In Vitro Localization of TIGR/MYOC in Trabecular Meshwork Extracellular Matrix and Binding to Fibronectin

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**PURPOSE.** To determine whether trabecular meshwork-inducible glucocorticoid response/myocilin (TIGR/MYOC) protein associates with the extracellular matrix (ECM) of human trabecular meshwork (HTM) cells.

**METHODS.** The extracellular localization of TIGR/MYOC was examined by immunofluorescence microscopy in HTM cultures treated with and without dexamethasone and ascorbate and in a transformed HTM cell line, TM-1, transiently transfected with TIGR/MYOC cDNA. Antibodies to TIGR/MYOC, fibronectin, laminin, type IV collagen, or thrombospondin were used to determine the extracellular localization of TIGR/MYOC. Solid phase binding assays using ¹²⁵I-recombinant TIGR/MYOC and types I and IV collagens, fibronectin, and laminin were done to examine the association of TIGR/MYOC with these proteins and to identify a specific TIGR/MYOC binding site within fibronectin. The domains of fibronectin tested were the fibrin/collagen binding domain, the RGD domain, and the Heparin II (Hep II) domain.

**RESULTS.** TIGR/MYOC colocalized with fibronectin, laminin, and type IV collagen, but not thrombospondin in both dexamethasone and dexamethasone/ascorbate-treated HTM cultures and in TM-1 cultures transfected with TIGR/MYOC cDNA. In solid phase binding assays, ¹²⁵I-TIGR/MYOC bound fibronectin but not laminin or type IV collagen. Binding to fibronectin could be competed with excess TIGR/MYOC or fibronectin. Specific binding was found for the Hep II domain of fibronectin.

**CONCLUSIONS.** TIGR/MYOC can associate with components of the ECM via interactions with the Hep II domain of fibronectin. The interactions with the Hep II domain of fibronectin could alter cell–matrix interactions in the TM and provide an interesting lead to explore the role(s) of TIGR/MYOC in both steroid-induced and primary open angle glaucoma. *(Invest Ophthalmol Vis Sci. 2002;43:151–161)*

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TIGR (trabecular meshwork-inducible glucocorticoid response), also known as myocilin (MYOC), encodes a novel 55- to 57-kDa protein whose sequence was cloned independently from human trabecular meshwork (TM) and photoreceptor cells.¹² The normal function and cellular localization of TIGR/MYOC is currently an active and controversial area of research. Recent data suggest that the protein has a role in glaucoma. The gene for TIGR/MYOC maps to a locus on chromosome 1 associated with juvenile open-angle glaucoma (JOAG), and its gene product is selectively induced in human trabecular meshwork (HTM) cell cultures by dexamethasone (DEX) treatment.³⁴ Furthermore, several mutations within TIGR/MYOC have been found in patients with juvenile or adult-onset OAG.⁵⁶

Although the function of TIGR/MYOC is still unknown, many investigators hypothesize that it may have a role in regulating aqueous outflow. One of the tissues expressing the highest level of TIGR/MYOC protein and its mRNA is the TM,⁷⁸ which is the major site of aqueous humor outflow from the eye. The expression of the TIGR/MYOC gene in the TM is especially high in DEX-perfused eyes,⁹ and TIGR/MYOC localization can be increased in glaucomatous eyes.¹⁰ Within TM cultures, its expression can also be induced in response to phorbol esters, heat shock, and oxidative stress.³¹¹² Furthermore, the perfusion of recombinant TIGR/MYOC into human eye organ cultures increases outflow resistance,¹³ whereas outflow resistance is decreased when the expression of a truncated form of the protein reduces endogenous TIGR/MYOC secretion.¹⁴ The role of TIGR/MYOC may not be restricted to the TM, outflow facility, and glaucoma, because TIGR/MYOC mRNA expression and protein are widely distributed throughout the eye,⁶⁻⁸,¹⁵,¹⁶ and in nonocular tissues as well.¹⁷ Thus, it is likely that TIGR/MYOC is a multifunctional protein.

Several reports have indicated that TIGR/MYOC can be secreted and therefore may have an extracellular function. In vitro, radiolabeled protein rapidly appears in the media of DEX-treated human TM cultures³ and can be recovered from Schlemm’s canal effluent after glucocorticoid treatment.¹ Additionally, the TIGR/MYOC amino acid sequence reveals a putative signal sequence required for most secretory proteins,² and it has been localized to the Golgi apparatus of Schlemm’s canal cells,¹⁷ which is consistent with its being a secreted protein. TIGR/MYOC has also been localized extracellularly in the corneal stroma, sclera, and vitreous body in vivo³ and has been found in the aqueous humor of several species.¹⁹,²⁰ Once outside the cell, TIGR/MYOC could interact with the extracellular matrix (ECM) as suggested in a recent study by Tawara et al.,²¹ or in the case of the corneal epithelium, it could interact with the inner mucous layer of the tear film.⁸

Several studies also suggest that TIGR/MYOC may have some intracellular function. A distinctive intracellular staining pattern for TIGR/MYOC has been found in the cytoplasm of cultured TM cells,²²,²³ the Golgi apparatus of Schlemm’s canal...
cells, the cilium of the photoreceptor cells, along microtubules in TM cells, and in tissues of the anterior eye such as the lens epithelium and cornea. A distinct cellular staining has also been found for TIGR/MYOC in the uveal and corneoscleral TM. It is not clear from these immunofluorescence microscopy studies, however, whether some of this staining represented extra- or intracellular staining and whether some of the intracellular staining represents cells synthesizing large amounts of TIGR/MYOC for secretion into the extracellular space.

It is generally accepted that the ECM plays an important role in maintaining normal aqueous outflow. The ECM may act as a filter that restricts aqueous humor outflow from the TM and provides the adhesive substrate that maintains cellular integrity against the shear force of aqueous outflow. The ECM may also govern a number of cellular processes including phagocytosis, metalloprotease expression, cell adhesion, and contractility, used by the cells of the TM-Schlemm’s canal system to regulate outflow facility. The extracellular localization of TIGR/MYOC in the ECM of the TM therefore could affect aqueous humor outflow by either physically obstructing outflow and/or affecting cell-mediated processes that control outflow.

To better understand the extracellular role of TIGR/MYOC in the TM, we set out to identify the localization of TIGR/MYOC in TM cell cultures and identify potential extracellular proteins that could interact with TIGR/MYOC. These studies indicate that TIGR/MYOC, via interactions with the Hep II domain of fibronectin, could become incorporated into the ECM of the TM. Investigating the interactions between TIGR/MYOC and the ECM may provide insights into the role that TIGR/MYOC plays in both steroid-induced and primary OAG as well as any potential role that it may play in regulating outflow under nonglaucomatous conditions.

**Materials and Methods**

**Cell Culture**

Seventh-passage HTM cells, isolated as previously described, were grown to confluence in 8-well LAB-TEK chamber slides (Nunc International, Naperville, IL) and maintained for 7 days in growth medium (low glucose DME [Sigma, St. Louis, MO], 15% fetal bovine serum [FBS; Summit Biotechnology, Ft. Collins, CO], 1 mg/ml FGF-2 [Intergen Company, Purchase, NY] together with Image Pro Plus ver. 1.3 software. After an additional 3 weeks, colonies of potential “immortalized” HTM flasks were spread and passaged once a week for approximately 4 months until the cultures showed marked slowing in their growth rate. After an additional 3 weeks, colonies of potential “immortalized” HTM lines were obtained. The immortalized HTM cells, designated TM-1, showed diploid features and morphology during growth that closely resembled the parent HTM cultures (not shown). TM-1 cells showed a high plating efficiency and excellent growth characteristics without the need for FGF-2 or other factors (not shown). To confirm that these immortalized cells contained the SV40 genome, PCR was performed on TM-1 DNA with primers located in the large T antigen.

**Establishment of the TM-1 Immortalized Cell Line**

Monolayer cultures of fifth-passage diploid HTM cells established from a 30-year-old nonglaucomatous individual and characterized as previously described by Polansky et al. were transfected for 6 hours with an SV40 origin defective vector together with Superfect transfection reagent (Qiagen, Valencia, CA) according to the supplier’s protocols. After transfection, cells were fed weekly with growth medium (without FGF-2), and colonies overgrowing the underlying cells were identified. Colonies of approximately 500 cells were isolated using glass cloning cylinders and trypsin and transferred to multwell plates. On reaching confluency these cells were subcultured into flasks, expanded, and passaged once a week for approximately 4 months until the cultures showed marked slowing in their growth rate. After an additional 3 weeks, colonies of potential “immortalized” HTM lines were obtained. The immortalized HTM cells, designated TM-1, showed diploid features and morphology during growth that closely resembled the parent HTM cultures (not shown). TM-1 cells showed a high plating efficiency and excellent growth characteristics without the need for FGF-2 or other factors (not shown). To confirm that these immortalized cells contained the SV40 genome, PCR was performed on TM-1 DNA with primers located in the large T antigen.

**Immunofluorescence Microscopy**

Normal differentiated HTM cells were grown for 12 to 14 days with or without DEX and/or ascorbate. In immunolocalization studies with TIGR/MYOC and fibronectin, laminin, or type IV collagen, undetected cells were incubated with 1:200 rabbit anti-TIGR/MYOC and either 1:100 antibovine (mAb 235, 25 µg/ml antimelanin [mAb LAM-89; Sigma]), or 1:500 mAb anti-type IV collagen (Chemicon International, Temecula, CA) for 30 minutes. Incubations and washes were done in phosphate-buffered saline (PBS), 1 mM CaCl₂ and 1 mM MgCl₂. Immunofluorescence microscopy studies to detect type IV collagen (see below), ascorbate was added to the medium at a final concentration of 25 µg/ml 24 hours before labeling with antibodies (see below). Preliminary experiments (not shown) found that under these conditions ascorbate significantly enhanced the deposition of type IV collagen within the ECM of TM-1 cells relative to untreated cells.

**Transient Transfections**

A full-length TIGR/MYOC cDNA was cloned into the 5′ BamHI and 3′ EcoRI sites of the pcDNA3 plasmid (Clontech, Palo Alto, CA) under the control of the human cytomegalovirus immediate early gene (CMV-IE) promoter. pcDNA3 without the TIGR/MYOC cDNA was used as the control vector. For the transfection, TM-1 cells were plated into 6-well Falcon plates (Becton Dickinson Labware, Lincoln Park, NJ) on uncoated, sterile glass coverslips and allowed to reach ~90% confluence (48 hours) by the day of transfection. Each well of cells was transfected...
with 3.5 μg of either control vector or vector containing the TIGR/MYOC cDNA using LipofectAMINE 2000 (Life Technologies, Inc., Rockville, MD) according to the manufacturer’s protocol. Cells were transfected for 4 hours after which time they were refed with normal growth medium. An additional control included treating cells with LipofectAMINE 2000 only. The transfection efficiency was determined by transfecting TM-1 cells with pEF1/Myc-His/LacZ (Invitrogen, Carlsbad, CA). The cells were then stained with X-gal 36 hours later. Under the conditions used for transfection, 60% to 80% of the cells were lacZ positive.

Preparation of Proteins

Recombinant human TIGR/MYOC was prepared as described1 and was iodinated with carrier-free Na 125I using the Chloramine-T method.41 Mouse laminin and pepsinized bovine type IV collagen were purchased from Chemicon International. Pepsinized bovine type I collagen was purchased from Collagen Corp. (Palo Alto, CA). Human plasma fibronectin, the 70-kDa fragment of fibronectin and the recombinant III7-10 and III12-14 proteins were made as previously described.41–44

TIGR/MYOC Solid Phase Binding Assay

Direct binding interactions between TIGR/MYOC and ECM proteins were measured in the absence of cells as described previously.41 Briefly, Costar 96-microtiter wells (Cat. 9102; Corning Inc., Corning, NY) were coated for 1 hour at room temperature with increasing concentrations of molar equivalents of plasma fibronectin, laminin, type I collagen, or type IV collagen. In some experiments, wells were coated with increasing concentrations of various domains of fibronectin. All the matrix proteins and fibronectin fragments were diluted in Hanks’ balanced salt solution containing 25 mM HEPES, pH 7.0 (HBSS/HEPES). The pH of the type I collagen and type IV collagen solutions was readjusted to pH 7.0 with 1N NaOH before plating these proteins onto the wells. Wells were then incubated with 5% nonfat milk in HBSS/HEPES for 2 hours at room temperature to block unbound regions in the wells. Afterward, the wells were washed three times with HBSS/HEPES and then incubated with 150,000 cpm of recombinant 125I-TIGR/MYOC for 2 hours at room temperature. The 125I-TIGR/MYOC was diluted in HBSS/HEPES and warmed to 37°C for 20 minutes before pipetting it into the wells. Wells were then washed three times, separated, and counted in a gamma counter. In competition assays, wells were coated with a single concentration of plasma fibronectin (17 μg/ml). Coated wells were incubated with 125I-TIGR/MYOC in the presence or absence of increasing concentrations of recombinant TIGR/MYOC, 70-kDa fragments, III12-14 or III7-10. In some instances, the 125I-TIGR/MYOC was preincubated with increasing concentrations of fibronectin or casein at room temperature for 2 to 3 hours before plating it into the wells. In all experiments described above nonspecific binding was determined as the amount of binding to wells coated with 5% nonfat milk and was subtracted from each value.

RESULTS

Localization of Extracellular Matrix Proteins and TIGR/MYOC in HTM Cultures Treated with DEX

The staining patterns observed for fibronectin, laminin, and type IV collagen are consistent with previous studies localizing these proteins in HTM cultures.45–48 As shown in Figures 1 and 2, fibronectin, laminin, and type IV collagen demonstrate a fibrillar pattern around cell edges and between individual groups of cells (Figs. 1C, 1E and 2A, respectively). Besides localizing around the periphery of the cells, the small amounts of laminin and type IV collagen can also be found diffusely distributed over the surface of some of the cells. TSP appears to have a similar distribution as fibronectin, laminin, and type IV collagen in both the DEX (Fig. 2E) and non-DEX–treated (not shown) HTM cultures and is found around and between cells in a fibrillar network. Additionally, TSP demonstrates a punctate localization pattern over the surface of many cells. This TSP localization pattern is similar to that reported previously for cultured HTM cells49 and other cell types.50 The level of TSP in these cultures, however, is not as extensive as that observed for the other three proteins. The labeling pattern for TIGR/MYOC and these matrix proteins is specific, because cells incubated with rabbit nonimmune serum (R-NIS), respectively. Arrows: areas of colocalization of each ECM protein and TIGR/MYOC; asterisks: a reference point in each pair of panels (A and B, C and D, E and F). Original magnification, ×400; scale bar, 20 μm.

![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932903/)

**Figure 1.** Colocalization of fibronectin or laminin and TIGR/MYOC in DEX-treated HTM cell cultures. Cultures were treated with 500 nM DEX for 12 to 14 days before double-labeling with antibodies against TIGR/MYOC (TIGR, D and F) and either fibronectin (FN, C) or laminin (LN, E). Cells in (A) and (B) were used as negative controls and were double-labeled with antigliarial fibrillary acidic protein (GFAP) and rabbit nonimmune serum (R-NIS), respectively. Arrows: areas of colocalization of each ECM protein and TIGR/MYOC; asterisks: a reference point in each pair of panels (A and B, C and D, E and F). Original magnification, ×400; scale bar, 20 μm.

In the DEX-treated HTM cultures, TIGR/MYOC appears to have two very distinct labeling patterns. As shown in Figures 1 and 2, TIGR/MYOC can be found around and between groups of cells as well as in a punctate pattern over the surface of some cells. Areas in each culture where TIGR/MYOC appears to localize in a punctate pattern appear to occur randomly within individual cells and most likely represent secreted TIGR/MYOC bound to the apical surface of these cells rather than TIGR/MYOC localized extracellularly to basal or basolateral sides of the cells, because the cells were not permeabilized.

TIGR/MYOC observed around the periphery of cells frequently, but not always, colocalizes with fibronectin (Fig. 1C vs. 1D), laminin (Fig. 1E vs. 1F), and type IV collagen (Fig. 2A vs. 2B, 2C vs. 2D) in the extracellular matrix. TIGR/MYOC does not colocalize with TSP (Fig. 2E vs. 2F). The localization of TIGR/MYOC with either fibronectin, laminin, or type IV collagen suggests that TIGR/MYOC could be an extracellular protein or interact with proteins in the ECM. This extracellular localization of TIGR/MYOC was sensitive to the fixation pro-
procedure. If cells were fixed before labeling, the staining intensity of extracellular TIGR/MYOC was reduced (not shown), suggesting that some of the antibodies are recognizing conformational determinants in TIGR/MYOC. This extracellular localization of TIGR/MYOC and its colocalization with fibronectin is not restricted to this particular cell strain, as it has been observed in at least two other HTM cell strains (data not shown).

Effect of Matrix Deposition on the Extracellular Localization of TIGR/MYOC in HTM Cultures Treated with DEX plus Ascorbate

Although TIGR/MYOC appears to colocalize with fibronectin or laminin better than with type IV collagen (Figs. 1C, 1E and 2A), this may reflect the fact that the staining for type IV collagen, relative to that for fibronectin or laminin, is not very strong because the cells were not treated with ascorbate. Given the overall light-to-moderate type IV collagen localization within our HTM cultures in the presence of DEX alone, we examined type IV collagen localization within the ECM of HTM cells treated with DEX and ascorbate. Studies have demonstrated that treatment of various cell types with ascorbate increases synthesis, secretion, and subsequent deposition of collagens into ECM.51,52

Combined treatment of DEX and ascorbate (Fig. 2C) dramatically increases the amount of type IV collagen localized in the matrix relative to DEX treatment alone (Fig. 2A), untreated cultures (Figs. 3A, 3C), or cultures treated with ascorbate alone (Figs. 3E, 3G). This suggests that DEX and ascorbate together may significantly enhance the production of type IV collagen in HTM cell cultures, because there is no increase in the deposition of type IV collagen in the presence of DEX alone (Fig. 2A) and only a modest increase in type IV collagen deposition is seen in cultures treated with ascorbate alone (Fig. 3) relative to untreated cultures.

Coincidental with the increase in matrix-associated type IV collagen, there is also a dramatic increase in the amount of TIGR/MYOC detected that is associated with the ECM of HTM cell cultures treated with DEX and ascorbate. The colocalization of TIGR/MYOC and type IV collagen is much better in the presence of DEX plus ascorbate (Figs. 2C, 2D) relative to DEX treatment alone (Figs. 2A, 2B). Ascorbate by itself does not induce TIGR/MYOC staining in HTM cell cultures (Figs. 3F, 3H), and it appears that the increased localization of TIGR/MYOC seen with DEX plus ascorbate treatment may involve an increased deposition of type IV collagen and possibly other matrix proteins.

![Figure 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932903/)

**Figure 2.** Colocalization of type IV collagen, but not thrombospondin, and TIGR/MYOC in DEX-treated HTM cell cultures. Cultures were treated with 500 nM DEX for 12 to 14 days before double-labeling with antibodies against TIGR/MYOC (TIGR, B, D, and F) and either type IV collagen (C-IV, A and C) or thrombospondin (TSP, F). Cells in (C) and (D) were treated with 500 nM DEX and 250 μg/ml ascorbate for 12 to 14 days. Arrows: areas of colocalization of type IV collagen and TIGR/MYOC; asterisks: a reference point in each pair of panels (A and B; C and D; E and F). Original magnification, ×400; scale bar, 20 μm.

![Figure 3](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932903/)

**Figure 3.** Type IV collagen localization in HTM cultures treated with or without ascorbate. Cultures were untreated (A and B) or treated with 250 μg/ml ascorbate for 12 to 14 days before double-labeling cells with antibodies against type IV collagen (A, C, and E) and TIGR/MYOC (B, D, and F). Asterisks: a reference point in each pair of panels (A and B; C and D; E and F; G and H). (C and D) Higher power views of the areas indicated by the asterisks in (A) and (D), respectively; (G and H) higher power views of areas indicated by the asterisks in (E) and (F), respectively. Original magnification, (A, B, E, and F) ×200; scale bar in (A), 50 μm; original magnification, (C, D, G, and H) ×400; scale bar in (C), 20 μm.
and each pair of panels (A and B, treated with 500 nM DEX (C and D), or DEX plus 250 μg/mL ascorbate (E and F) for 14 days. Cells were then double-labeled with antibodies against laminin (LN, 3A, C, and E) and TIGR/MYOC (TIGR, B, D, and F). Arrows: areas of colocalization of laminin and TIGR/MYOC; asterisks: a reference point in each pair of panels (A and B, C and D, E and F). Original magnification all panels, ×200; scale bar, 50 μm.

To examine this possibility further, the combined effects of DEX and ascorbate treatment on the colocalization of TIGR/MYOC with laminin (Fig. 4) or fibronectin (Fig. 5) in HTM cultures were also assessed and compared with either no treatment or treatment with DEX alone. A significant increase in laminin staining is observed in the presence of DEX and ascorbate (Fig. 4E). This effect is likely mediated by ascorbate because we found no significant changes in laminin localization in cultures treated with DEX alone (Fig. 4A vs. 4C), which is consistent with Li et al., although it is contrary to others. In contrast to laminin and type IV collagen, fibronectin staining is clearly increased by DEX alone (Fig. 5C), which is consistent with Steely et al. A further increase in fibronectin staining is observed in the presence of DEX and ascorbate. This was expected as ascorbate has been reported to increase fibronectin staining in bovine TM cultures.

The increased localization of type IV collagen, laminin, and fibronectin in ascorbate treated cells is apparently not due to increased accessibility of these proteins to antibody detection. No changes in cell shape or separation of neighboring cells that might expose matrix proteins found on the basal surface of individual cells are seen in cultures treated with both reagents relative to untreated cells (not shown).

Combined DEX and ascorbate treatment does not change the colocalization of TIGR/MYOC with fibronectin or laminin, but does cause a clear and consistent increase in the staining intensity of TIGR/MYOC under such conditions relative to DEX treatment alone (cf. Fig. 4D vs. 4F and 5D vs. 5F). A twofold lower magnification is shown in Figures 4 and 5 relative to Figure 2 to better demonstrate the increase in TIGR/MYOC staining. These observations confirm the initial findings for type IV collagen and TIGR/MYOC shown in Figure 2 and show that treatments that increase matrix deposition also increase the colocalization and/or the amount of TIGR/MYOC deposited within the matrix.

Localization of Extracellular Matrix Proteins and TIGR/MYOC in Transiently Transfected TM-1 Cells

Because DEX treatment, in addition to inducing TIGR/MYOC expression, increases the amount of matrix-associated fibronectin, we sought to examine the localization of TIGR/MYOC within the ECM in a system where the expression of TIGR/MYOC is uncoupled from matrix production and/or deposition. For these studies, an SV-40-immortalized TM cell line, TM-1, was transiently transfected with a TIGR/MYOC cDNA under the control of the CMV-IE promoter. Under these conditions, DEX induction is not required for TIGR/MYOC expression. Figure 6 shows the immunolocalization of TIGR/MYOC, fibronectin, type IV collagen, and laminin performed 36 hours posttransfection. TIGR/MYOC protein is detected by immunofluorescence in all cultures transfected with the TIGR/MYOC expression vector (Figs. 6D, 6F, 6H) and, as with the DEX-treated HTM cells, exhibits two distinct localization patterns. Within small groups of cells, TIGR/MYOC is organized around the perimeter of individual cells and colocalizes quite well with fibronectin, type IV collagen, and laminin (cf. Figs. 6C vs. 6D, 6E vs. 6F, and 6G vs. 6H, respectively), similar to what was observed in DEX-treated normal HTM cell cultures. Double-labeling of TM-1 cells transfected with the TIGR/MYOC expression vector with mAb MF-20 and R-NIS failed to demonstrate any significant staining (Figs. 6A, 6B). Likewise, cells transfected with a control vector and labeled with rabbit anti-TIGR/MYOC show no significant staining (not shown). Together,
these data demonstrate the specificity of the staining observed in panels C through H of Figure 6. These data support our results with the DEX-treated HTM cells that TIGR/MYOC can interact with the ECM elaborated by TM cells around the cell perimeters.

Although it is possible that the deposition of TIGR/MYOC along the periphery of cells in these cultures could be mediated via interactions with cell surface proteins along the periphery of each cell, this interpretation appears unlikely. TIGR/MYOC is rarely observed around the perimeter of TM-1 cells that have not elaborated a detectable ECM. As shown in Figure 7, areas of transfected TM-1 cultures that demonstrate negative or weak staining for fibronectin (Figs. 7A, 7E) or laminin (Figs. 7B, 7F) did not show any organized TIGR/MYOC staining around cell perimeters. Rather, TIGR/MYOC on these cells demonstrates a second localization pattern in that the protein is scattered randomly over and around individual cells. This is in contrast to TIGR/MYOC that colocalizes with fibronectin or laminin around cell perimeters (see Fig. 6). A similar finding was observed with cultures stained for type IV collagen (data not shown).

The lack of TIGR/MYOC staining in these areas is not due to the absence of cell–cell contacts. As seen in the phase contrast images of fibronectin or laminin negative cells (Figs. 7I and 7J, respectively), the cell layers are confluent and cell–cell con-
contacts are present. This supports the findings in Figures 1, 2, 4, and 5 that the organization of TIGR/MYOC around cell perimeters is driven by the presence of matrix proteins rather than by the presence of cell–cell contacts. Nevertheless, this does not rule out the possibility that once localized to the cell periphery, TIGR/MYOC could interact with proteins at cell-cell contacts.

**Binding Preference of TIGR/MYOC to Purified Matrix Proteins**

The DEX-treated HTM and the TIGR/MYOC transient transfection data strongly suggest that TIGR/MYOC is an extracellular protein that is incorporated either actively or passively into the ECM of TM cell cultures and that it does so through specific interactions with one or more ECM proteins. To examine if TIGR/MYOC can specifically interact with one or more of these ECM proteins, microtiter wells were coated with fibronectin, laminin, type I collagen, or type IV collagen, and the ability of TIGR/MYOC to bind these proteins was measured in solid phase binding assays using $^{125}$I-labeled TIGR/MYOC. As seen in Figure 8A, TIGR/MYOC clearly shows a binding preference for fibronectin over the other matrix proteins. Binding to fibronectin is dose dependent and increases as the concentration of adsorbed fibronectin is increased. In contrast, little binding to either laminin, type I collagen, or type IV collagen is observed. The binding interaction between fibronectin and TIGR/MYOC is specific and can be competed by 60% with a molar excess of soluble fibronectin (Fig. 8B). In contrast, a similar molar concentration of casein (Fig. 8B) has no effect on the binding interaction between fibronectin and TIGR/MYOC. Casein, rather than another matrix protein, was used as a control protein because laminin, type IV collagen, and type I collagen bind fibronectin.

Because fibronectin contains a number of matrix protein- and cell-binding domains that could potentially influence aqueous outflow, we were interested in determining if this TIGR/MYOC–fibronectin interaction involved any specific domains of fibronectin. Two of the cell-binding domains examined were the III7-10 and III12-14 domains of fibronectin (Fig. 9A). The III7-10 domain contains the RGD integrin-binding site in fibronectin. The III12-14 domain, also known as the heparin II (Hep II)-binding domain, contains an $\alpha$4-integrin binding site and the syndecan-4 heparan sulfate chain-binding site. We also used the amino-terminal 70-kDa heparin-binding fragment (Hep I), which lacks a cell-binding domain but does contain matrix protein-binding sites. This fragment contains the collagen-binding domain of fibronectin and has been shown to control the assembly of fibronectin fibrils. Of the domains tested, only the recombinant III12-14 repeats bind the $^{125}$I-labeled TIGR/MYOC in a dose-dependent fashion (Fig. 9, B). Binding of $^{125}$I-labeled TIGR/MYOC to the rIII12-14 domain was at least two- to threefold less than the level of binding observed to intact fibronectin at equivalent molar concentrations (Fig. 9B, C). This may be due to the fact that as a dimer each fibronectin molecule contains two III12-14 domains. In contrast, TIGR/MYOC fails to demonstrate any dose-dependent binding to either the rIII7-10 repeats (Fig. 9B, D) or the 70-kDa fragments (Fig. 9B, E). In both these instances, the same level of binding is obtained whether the wells are coated with $10^{-7}$ or $10^{-12}$M fibronectin fragments.

The rIII12-14 domain was also able to compete for $>50\%$ of the $^{125}$I-labeled TIGR/MYOC binding to adsorbed fibronectin (Fig. 9C, D) with an $IC_{50}$ of $10^{-7}$ M. This is comparable to the $IC_{50}$ of soluble, unlabeled TIGR/MYOC in this assay ($7 \times 10^{-7}$ M; Fig. 9C, D). In contrast, a $10^{-5}$ molar concentration of the 70-kDa fragments (Fig. 9C, D) fails to compete for the binding of $^{125}$I-labeled TIGR/MYOC to adsorbed fibronectin, and the rIII7-10 domain (Fig. 9C, E) only competes for 10% of the binding of $^{125}$I-labeled TIGR/MYOC to adsorbed fibronectin at this concentration. At higher concentrations, the rIII7-10 domain was able to compete for the binding of $^{125}$I-labeled TIGR/MYOC to adsorbed fibronectin. The $IC_{50}$ ($10^{-7}$ M) of this interaction, however, was 100-fold higher than with the rIII12-14 domain.

**DISCUSSION**

In this article we show that TIGR/MYOC appears as an extracellular protein around the periphery of cultured TM cells. The data are consistent with previous reports that TIGR/MYOC is an extracellular protein secreted into the media of HTM cell cultures and localized extracellularly in vivo. The extracellular localization pattern of TIGR/MYOC in HTM cultures suggests that it may be an ECM protein or at least associates with the ECM. TIGR/MYOC often colocalized in a pericellular pattern with fibronectin, laminin, and type IV collagen, which are all well-known ECM proteins. The pericellular localization of TIGR/MYOC was dependent on matrix deposi-
separate experiments done in duplicates. Bars, SEM. (soluble 70-kDa fragments (well) in the presence or absence of increasing concentrations of intact fibronectin, the rIII12-14 or rIII7-10 domains were incubated wells coated with increasing concentrations of the 70-kDa fragment, rIII12-14 domains and TIGR/MYOC compete for binding of 125I-TIGR/MYOC binds to the Hep II domain of fibronectin. Microtiter wells coated with 0.06 nM 125I-TIGR/MYOC (1.5 × 10^5 cpm/well). (C) Soluble rIII12-14 domains and TIGR/MYOC compete for binding of 125I-TIGR/MYOC to adsorbed fibronectin. Microtiter wells coated with fibronectin were incubated with 0.06 nM 125I-TIGR/MYOC (1.5 × 10^5 cpm/well) in the presence or absence of increasing concentrations of soluble 70kDa fragments (●), rIII12-14 domains (□), rIII7-10 domains (△), or TIGR/MYOC (○). The data represent the means of three separate experiments done in duplicates. Bars, SEM.

Conversely, when there was little or no matrix deposition, TIGR/MYOC was not observed around the perimeter of the cell and the only extracellular TIGR/MYOC present assumed a punctate distribution over the apical surface of the cell. Thus, it seems likely that the pericellular localization of TIGR/MYOC was due to matrix interactions and not interactions with other cell surface proteins found around the perimeter of the cell such as the cell adhesion proteins in adherens junctions. The punctate distribution of TIGR/MYOC in the absence of ECM suggests that extracellular TIGR/MYOC can participate in multiple binding interactions and therefore can interact with both matrix proteins and cell surface proteins. The identity of these cell surface proteins is currently unknown.

The fact that ascorbate enhanced TIGR/MYOC localization within the ECM but did not appear to affect its production is significant, given the high concentration of ascorbate and the significant levels of TIGR/MYOC within the aqueous humor.35-36 This suggests that enhanced matrix deposition mediated by ascorbate in the aqueous humor could lead to the increased deposition of TIGR/MYOC from the aqueous humor into the ECM of the TM in vivo.

TIGR/MYOC appears to interact with specific ECM proteins, because we did not observe any colocalization between TIGR/MYOC and the matrix protein TSP. The localization of TIGR/MYOC in the ECM may involve additional binding interactions with a protein(s) other than those examined in this study, because there were regions within HTM cultures where the TIGR/MYOC labeling assumed a fibrillar pattern that did not overlap with fibronectin, type IV collagen, or laminin. This is not to be unexpected because many matrix proteins contain similar binding and or structural motifs such as heparin binding domains, EGF repeats, fibronectin type III repeats, etc.67-68 Thus, TIGR/MYOC may be interacting with several matrix proteins via a common motif.

The specificity of a TIGR/MYOC interaction with matrix proteins was confirmed by the solid phase binding assay data, which demonstrated specific binding interactions between TIGR/MYOC and fibronectin. Interestingly, we were not able to obtain solid-phase binding data to explain the observed colocalization of TIGR/MYOC with either type IV collagen or laminin. Several possible explanations may be involved, including the potential central role for fibronectin interactions with other ECM proteins. Fibronectin is known to have its own binding interactions with both laminin and type IV collagen,58,69 and the observed TIGR/MYOC colocalization with type IV collagen and laminin may be via its interactions with fibronectin in HTM cell cultures. It is also possible that interactions between TIGR/MYOC and either type IV collagen or laminin could involve unique TIGR/MYOC binding sites generated when these proteins are organized within the three-dimensional matrix of HTM cell cultures. Because our binding studies represent a two-dimensional matrix, these sites may not be present. Additional possibilities could involve roles for internal, pepsin-sensitive, noncollagenous domains that are absent in commercially prepared type IV collagen.70 This is not a concern for type I collagen.11 For laminin, we used laminin-1 (the form found in the TM72) in our binding assay, but one may speculate that other forms could be involved that for some reason have not been detected and/or evaluated in the TM.

How extracellular TIGR/MYOC may function in the TM is unknown. It is also unknown how its expression in stimulated conditions or with glaucoma-associated mutations may be involved in glaucoma pathogenic mechanisms. Specifically, no consensus has been achieved as to how to reconcile proposals that the increased production of the protein (seen in DEX-treated TM cells and tissues) may directly contribute to outflow obstruction1-3 with suggestions/observations that decreased endogenous TIGR/MYOC expression occurs in certain experimental studies after transfection of TIGR/MYOC mutant constructs.

There does appear to be a consensus that many of the mutant forms of TIGR/MYOC are retained within cells.71,72 This observation supports a suggestion made by Polansky et al.73 in
which it was suggested that retention of the abnormal protein might produce a "stress response" resulting in a chronic increase in normal TIGR/MYOC due to activation of internal signaling pathways analogous to the unfolded protein response within the endoplasmic reticulum. Another suggestion, potentially relevant to a number of TIGR/MYOC mutations, has involved a role for abnormal oligomers within the TM.74,75 A cellular response to abnormal TIGR/MYOC forms could also produce changes in the expression of other extracellular gene products.

Another study has emphasized a role for reduced TIGR/MYOC as a means to explain a rapid decrease in IOP seen in a transfection overexpression system of a mutant form of TIGR/MYOC.14 This latter study suggests a role for extracellular TIGR/MYOC in maintaining normal aqueous outflow in the TM and as a potential regulator of physiological outflow. As part of the ECM, it is certainly possible that TIGR/MYOC could function, in part, to restrict the flow of aqueous humor. The ECM acts as a filter that restricts outflow and therefore the deposition of TIGR/MYOC into the ECM could be helping to regulate outflow by acting as a physical barrier. In support of this, recent studies by Fautsch et al.15 have shown that the perfusion of excessive recombinant TIGR/MYOC into eye organ cultures decreases outflow facility. In addition, four shear stress response elements (SSRE) located upstream of the TIGR/MYOC transcriptional start site1 could help regulate TIGR/MYOC expression in response to changes in the rate of aqueous humor outflow through the TM. Thus, the deposition of normal TIGR/MYOC into the ECM of the TM may be a physical means to regulate outflow facility.

Alternatively, extracellular normal TIGR/MYOC could be affecting IOP levels by altering the signaling events modulated by the ECM. As indicated by the solid phase binding assay, TIGR/MYOC specifically interacts with the Hep II (III12-14 repeats) domain of fibronectin. This domain in fibronectin is well known for its biological role in adhesion, organization of the cytoskeleton, signal transduction, and phagocytosis,57 all of which are believed to play a role in the regulation of aqueous outflow.25,76–79 Of particular importance would be the effect of TIGR/MYOC and fibronectin interactions on the organization of the actin cytoskeleton. The cytoskeleton has been shown to play an important role in modulating outflow facility.79 and the Hep II binding domain of fibronectin plays a major role in controlling the organization and contractility of the actin cytoskeleton.51,80 Thus, TIGR/MYOC interactions between fibronectin and TIGR/MYOC could affect the contractility of the TM cells, thereby altering outflow facility.

Finally, interactions between fibronectin and TIGR/MYOC in the ECM could regulate the formation of the matrix. The Hep II domain has been shown to regulate matrix metalloproteinase (MMP) expression via α5β1 integrin mediated signaling events.81 Because MMPs and their inhibitors help to regulate the turnover of ECM components within the TM,25 it is conceivable that interactions between TIGR/MYOC and fibronectin could modulate the amount of ECM present within the TM at any given time, thereby influencing aqueous outflow.

Clearly there are a number of ways TIGR/MYOC interactions with fibronectin could affect aqueous outflow, and because many of these activities of the Hep II domain of fibronectin involve interactions with known cell surface proteins such as integrins and syndecans,82,83 it is tempting to speculate that interactions between TIGR/MYOC and the Hep II domain of fibronectin could conceivably modulate aqueous outflow by interfering with integrin- and/or syndecan-mediated events. Whether integrins or syndecans could play a role in aqueous outflow, however, is currently unknown.

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References


