFCM alone (Fig. 3A) or 0.5 μM of TAT-HA-β-gal (Fig. 3C) displayed a weak, diffuse cytoplasmic staining pattern for myocilin, representing the basal endogenous myocilin expression in TM cells. The myocilin staining in cells became much stronger after transduction with 0.5 μM TAT-HA-myocilin (Fig. 3E). Confirming these results, cells transduced with TAT-HA-β-gal (Fig. 3D) and TAT-HA-myocilin (Fig. 3F), but not those treated with the SFCM control (Fig. 3B), nontatylated β-gal, or EGFP (data not shown), exhibited positive immunoreactivity when stained with anti-HA. The intensity of immunostaining was increased in a dose-dependent manner (data not shown). The pattern of staining appeared to vary, perhaps relating to the extent of the protein transduction. For both myocilin and HA, there was an indication of perinuclear staining, but most of the staining products distributed diffusely in the cytoplasm.

In addition, green fluorescence, representing HA-myocilin-EGFP, was observed in TM cells transduced with TAT-HA-myocilin-EGFP (Fig. 4B), but not in those with TAT-HA-β-gal (Fig. 4A) or EGFP (data not shown). The transduction was seen in almost all cells after treatment of TAT-HA-myocilin-EGFP.

Although the fluorescent protein was found mainly in the cytoplasm, its presence in the nucleus was also noted occasionally. Nuclear labeling, however, was not further investigated.

Transduction of TAT-HA-Myocilin into TM Tissues in Conventional and Perfusion Organ Cultures

TAT-HA-myocilin was transduced into human and bovine TM tissues in organ-culture systems, as described for TAT-HA-β-gal experiments. Immunofluorescence experiments showed that the green fluorescent myocilin staining was enhanced in the TM, Schlemm’s canal, and ciliary body of 0.5 μM TAT-HA-myocilin–treated human tissues compared with DMEM-, β-gal-, EGFP-, or TAT-HA-β-gal–transduced controls (data not shown). The successful transduction was again verified by HA immunostaining. Tissues incubated with DMEM alone (Fig. 2E), nontatylated β-gal, or EGFP (data not shown) yielded only background staining, whereas tissues transduced with TAT-HA-myocilin (Fig. 2F) and TAT-HA-β-gal (data not shown) demonstrated distinct HA immunostaining in the TM, Schlemm’s canal, and ciliary body. Similar results were ob-
FIGURE 4. Transduction of TAT-HA-myocilin-EGFP into normal human TM cells in culture. Cells were transduced for 60 minutes with 0.5 μM of TAT-HA-β-gal (A) or TAT-HA-myocilin-EGFP (B). Green fluorescence of EGFP shows TAT-HA-myocilin-EGFP protein in TM cells after transduction, but not in the TAT-HA-β-gal control. Blue: DAPI-stained nuclei. Bar, 20 μm. T-tained in bovine anterior segments in perfusion organ cultures (data not shown).

Actin and Immunofluorescence Staining on TM Cells in Tissue Culture and Organ-Culture Systems

The actin structure, focal contact formation, and/or MLC phosphorylation were examined after myocilin transduction. In TAT-HA-β-gal-transduced control TM cultures, cells exhibited robust actin stress fibers (Fig. 5A), prominent paxillin-positive focal contact formation (Fig. 5B), and marked phosho-MLC staining (Fig. 5D). Most of the actin was in the form of F-actin, not the unpolymerized G-actin (Fig. 6A, 6B). By contrast, TAT-HA-myocilin transduction resulted in a substantial reduction in actin stress fiber, focal contact, and phospho-MLC staining (Figs. 5E–H). Rather than the long, parallel stress fibers, shorter actin bundles were seen (Fig. 5E versus 5A). Paxillin, a protein localized in focal contacts along with vinculin, changed from a dotlike pattern widely scattered over the cells to a more peripheral distribution (Fig. 5F versus 5B). The staining of phospho-MLC became diffuse and less fiber associated (Fig. 5H versus 5D).

Accompanying the loss of the polymerized F-actin stress fibers (Fig. 6D versus 6A), an increase in the G-actin monomer staining was observed in TM cells transduced with TAT-HA-myocilin, compared with the control (Fig. 6, E versus B). The β-tubulin architecture was intact regardless of protein transduction (data not shown). Nor was a difference found in the fibrillin-1 staining pattern (data not shown).

Similar to that observed with tissue cultured cells, TAT-HA-myocilin transduction caused a decrease in the staining intensity for F-actin (Fig. 7C versus 7A) and paxillin (Fig. 7D versus 7B) in cells of the TM, Schlemm’s canal, and ciliary body in organ-cultured human tissues. In bovine perfusion organ cultures, the staining intensity for F-actin (Fig. 8C versus 8A) and vinculin (Fig. 8D versus 8B) in TAT-HA-myocilin-treated eyes was likewise found to be lowered in the TM, Schlemm’s canal, ciliary body, and corneal endothelium. The immunostaining pattern for fibrillin-1, tested as a control, was unchanged (data not shown). The actin and vinculin staining in the bovine corneal epithelium (Fig. 8A, 8B, insets), not in the perfusion route, was not affected. The actin staining in the TM and Schlemm’s canal was associated mostly with the cells (Fig. 8A, yellow-bordered inset). The actin appeared as filamentous fibers at or around the cell periphery, similar to that described previously by Ethier et al.32 Staining for phosphorylated MLC was attempted on both human and bovine TM tissues. The staining, however, was too faint to allow valid conclusions.

DISCUSSION

The present study demonstrates the feasibility of applying protein transduction technology to TM cells and to tissues in the aqueous humor outflow pathway. Fusion proteins TAT-HA-β-gal, TAT-HA-myocilin, and TAT-HA-myocilin-EGFP were efficiently transduced into cells in tissue culture and organ cultures. When optimized, the transduction into cultured TM cells and tissues in organ culture achieved a nearly 100% efficiency, comparable to that reported for the TAT technology in previous studies of other systems.5–8

The TAT-HA-myocilin fusion protein we used was expressed in bacteria and isolated in 8 M urea in a denatured form. As noted in the literature, denatured proteins are transduced more efficiently into cells than are the properly folded ones.5,26 Once inside the cell, transduced denatured proteins...
may be correctly folded by chaperons such as heat shock protein 90.26,33 Our study showed that the urea-denatured TAT-HA-myocilin, after desalting and ferrying into TM cells, elicited biological effects that caused a loss of actin stress fibers and focal contact formation, a phenotype similarly observed when myocilin is transfected into cultured TM cells.24 Such a demonstration indicates that the myocilin transduced into cells is functionally active. Like TAT-p27Kip1,3, TAT-P15INK4b,9, TAT-demonstration indicates that the myocilin transduced into cells to effect biological action.

To the list as another TAT fusion protein that can be transduced into cells appears to be functionally active, glycosylation per se may not be a critical determinate of the biological consequences of myocilin. It is unclear at this point whether some of the proper glycosylation. However, as the fusion protein in TM typically lacking in proteins produced in bacterial systems. Thus, the TAT-HA-myocilin fusion protein we used may not be correctly glycosylated. However, as the fusion protein in TM cells appears to be functionally active, glycosylation per se may not be a critical determinate of the biological consequences of myocilin. It is unclear at this point whether some of the myocilin fusion protein after transduction was secreted. A more careful and systematic study is needed to address this question.

The mechanism that leads to the myocilin phenotype is still unknown. The alteration in F-actin stress fibers and focal contacts has led to the speculation that myocilin’s effects may be mediated via Rho GTPase signaling (Shen et al. IOVS 2004;45: ARVO E-Abstract 4418). It is well documented that members of the Rho family, especially RhoA, Rac1, and Cdc42, coordinate many cellular responses, including adhesion and morphologic change, by regulating formation of different actin assemblies.35–37 Indeed, preliminary data (Shen et al., unpublished results, 2005) revealed a decreased RhoA activity in myocilin-transfected TM cells. Concomitant with the decreased F-actin, enhanced G-actin staining was seen in TM cells transduced with TAT-HA-myocilin. It appears that the actin existing in the F, or polymerized form, was switched to the G monomer form enhanced in TAT-HA-myocilin-transduced cells. (C, F) Merged images for F- and G-actin. Blue: DAPI-stained nuclei. Bar, 20 μm.

Figure 6. F- and G-actin staining in myocilin-transduced human TM cells in culture. Cells were transduced with 0.5 μM of TAT-HA-β-gal (A–C) or TAT-HA-myocilin (D–F). Compared with the control, staining for F-actin stress fibers (green) was reduced (D versus A), and that for unpolymerized G-actin monomers (red, E versus B) was much enhanced in TAT-HA-myocilin-transduced cells. (C, F) Merged images for F- and G-actin. Blue: DAPI-stained nuclei. Bar, 20 μm.

Figure 7. Actin and paxillin double staining in human tissues in organ cultures. Human tissues from the same donor were transduced for 6 hours with 0.5 μM of TAT-HA-β-gal (A, B) or TAT-HA-myocilin (C, D) and double stained. Green: actin staining (A, C) in the trabecular meshwork (TM), Schlemm’s canal (★) and ciliary body (CB); red: paxillin staining (B, D). Staining was weaker in the TM in TAT-HA-myocilin-transduced (C, D) tissues than that in the control (A, B). Inset: intact actin staining in the corneal epithelium in TAT-HA-myocilin-transduced eyes. Bar, 50 μm.

Figure 8. Actin and vinculin staining in bovine tissues in perfusion organ cultures. Bovine anterior segments in perfusion organ cultures were transduced with 0.5 μM of TAT-HA-β-gal (A, B) or TAT-HA-myocilin (C, D) and stained for either actin or vinculin. Green: actin staining in the trabecular meshwork (TM), Schlemm’s canal (★), and corneal endothelium (arrow) in one eye; red: vinculin staining (B, D) in another bovine tissue. Both actin and vinculin staining decreased in the TM in TAT-HA-myocilin-transduced specimens. Actin staining in the corneal endothelium was also reduced. The corneal epithelium (white-bordered insets), not in the perfusion route, retained strong actin and/or vinculin staining. (A, yellow-bordered inset) Higher magnification of actin staining in cells at the bovine TM. Bar, 50 μm.
previously in myocilin-transfection experiments (Wentz-Hunter et al, unpublished results, 2004). This is therefore a new piece of evidence that myocilin may have an inhibitory role in actin polymerization or assembly. Furthermore, the level of phospho-MLC was lowered on myocilin transduction, implying reduced TM cell contractility. This new finding is consistent with the previous data that inhibitors to Rho kinase, a downstream effector of activated RhoA, induced MLC phosphorylation in TM cells. Loss or disturbance of actin cytoskeleton and inhibition of MLC phosphorylation in TM cells have been shown to increase outflow facility in perfusion organ cultures and living monkey eyes.33–34

The present study demonstrates that studies of myocilin, like those of proteins such as profilin I,35 may be facilitated by the protein transduction technology. TAT fusion proteins are efficiently transduced into TM cells in tissue culture. The high efficiency of the protein transduction methodology is an obvious advantage over methods currently used for transfection of DNA constructs into TM cells. Transfection methods such as that employing FuGene 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN) often result in only 10% to 30% targeting efficiency, yielding high background from nontransfected samples35 and masking transfection effects. The TAT protein transduction also circumvents the need of laborious microinjection of molecules into individual cells. In addition, this methodology can be extended to deliver proteins into perfusion organ cultures and possibly in vivo animal models as well. Gene transfer to the TM has been attempted previously using viral vectors including adenovirus,36 herpes simplex virus,37 and lentivirus.38 Apart from issues related to gene therapy, the nonviral approach of TAT protein transduction may still be better than that using viral vectors, which, although highly efficient, tends not to disperse well in tissues. Of note, however, is that the protein transduced by TAT is short-lived whereas gene transfer with viral vectors is more sustained. To ever, is that the protein transduced by TAT is short-lived whereas gene transfer with viral vectors is more sustained.

From the study the outflow physiology over a course of several days or weeks, relatively long-term perfusion of TAT fusion proteins may be necessary. In any case, studies of myocilin and other proteins in the TM and other tissues can be greatly fostered by the efficient transduction of TAT fusion proteins.

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