Fluorescent Protein–Labeled Glucocorticoid Receptor alpha Isoform Trafficking in Cultured Human Trabecular Meshwork Cells

Adnan Dibas,^{1,2} Ming Jiang,^{1,2} Rafal Fudala,³ Ignacy Gryczynski,⁴ Zygmunt Gryczynski,³ Abbot F. Clark,^{2,4} and Thomas Yorio^{1,2}

PURPOSE. To characterize the roles of the cytoskeleton and heat shock protein 90 (HSP90) in steroid-induced glucocorticoid receptor alpha (GR α) translocation in cultured human trabecular meshwork cells.

METHODS. Stably transfected red fluorescent protein (RFP)-GR α NTM5 cell lines were developed. Nuclear localization of RFP-GR α in NTM5 cells treated with vehicle (ethanol), dexamethasone (DEX), or RU486 was measured in cytosolic and nuclear fractions by western blotting and laser confocal microscopy. Cytochalasin D, colchicine, and 17-demethoxygeldanamycin (17AAG, an HSP90 inhibitor), were tested for their abilities to affect GR α trafficking. Nuclear export of RFP-GR α was studied using confocal microscopy following DEX or RU486 removal.

RESULTS. NTM5 cells transfected with RFP-GR α showed a clear cytosolic localization of receptor that underwent nuclear localization after DEX treatment. RFP-GR α translocation was temperature sensitive, occurring at 37°C but not at room temperature. Neither cytochalasin D nor colchicine blocked DEX-induced or RU486-induced RFP-GR α nuclear translocation; however, 17AAG prevented DEX-induced RFP-GR α nuclear translocation. Both nuclear import and export of DEX-induced RFP-GR α were faster than RU-486-induced nuclear shuttling.

CONCLUSIONS. RFP-GR α receptor behaves similarly to the wild-type GR α with its cytosolic localization and shuttling to nucleus after DEX or RU486 treatment. HSP90 is required for nuclear translocation, but the disruption of cytoskeleton had no effect on nuclear translocation of RFP-GR α . (*Invest Ophthalmol Vis Sci.* 2012;53:2938–2950) DOI:10.1167/iov.11-8331

The glaucomas represent a heterogeneous group of diseases that result in a progressive optic neuropathy characterized by functional and structural impairment of ocular tissues.

From the Departments of ¹Pharmacology and Neuroscience, ³Molecular Biology and Immunology, and ⁴Cell Biology and Anatomy, and the ²North Texas Eye Research Institute, University of North Texas Health Science Center, Fort Worth, Texas.

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Corresponding author: Adnan Dibas, Department of Pharmacology and Neuroscience, University of North Texas Health Science Center at Fort Worth, 3500 Camp Bowie Boulevard, Fort Worth, TX 76107; adnan.dibas@unthsc.edu.

Particularly affected in open-angle glaucoma are the trabecular meshwork (TM), a reticulated tissue located at the corneal-iridial junction regulating aqueous outflow resistance, the optic nerve head, and retinal ganglion cells; the loss of the latter results in blindness. It is estimated that more than 4 million Americans have glaucoma and additional individuals have the disease but are undiagnosed because there are no symptoms until peripheral vision deteriorates.¹ Although a number of the risk factors have been identified (i.e., family history, elevated intraocular pressure [IOP], age, race, and sensitivity to glucocorticoids [GCs]), elevated IOP remains the key risk factor for the development and progression of primary open-angle glaucoma (POAG). Therefore, IOP-lowering drugs are the first line of defense in delaying the progression of the disease. The elevation of IOP in POAG results from increased aqueous humor outflow resistance and is associated with changes in TM cells, accompanied by increased deposition of extracellular matrix material (ECM).^{2,3} GCs can induce similar changes in the TM ECM.^{4,5}

Because of their anti-inflammatory properties, GCs are often used topically and/or intravitreally to treat ocular inflammation conditions or edema associated with macular degeneration and diabetic retinopathy.^{6,7} Unfortunately, ocular GC therapy can lead to severe side effects. Serious and sometimes irreversible eye damage can occur as a result of the development of GC-induced ocular hypertension causing secondary open-angle glaucoma.^{8–15} There are differences in steroid responsiveness among the population, however, where topical ocular administration of GCs elevates IOP in approximately 30% to 40% of the general population (also known as “steroid-responders”). In contrast, nearly all of POAG patients are steroid responders.^{16–20}

The clinical and cellular actions of GCs are mediated via the α isoform of the glucocorticoid receptor (GR α), a member of the nuclear receptor family of ligand-dependent transcription factors. Multiple translational initiation sites can generate up to eight different subtypes of GR α (i.e., GR α -A, GR α -B, GR α -C1, GR α -C2, GR α -C3, GR α -D1, GR α -D2, and GR α -D3).^{21,22} Another alternatively spliced form of the receptor termed GR β has been discovered in humans, which also has eight different subtypes owing to alternative translational sites (termed GR β -A, GR β -B, GR β -C1, GR β -C2, GR β -C3, GR β -D1, GR β -D2, and GR β -D3).²³ This means that there may be 256 different combinations of GR α /GR β heterodimer receptors depending on different subtypes expressed. The human GR β shares with GR α the first 727 amino acids, but has a unique 15-amino acid carboxyl terminus.²⁴ This alternative splicing of the GR β receptor eliminates the GC binding domain. Instead, GR β has been shown to act as a dominant negative regulator of GC-induced activation of GR α ,^{23–25} and elevated levels of GR β have been implicated in the development of several steroid-resistant

diseases, such as asthma, inflammatory bowel diseases, and rheumatoid arthritis.^{24,25}

The ligand-free GR α is sequestered in the cytoplasm of cells as part of a large multiprotein complex that includes various chaperone proteins, such as heat shock chaperone protein 90 (HSP90), HSP70, and immunophilins (FK506-binding proteins FKBP51 and FKBP52).²⁶ GC binding induces an exchange of FKBP51 for FKBP52,^{27,28} and a conformational change in the GR α that results in dissociation of the multiprotein complex, exposure of the masked nuclear localization signals for subsequent nuclear shuttling, and entry via importin proteins.²⁹ RU486 is a GC antagonist that also binds GR α and paradoxically also translocates the RU486-GR α complex to the nucleus.³⁰ The GR α -complex associates with tubulin and the microtubule motor protein dynein, suggesting microtubule network involvement in the shuttling of the complex to the nucleus.^{31,32} Once in the nucleus, GR α binds directly to GC response elements (GREs), which are DNA motifs to stimulate or silence the expression of GC target genes.^{33,34} The translocation mechanism of GR α is complex and may involve microtubules, HSP90, and GR phosphorylation.^{21–23}

In this study, we successfully generated a human trabecular meshwork cell line overexpressing the GR alpha isoform fused with a fluorescent protein. We used this fusion protein of human GR α with red fluorescent protein (RFP-GR α) to determine the potential involvement of HSP90 and cytoskeletal elements (microtubule and microfilaments) in steroid-induced GR α translocation. Here, we find that RFP-GR α fluorescence is randomly distributed throughout the cytoplasm of TM cells, and we provide evidence that rapid GC-dependent movement of the RFP-GR α through the cytoplasm uses transport machinery that is dependent on HSP90 chaperone activity, but is independent of the cytoskeleton.

MATERIALS AND METHODS

Dexamethasone (DEX), cytochalasin D, phalloidin-FITC, FITC-conjugated monoclonal anti- α -tubulin antibody, and colchicine were obtained from Sigma-Aldrich Corp (St. Louis, MO), and 17AAG (17-allylamino, 17-demethoxygeldanamycin, a geldanamycin derivative) was a kind gift from Dr Thomas Mueller (Kosan Biosciences, Inc., Hayward, CA). Polyclonal GR α antibodies were custom-made by Antibody Research Inc. (St. Charles, MO). Monoclonal anti-RFP antibodies were purchased from Santa Cruz Inc. (Santa Cruz, CA). The transformed human NTM5 trabecular meshwork cell line was obtained from Alcon Research, Ltd. (Fort Worth, TX) and was derived from an 18-year-old male donor with no reported history of glaucoma.³⁵ The cells were cultured at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (1000 units/mL), streptomycin (1 mg/mL), and glutamine (4 mM) (Invitrogen, Grand Island, NY). Confluent cells were used in most studies as previously described.³⁶

Production of RFP-GR α NTM5 Cell Line

The RFP-GR α lentivirus was custom-made by GenTarget, Inc. (San Diego, CA). The lentivirus promoter region has the Ψ (Psi) encapsidation signal sequence for efficient encapsulation of viral RNA into particles that are adjacent to the 5' long terminal repeat (LTR), which has sequences necessary for reverse transcription of the genome (the tRNA primer binding site). The vector has the Rev-responsive element (RRE), a cis-acting RNA regulatory element that is essential in replication of all lentiviruses. The RRE binds the viral transacting regulatory protein, Rev, to mediate nuclear export of incompletely spliced viral mRNA via the exportin-1 nuclear export pathway. A central polypurine tract (cPPT), known to protect the viral genome from DNA editing, is also included in the vector with a

FIGURE 1. A schematic representation of the RFP-GR α lentivirus vector. The lentivirus promoter region has the Ψ (Psi) encapsidation signal sequence that is adjacent to the 5' LTR and for efficient encapsidation of viral RNA into particles. An internal proprietary engineered suCMV promoter (GenTarget, Inc.) drives expression of RFP-GR α . The vector carries a blasticidin deaminase (Bsd) gene under the control of a RSV/PGK (Rous sarcoma virus/phosphoglycerate kinase) promoter to provide resistance to blasticidin and to allow selection of infected cells. Also indicated are the positions of the Rev-responsive element (RRE), the central polypurine tract (cPPT), and the Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE). The RFP protein is fused at the amino-terminus of GR α with a seven-amino acid linker-span. To enhance its biosafety, the lentivector has the self-inactivation (SIN) feature at its 3' LTR region, which only produces replication incompetent lentivirus.

proprietary engineered suCMV promoter (GenTarget Inc.) driving the transcription of the viral genome. The RFP protein is fused at the amino-terminus of GR α with a seven-amino acid linker-span. The vector carries a blasticidin deaminase (Bsd) gene under the control of an RSV/PGK (Rous sarcoma virus/phosphoglycerate kinase) promoter to provide resistance to blasticidin and to allow selection of transduced cells. Insertion of the Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) in the 3' untranslated region of coding sequences of the lentiviral vector substantially increased its levels of expression. To enhance its biosafety, the lentivector encodes the self-inactivation (SIN) feature at its 3' LTR region, which only produces replication-incompetent lentivirus. A map of the RFP-GR α construct is shown in Figure 1. For transduction of cells, 100,000 cells were mixed with virus (10^4 pfu) and plated. After 3 days, media was removed and complete DMEM was added. Following 48 hours, positive clones were selected using blasticidin S antibiotic (100 μ g/mL).

Immunoblotting and Coimmunoprecipitation

Cells were subcultured in 100-mm dishes and serum starved overnight once they reached 80% to 90% confluency. To determine the effects of the cytoskeleton and HSP90 on GR α nuclear translocation, cells were treated with vehicle (dimethyl sulfoxide [DMSO]) or drugs (20 μ M of cytochalasin, colchicine, or 17AAG) for 90 minutes before stimulation with DEX (100 nM), RU486 (100 nM), or vehicle (ethanol 0.1%) for 2 to 6 hours. Nuclear and cytoplasmic extracts were prepared from cells receiving vehicle, DEX, RU486, colchicine, cytochalasin D, DEX/colchicine, and DEX/cytochalasin using a kit from Thermo Fisher Scientific Inc. (Rockford, IL). Immunoprecipitation of cytoplasmic or nuclear fractions was performed with 10 μ g of anti-RFP or anti-GR α

antibodies. Immunoprecipitated RFP-GR α or GR α receptors were separated by 7.5% SDS-PAGE, and proteins were transferred overnight at 50 volts onto nitrocellulose membranes. Membranes were blocked in skim milk for 30 minutes followed by incubation with anti-GR α (90 minutes) at room temperature. Membranes were washed twice (5 minutes each), then incubated with the secondary antibodies horseradish peroxidase-conjugated anti-rabbit IgG for 30 minutes (1:10,000; GE Healthcare, Piscataway, NJ). Immunoreactivity was detected by enhanced chemiluminescence (GE Healthcare) using Bio-Rad bio-imaging system (Hercules, CA).

Analysis of Actin Cytoskeleton by Laser Confocal Microscopy

RFP-GR α -NTM5 cells were grown on glass coverslips in complete DMEM media, and once they reached 80% to 90% confluency, they were placed overnight in serum-free DMEM media. Cells were treated with vehicle (DMSO) or cytochalasin D (20 μ M) for 90 minutes before stimulation with DEX for 2 hours. Cells were then fixed with 3.7% formaldehyde in 1X PBS at 4°C, permeabilized with 0.1% Triton in 1X PBS, and then incubated with phalloidin-FITC (10 ng/ μ L) for 50 minutes in the dark at room temperature followed by three washes in 1X PBS. Coverslips were mounted on glass slides in antifade medium containing DAPI stain for nuclear visualization (FluorSave; Calbiochem, La Jolla, CA) and allowed to dry in the dark. Cells were viewed and images of actin filaments were captured and analyzed with a Zeiss LSM 510 confocal microscope (Thornwood, NY).

Analysis of Microtubule Cytoskeleton by Laser Confocal Microscopy

RFP-GR α -NTM5 cells grown on glass coverslips were treated with vehicle (DMSO) or colchicine (20 μ M) for 90 minutes before stimulation with DEX for 2 hours. Cells were fixed in ice-cold acetone:methanol (1:1) for 4 minutes at 4°C followed by three washes in 1X PBS. To minimize nonspecific binding, coverslips were immersed in blocking solution (1% bovine serum albumin + 3% normal horse serum in 1X PBS) for 60 minutes. Microtubules were specifically identified using mouse monoclonal anti- α -tubulin conjugated to FITC (1:50 in 1% bovine serum albumin + 3% normal horse serum in 1X PBS) for 1 hour at room temperature. Coverslips were washed three times in 1X PBS and once in de-ionized water. Coverslips were mounted on microscope slides in antifade medium containing DAPI stain for nuclear visualization (FluorSave; Calbiochem) and allowed to dry in the dark. Images of microtubules were captured and analyzed with a Zeiss LSM 510 confocal microscope.

Analysis of Nuclear Translocation

Time-resolved images were obtained on a confocal MicroTime 200 (Picoquant GmbH, Berlin, Germany) system coupled with an Olympus IX71 microscope (Center Valley, PA). Fluorescence photons were gathered from different places of the sample using a $\times 60$ water-immersed objective (N.A 1.2, Olympus). To remove scattered light, a 500-nm long-pass filter, with an additional 510/23 band-pass filter or a 650-nm long-pass filter (Shemrock, Rochester, NY) was applied. As a light source, a pulsed laser (470 nm LDH-P-C470B) with a repetition rate of 20MHz was used. Fluorescence photons were collected with the photon-counting module (SPCM-AQR-14; PerkinElmer, Waltham, MA) and processing using the PicoHarp300 time-correlated single photon counting (TCSPC) module. Data analysis was performed using SymPhoTime (5.2.4) software package (Picoquant GmbH). In studies characterizing nuclear export following DEX or RU486 treatments, cells were washed three times in 1X PBS followed by fixation in ice-cold acetone: methanol (1:1) for 5 minutes at -20°C . Cells were washed again three times in 1X PBS, then coverslips were mounted on glass slides in antifade medium (FluorSave; Calbiochem) and allowed to dry for 20 minutes in the dark.

MTT Assay to Check the Viability of RFP-GR α NTM5 Cells Following Geldanamycin Treatment

MTT is a colorimetric assay that was used to assess the viability of RFP-GR α NTM5 cells following geldanamycin treatment (5 μ M, 90 minutes before DEX treatment and another 90 minutes after DEX addition) and measures the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria, where it is reduced to an insoluble, colored formazan product. The cells are then solubilized with an organic solvent and the released, solubilized formazan reagent is quantified spectrophotometrically by measuring the absorbance at 570 nm. Because reduction of MTT can occur only in metabolically active cells, the level of activity is a measure of the viability of the cells.

RESULTS

Characterization of Trafficking of RFP-GR α by DEX, RU486, and Temperature Dependence

NTM5 cells were transduced with lentiviruses encoding the RFP-GR α chimera, and fluorescence was examined in living cells. Under serum-free conditions, the RFP-GR α chimera was retained predominantly in the cytoplasm in the absence of DEX (Fig. 2A), and it moved to the nucleus when cells were treated with DEX (Fig. 2A) but not with vehicle (ethanol). The RFP-GR α moved to the nucleus rapidly in a DEX-dependent manner; therefore, the RFP-GR α chimera behaved similarly to the wild-type GR α . Surprisingly, when translocation was studied at room temperature (25°C), RFP-GR α appeared to accumulate in clusters around the nucleus without entering it (see arrows in Fig. 2B); however, once cells were shifted to 37°C, RFP-GR α rapidly entered the nucleus (Fig. 2B). Also, in the absence of ligand, the cytoplasmic distribution of the receptor at room temperature was not affected.

The western blot data are identical to our immunofluorescence results of DEX-induced RFP-GR α nuclear translocation. Following DEX (100 nM) treatment for 120 minutes, cytosolic and nuclear fraction proteins were prepared followed by immunoprecipitation with anti-RFP or anti-GR α antibodies. Western blot analysis was performed to assess GR α cellular distribution in RFP-GR α -NTM5 cells. As shown in Fig. 2C, there was a clear translocation of RFP-GR α (125 kDa) from cytosol to nucleus after DEX treatment.

Next we tested RU486, a GR α antagonist that has been previously shown to induce GR α nuclear translocation.^{30,37} RU486 (100 nM) mimicked DEX in inducing RFP-GR α nuclear translocation as judged by confocal microscopy (Figs. 3A-D) and western immunoblotting (Fig. 3E). Similar to previously published reports, the RU486 translocation time was slower for the RFP-GR α movement into the nucleus compared with DEX treatment.³⁷ In fact, after 2 hours, $1.5\% \pm 1.0\%$ of cells (4 of 263 cells) showed an absolute nuclear localization, $7.0\% \pm 2.0\%$ (18 of 263 cells) showed an absolute cytosolic location, and $91.5\% \pm 2.0\%$ (241 of 263 cells) showed both cytosolic and nuclear localization (Fig. 3B). At 4 hours after RU486 treatment, $47\% \pm 5\%$ of cells (111 of 235 cells) showed an absolute nuclear localization, 0% (0 of 235 cells) showed an absolute cytosolic location, and $53\% \pm 5\%$ (124 of 235 cells) showed both cytosolic and nuclear localization (Fig. 3C). At 6 hours after RU486 treatment, $67\% \pm 3\%$ of cells (175 of 263 cells) showed a 100% nuclear localization and $33\% \pm 3\%$ (88 of 263 cells) were still showing both cytosolic and nuclear localization (Fig. 3D). In contrast, at 1 hour after DEX treatment, 100% of cells showed an absolute nuclear localization.

FIGURE 2. Dexamethasone-induced RFP-GR α translocation from cytoplasm to nucleus in NTM5 cells is temperature-dependent. NTM5 cells were transduced with RFP-GR α lentivirus as described in Materials and Methods. (A) Cells expressing RFP-GR α were incubated for 60 minutes with 0.1% ethanol (CON = control) or 100 nM dexamethasone (DEX) at 37°C and the fluorescence was photographed from the living cells. (B) Cells treated with DEX at room temperature (RT) showed clustering of RFP-GR α around nucleus without entering the nucleus (see arrows); however, when cells were incubated at 37°C for 60 minutes, RFP-GR α entered the nucleus, suggesting that RFP-GR α nuclear shuttling is temperature-dependent. (C) NTM5 cells expressing RFP-GR α were treated with ethanol or DEX for 60 minutes and nuclear and cytosolic fractions were isolated followed by immunoprecipitation using mouse anti-RFP antibodies. Anti-RFP immunoprecipitates of cytoplasmic and nuclear proteins were subjected to electrophoresis in 7.5% SDS-polyacrylamide followed by western blot analysis with anti-GR α antibodies. DEX induced the nuclear translocation of RFP-GR α (western blot). RFP-GR α was detected with a molecular weight of approximately 125 kDa.

Role of Microtubules and Microfilaments in DEX-Induced RFP-GR α Nuclear Trafficking

Tubulin is the major building block of microtubular tracks present in all mammalian cells, and microtubules have been shown to play an important role in the intracellular transport of cargo as well as in steroid-induced GR α nuclear transport.³⁸ However, there are conflicting reports on the involvement of microtubules in GR α translocation.^{39,40} Therefore, the role of microtubules and microfilaments in steroid-induced GR α nuclear transport was investigated using our RFP-GR α -labeled cells. As an initial test, we attempted to measure the effects of the microtubule-destabilizing agent colchicine and the microfilament-destabilizing agent cytochalasin D on DEX-induced RFP-GR α nuclear translocation. Cells treated with 20 μ M of either drug for 90 minutes showed

morphological changes by light microscopy owing to cytoskeletal structural changes.⁴¹ However, DEX (100 nM) treatment still induced nuclear translocation of RFP-GR α (Figs. 4A–F). To further confirm this observation, cells were treated similarly (20 μ M drug for 90 minutes then DEX for an additional 90 minutes), and cells were fractionated to isolate nuclear and cytosolic fractions. RFP-GR α receptors were isolated by immunoprecipitation using the RFP monoclonal antibody followed by western blot analysis using the rabbit polyclonal antibody against GR α . RFP-GR α still translocated to the nucleus in a DEX-dependent manner in cells treated for 2 hours with the cytoskeletal disrupting agents colchicine or cytochalasin D (Fig. 4G). In addition, both colchicine and cytochalasin D failed to block RU-486-induced nuclear translocation of RFP-GR α (Figs. 5A–F).

FIGURE 3. RU-486-induced RFP-GR α translocation from cytoplasm to nucleus. NTM5 cells were transduced with RFP-GR α lentivirus as described in Materials and Methods. Cells expressing RFP-GR α were incubated for 2 hours, 4 hours, and 6 hours with 0.1% ethanol or 100 nM RU486 at 37°C, and the fluorescence was photographed from the living cells. (A) RFP-GR α was located in the cytoplasm of control cells. Cells treated with RU486 for 2 hours (B) or 4 hours (C) showed partial nuclear translocation of RFP-GR α . (D) Even after 6-hour treatment, only 68% $\pm$ 3% of cells showed a 100% nuclear translocation. (E) NTM5 cells expressing RFP-GR α were treated with ethanol or RU486 for 120 minutes and nuclear and cytosolic fractions were isolated followed by immunoprecipitation using mouse anti-RFP antibodies. Immunoblotting was done using rabbit anti-GR α antibodies. RU486 induced partial nuclear translocation of RFP-GR α .

Colocalization with Cytoskeletal Elements Does Mean Involvement in DEX-Induced RFP-GR α Nuclear Translocation

RFP-GR α appeared to colocalize with α -tubulin microtubules and F-actin microfilaments (Figs. 6 and 7). Staining with α -tubulin antibodies showed clear filamentous structures spanning the cytosol that partially colocalized with RFP-GR α (Fig. 6A). Following DEX treatment, RFP-GR α moved to the nucleus. Following colchicine treatment, there was an obvious retraction of microtubules as judged by the disappearance of filamentous structures, and the microtubules appeared to be densely packed around the nucleus and plasma membrane (Fig. 6C). In spite of this distortion of the microtubular tracks, DEX treatment successfully induced RFP-GR α nuclear localization (Fig. 6D). Also, GR α was immunoprecipitated from untreated cells and probed for presence of α -tubulin by western immunoblotting. As shown in Fig. 6E, α -tubulin was detected in the GR α immunoprecipitation fraction.

We also investigated the actin filament network colocalization with RFP-GR α . The relative amount of filamentous actin (F-actin) was monitored using fluorescence of FITC-conjugated phalloidin, a phallotoxin from *Amanita phalloides*, that selectively binds polymerized F-actin. As shown in Figure 7A,

most actin filaments are aligned along the axis of the cells, which partially colocalized with RFP-GR α . Following cytochalasin D treatment, there was a complete disruption of the actin network, and actin showed a densely round structure outside the nucleus (Fig. 7C). Even with such complete disruption of the actin network, DEX still induced RFP-GR α nuclear translocation (Fig. 7D). In addition, the GR α was immunoprecipitated from untreated cells and probed for the presence of actin by western immunoblotting. As shown in Figure 7E, actin was detected in the GR α immunoprecipitation fraction.

Role of HSP90 in DEX-Induced RFP-GR α Nuclear Trafficking

The effect of geldanamycin (17AAG), a selective HSP90 inhibitor, on RFP-GR α translocation in NTM5 cells is shown in Figure 8. Cells were first incubated for 3 hours with 5 μ M 17AAG followed by adding DEX (100 nM) for 60 minutes. The 17AAG interacts with HSP90⁴² and blocks the formation of mature receptor-HSP90 complexes.⁴³ Under steroid-free conditions, RFP-GR α expressed in NTM5 cells is predominantly cytoplasmic (Fig. 8A). Treatment with 17AAG alone led to reduced RFP-GR α levels (Fig. 8B), most likely owing to proteasome degradation of the destabilized GR α complex,

FIGURE 4. Disruption of cytoskeletal networks in NTM5 cells does not inhibit DEX-induced RFP-GR α translocation. Cells were incubated for 90 minutes in the presence or absence (CON = control) (A, D) of the cytoskeletal disrupting agents colchicine (Col, 20 μ M) or cytochalasin D (Cyt-D, 20 μ M) as described in Materials and Methods. Col pretreatment had no effect on cellular distribution of RFP-GR α (B). Subsequent addition of DEX induced translocation to nucleus as shown by confocal microscopy (C) and western immunoblot analysis (G). Similarly, Cyt-D treatment failed to affect cytosolic location of RFP-GR α (E). The addition of DEX induced nuclear translocation of receptor as shown by fluorescence microscopy (F) and western immunoblot of isolated nuclear and cytosolic fractions (G).

because concomitant treatment with the proteasome inhibitor MG132 restored the cytoplasmic expression of RFP-GR α (Fig. 8D). The 17AAG blocked the DEX-induced nuclear translocation (Fig. 8F); however, MG132 did not block DEX-induced RFP-GR α nuclear translocation (Fig. 8G). The viability of geldanamycin-treated cells was assessed using the MTT assay, and cells were viable without any detected cytotoxicities (data not shown).

Characterization of Nuclear Export of RFP-GR α Overexpressing NTM5 Cells Following DEX and RU486 Washout

NTM5 cells treated with DEX (100 nM) or RU486 (100 nM) were washed to remove steroids, and the export of RFP-GR α out of the nucleus was studied 6, 13, 18, and 22 hours post washout (Fig. 9). Interestingly, the export of RFP-GR α

FIGURE 5. Disruption of cytoskeletal networks in NTM5 cells does not inhibit RU486-induced RFP-GR α translocation. Cells were incubated for 90 minutes in the presence or absence (CON = control) (A, D) of colchicine (Col) (B, C) and cytochalasin D (Cyt-D) (E, F) as described in Materials and Methods. (B) Col had no effect on cellular distribution of RFP-GR α . (C) Subsequent addition of RU486 induced translocation to nucleus as shown by confocal microscopy. (E) Similarly, Cyt-D treatment failed to affect cytosolic location of RFP-GR α . (F) The addition of RU486 induced nuclear translocation of receptor as shown by fluorescence microscopy.

following DEX removal was also much faster than RU486. At 13 hours post DEX withdrawal, $54\% \pm 5\%$ of cells (246 of 462 cells) showed an absolute cytosolic localization, $15\% \pm 2\%$ (71 of 462 cells) still showed nuclear localization, and $31\% \pm 4\%$ (145 of 462 cells) showed mixed cytosolic and nuclear localization. In contrast, at 13 hours post RU486 withdrawal, $17\% \pm 5\%$ of cells (59 of 345 cells) showed an absolute cytosolic localization, $61\% \pm 4\%$ (210 of 345 cells) still showed nuclear localization, and $22\% \pm 4\%$ (76 of 345 cells) showed mixed cytosolic and nuclear localization. Therefore, both the nuclear import and export of RFP-GR α are faster following DEX treatment/removal than RU486 (Figs. 9A, 9B).

DISCUSSION

GR α is a nucleocytoplasmic shuttling protein that has the capacity to bi-directionally cross nuclear membranes. It is considered a hormone-dependent transcription factor responsible for regulating a variety of biological pathways. The regulation of GR α nuclear import/export is a key mechanism regulating gene transcription that is either activated or silenced in response to specific extracellular stimuli.

In our previous studies, we have shown that NTM5 cells express endogenous levels of GR α and GR β ^{36,44,45}; however, we generated a fluorescent protein-GR α construct to more easily evaluate the mechanisms involved in steroid-induced

FIGURE 6. Colocalization of RFP-GR α with α -tubulin does not mean microtubule network involvement in DEX-induced RFP-GR α translocation. Cells were incubated for 90 minutes in the presence or absence (CON = control) of the indicated microtubule disrupting agent colchicine (Col) or vehicle (DMSO) as described in Materials and Methods. Treatment with vehicle had no effect on RFP-GR α in the cytosol that clearly colocalized with α -tubulin (green) (A), but translocated to the nucleus following DEX addition (B). Incubation of cells with 20 μ M Col caused retraction of microtubules and collapse around the nucleus but did not affect cytosolic RFP-GR α (C) that underwent nuclear translocation following DEX stimulation (D). (E) Immunoprecipitated GR α fractions from untreated, serum-starved TM cells were run on a 7.5% SDS-PAGE followed by western immunoblotting. The upper part of the membrane was probed for GR α using rabbit anti-GR α antibodies, whereas the lower part was probed using mouse-anti-tubulin antibodies. Not surprisingly, the GR α immunoprecipitated fractions are enriched with α -tubulin (lanes 1 and 2 are from 2 different immunoprecipitation experiments of control cells).

FIGURE 7. Colocalization of RFP-GR α with F-actin does not mean microfilament network involvement in DEX-induced RFP-GR α translocation. Cells were incubated for 90 minutes in the presence or absence (CON = control) of the microfilament disrupting agent cytochalasin D (Cyt-D) as described in Materials and Methods. RFP-GR α was located in the cytoplasm of control cells (**A**), but was translocated to the nucleus on DEX treatment (**B**). Although vehicle had no effect on F-actin filaments (green) (**A**), Cyt-D (20 μ M) clearly disrupted microfilaments and changed cell shape (**C**). Even with the collapse of the microfilaments, DEX still induced nuclear translocation of RFP-GR α (**D**). (**E**) Immunoprecipitated GR α

FIGURE 8. DEX-induced translocation of RFP-GR α from cytoplasm to nucleus is HSP90-dependent and is inhibited by geldanamycin. NTM5 cells expressing RFP-GR α were treated with or without 5 μ M geldanamycin (17AAG) for 3 hours, followed by 100 nM Dex. (A) In control (CON) cells, RFP-GR α was localized to the cytoplasm. (B) Treatment with 17AAG alone reduced RFP-GR α expression, demonstrating a need for HSP90 to stabilize the GR α complex. (C) Addition of the proteasome inhibitor MG132 alone had no apparent effect on RFP-GR α expression, which remained cytoplasmic. (D) The addition of MG132 and 17AAG restored cytoplasmic RFP-GR α expression suggested that the loss of RFP-GR α in 17AAG-treated cells was attributable to proteosomal degradation. (E) DEX treatment caused the nuclear translocation of RFP-GR α . (F) The 17AAG prevented the DEX-mediated nuclear translocation of RFP-GR α in MG132-treated cells. (G) MG132 did not block DEX-induced RFP-GR α nuclear translocation.

nuclear import of GR α . We used immunofluorescence microscopy to track the movement of RFP-GR α in trabecular meshwork cells in the absence and presence of DEX, a synthetic GC. In the absence of GCs, RFP-GR α localized mainly in the cytoplasm, whereas treatment with DEX resulted in the efficient translocation of RFP-GR α into the nucleus. In addition, the GR α antagonist RU486 also induced RFP-GR α nuclear translocation. Thus, RFP-GR α maintained ligand-dependent cytoplasm to nuclear shuttling, exactly like the wild-type GR α . Surprisingly, RFP-GR α nuclear entry was suppressed at room temperature with the receptor clustering in small round structures outside the nuclear membrane. The requirement for a 37°C temperature for nuclear entry is consistent with a previous study by Moguilewsky and Philibert.³⁷ These authors demonstrated that nuclear uptake of ³H-DEX was approximately 10-fold higher at 37°C than at 25°C. In our study, the RU486-induced nuclear translocation of RFP-GR α took longer than with DEX. Our observations are also in agreement with the Moguilewsky and Philibert study,³⁷ which demonstrated

that the nuclear uptake of ³H-DEX was greater than 20-fold higher than ³H-RU486.³⁷

The cytoskeleton, which includes actin microfilaments, tubulin microtubules, and intermediate filaments, is very important in regulating a variety of cellular functions. To mediate the cellular effects of extracellular and intracellular stimuli, rearrangements of the cytoskeleton are often required. Our current study shows partial localization of α -tubulin and actin with RFP-GR α (Figs. 6 and 7), similar to previous studies showing colocalization of these cytoskeletal elements with GR α using immunofluorescence and immunoprecipitation.^{31,32} Harrell et al.³⁸ have shown that the overexpression of dynactin (a 50-kDa subunit of the dynein-associated dynactin complex), which competes for the dynein motor with its cargo, inhibited DEX-induced translocation of GR α to the nucleus in 3T3 mouse fibroblasts. The same group reported similar results using geldanamycin or overexpression of the peptidylprolyl isomerase (PPIase) domain fragment of FKBP52.³⁸ In our study, depolymerization of the microtubules

fractions from untreated, serum-starved TM cells were run on a 7.5% SDS-PAGE followed by western immunoblot analysis. The upper part of the membrane was probed for GR α using rabbit anti-GR α antibodies and the lower part was probed using mouse anti-actin antibodies. Not surprisingly, the GR α immunoprecipitated fractions are enriched with actin (lanes 1 and 2 are from 2 different immunoprecipitation experiments of control cells).

FIGURE 9. Nuclear export of RFP-GR α after DEX or RU-486 removal. RFP-GR α NTM5 cells treated with DEX (100 nM, 2 hours) or RU-486 (100 nM, overnight) were washed 3 times and media was replaced with serum-free DMEM. Cells were fixed after 6, 13, 18, and 22 hours post wash and processed as described in Materials and Methods. As shown, cells treated with DEX (A) showed a quicker nuclear export of RFP-GR α than cells treated with RU-486 (B).

by colchicine disrupted the intracellular organization of the microtubule network, but to our surprise failed to inhibit DEX-induced GR α translocation, suggesting that the microtubule track may not be needed for nuclear trafficking in TM cells. Alternatively, the existence of both microtubule-dependent and -independent pathways for GR α nuclear shuttling may be present. Similarly, cytochalasin D treatment failed to block DEX-induced RFP-GR α nuclear translocation, suggesting the presence of a microfilament-independent mechanism for GR α translocation. Also, neither colchicine nor cytochalasin D prevented RU-486-induced nuclear translocation of RFP-GR α (Figs. 5C and 5E, respectively).

The association of tubulin and actin with GR α may suggest that microfilaments and microtubules play yet unidentified roles, such as depolymerization of microtubules and or microfilaments. For example, Garnier et al. have shown that HSP90 binding to tubulin dimer prevented microtubule polymerization,⁴⁹ an effect reproduced by FKBP52 protein.⁵⁰ Both of these proteins are key components of the GR α complex and both inhibited microtubule polymerization.^{49,50} In addition, Akner et al.⁵¹ have shown that the GR α receptor inhibited microtubule polymerization. A number of investigators have shown that the cytoskeleton may not be required for GR α nuclear translocation. Szapary et al.³⁹ reported that microtubules are not required for either the nuclear translocation or biological activity of glucocorticoid receptors, as steroid-induced gene expression was unchanged in the absence of intact microtubules. Vorgias et al.⁴⁰ confirmed these findings and also showed that the microfilament network is not involved in GR α nuclear translocation in thymocytes. Galigniana et al.⁴¹ confirmed the lack of involvement of microtubules, microfilaments, and intermediate filaments in GFP-GR α nuclear translocation by DEX.

Studies on other nuclear receptors have indicated both microtubule-dependent and -independent nuclear translocation. For example, although Campbell et al.⁵² have shown that

the estrogen receptor nuclear translocation was inhibited by microtubule-disrupting agents, while Kalimi et al. reported the lack of an effect.⁵³ The first group used a relatively high concentration of colchicine (100 μ M), which may have been toxic and killed the cells. Perrot-Appianat et al.⁵⁴ reported that the progesterone receptor (PR) nuclear trafficking is microtubule independent. The same group demonstrated that the PR exited the nucleus into the cytoplasm by the administration of energy-depleting drugs and observed reaccumulation of the receptor in the nucleus on removal of the drugs, regardless of whether the cytoskeleton was intact or disrupted. They suggested that the "karyophilic or importin signals and interactions with the nuclear pore seem to be the primary determinants of the cellular traffic of the progesterone receptor." Finally, HSP90 nuclear translocation appears also to be microtubule and microfilament independent⁵⁵; however, it is possible there are other yet unidentified roles for the microtubules/microfilaments in GR α translocation.

Similar to earlier reports, the nuclear translocation in our study was HSP90 dependent because geldanamycin blocked DEX-induced GFP-GR α nuclear translocation. Geldanamycin, a benzoquinone ansamycin that binds to HSP90 and disrupts its function, inhibited DEX-dependent translocation from the cytoplasm to the nucleus. GR α uses at least two distinct pathways for nuclear trafficking: HSP90-dependent (very fast <10 minutes),²⁸ and HSP90-independent (~1 hour)⁵⁶ pathways. However, in NTM5 cells, GR α nuclear translocation is absolutely HSP90 dependent. Geldanamycin-treated cells showed lower levels of fluorescent RFP-GR α , suggesting degradation of the destabilized complex, which is consistent with previous reports showing proteolysis of GR α by proteasomal degradation following geldanamycin treatment.^{57,58} We previously showed that the nuclear translocation of endogenous GR β in primary TM cells is HSP90 dependent, and that inhibition of this nuclear translocation induced the proteasomal degradation of cytosolic GR β .⁴⁷

In summary, we have used the RFP-GR α chimera to study cytoplasmic-nuclear translocation in living cells, and this construct behaves like wild-type GR α in that both DEX and RU486 enhance translocation to the nucleus. The translocation of RFP-GR α is inhibited by the inhibitor of the HSP90 chaperone function, geldanamycin; however, whenever NTM5 cells were treated with cytoskeletal disrupting agents, translocation of RFP-GR α proceeded uninterrupted. Although these observations argue against the model in which the RFP-GR α normally moves along cytoskeletal tracts,³⁷ they are consistent with the model suggesting that the transport mechanism is HSP90 chaperone dependent.^{21–22,35} The exact mechanism for GR α nuclear translocation remains unclear, as inhibitors of both microtubules and microfilaments failed to block the relocation of GR α from cytosol to nucleus following activation by glucocorticoid. The presence of alternative pathways for nuclear import might facilitate the uninterrupted and efficient translocation of proteins under different physiological conditions where importin-dependent and/or cytoskeleton-dependent pathways may be compromised.⁵⁹ Although studies presented in the current report investigated the role of cytoskeleton and heat shock proteins and suggested the lack of cytoskeletal involvement, the identity of importins (proteins responsible for nuclear trafficking) involved in GR α translocation in TM is currently unknown. The use of inhibitors or siRNA against different importins (>13 members), exportins (>5 members), and nuclear pore complex subunits, should be carried out to identify the players involved in GR α translocation. Such studies are of great importance because trabecular meshwork cell lines isolated from normal and glaucomatous donors retain their phenotype and behave like parent tissues in that they differ in levels of GR β and phagocytic activity, and respond differently to GCs.^{36,48} Therefore, understanding GR α transport could explain some of the differences in responsiveness between normal and glaucomatous TM cells, which will lead to a better understanding of the molecular mechanisms involved in GC-induced ocular hypertension and glaucoma.

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