Rho GDP dissociation inhibitor α interacts with estrogen receptor α and influences estrogen responsiveness

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Abstract

Estrogen receptor α (ER α) is a ligand-activated transcription factor that regulates expression of estrogen-responsive genes. Upon binding of the ligand-occupied ER α to estrogen response elements (EREs) in DNA, the receptor interacts with a variety of coregulatory proteins to modulate transcription of target genes. We have isolated and identified a number of proteins associated with the DNA-bound ER α . One of these proteins, Rho guanosine diphosphate (GDP) dissociation inhibitor α (RhoGDI α), is a negative regulator of the Rho family of GTP-binding proteins. In this study, we demonstrate that endogenously expressed RhoGDI α is present in the nucleus as well as the cytoplasm of MCF-7 breast cancer cells, and that RhoGDI α binds directly to ER α , alters the ER α –ERE interaction, and influences the ability of ER α to regulate transcription of a heterologous estrogen-responsive reporter plasmid in transient transfection assays as well as endogenous, estrogen-responsive genes in MCF-7 cells. Our studies suggest that, in addition to the activity of RhoGDI α in the cytoplasm, it also influences ER α signaling in the nucleus.

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Introduction

Individual cells in multicellular organisms rely on a variety of signals from their surrounding environment to survive and function effectively. Neighboring and more distant cells produce a wide range of ligands including lipophilic hormones, peptides, and neurotransmitters that control various cellular functions ranging from metabolism, cell division, and differentiation to communication among cells from the same or different tissues. Once sensed by a cell, these ligands can initiate a cascade of intracellular events that oftentimes concludes with transcription factor activation and modulation of target gene expression. The receptors targeted by ligands can be intracellular such as steroid receptors or embedded in the cell membrane such as ligand-gated ion channels, receptor tyrosine kinase family members, or G protein-coupled receptors.

Rho GTP-binding proteins (RhoGTPases) are members of the Ras superfamily of GTP-binding proteins and are considered key players in the intracellular transmission of signals initiated by cell-surface receptors. RhoGTPases act as molecular switches cycling between an active, GTP-bound form, which is anchored to the cell membrane, and an inactive, GDP-bound form, which is present in the

cytoplasm (Koch *et al.* 1997). When bound to GTP, RhoGTPases interact with downstream effector proteins and foster signal propagation. Once GTP is hydrolyzed, RhoGTPase activity ceases and signal transduction is halted. Proteins that regulate the activity of these molecular switches include GTPase-activating proteins (GAPs), which enhance the rate of GTP hydrolysis, GDP dissociation inhibitors (GDIs), which inhibit the release of GDP from the GTPase, and GDP exchange factors (GEFs), which replace GTPase-bound GDP with GTP. While GAPs and GDIs are negative regulators, GEFs are positive regulators of GTPase activity (Hart *et al.* 1992).

In addition to the signals initiated at the plasma membrane and propagated through the cytoplasm, some ligands, mainly lipophilic hormones, elicit their effects by targeting nuclear receptor superfamily members. In their classical mode of action, ligand-occupied nuclear receptors undergo a conformational change and interact with their cognate response elements in DNA to fulfill their function as transcription factors that regulate the expression of target genes. Through binding to specific nuclear receptors, lipophilic hormones regulate a variety of physiological processes. In addition to the traditional mode of nuclear receptor transactivation, there is accumulating evidence that receptors on the cell surface initiate

signals that alter nuclear receptor activity. For example, epidermal growth factor (Kato *et al.* 1995, Bunone 1996 #2283), transforming growth factor α (Ignar-Trowbridge *et al.* 1993), and insulin-like growth factor-I (Ma *et al.* 1994) are able to alter the capacity of nuclear receptors to activate gene expression. This nonclassical mode of nuclear receptor regulation can be hormone independent or hormone dependent and provides a link between various cues received by receptors on the cell surface and the activity of receptors in the nucleus.

Our laboratory has been interested in identifying proteins that influence estrogen-responsive gene expression. Using agarose gel mobility shift assays and mass spectrometry analysis, we identified novel HeLa nuclear proteins associated with the DNA-bound estrogen receptor α (ER α ; Schultz-Norton *et al.* 2007). One of these ER α -associated proteins was the 28 kDa protein Rho GDP dissociation inhibitor α (RhoGDI α), which was originally characterized as a negative regulator of the RhoGTPase family members RhoA, Rac1, and Cdc42 (Fukumoto *et al.* 1990, Leonard *et al.* 1992, Masuda *et al.* 1994, Koch *et al.* 1997, Olofsson 1999). Although the ability of RhoGDI α to enhance transcription of an estrogen response element (ERE)-containing reporter plasmid has been reported previously, it was thought that this enhanced activity was due to the cytoplasmic actions of RhoGDI α (Su *et al.* 2001, 2002). Because we found RhoGDI α associated with the DNA-bound ER α , we investigated the localization of endogenously expressed RhoGDI α in MCF-7 breast cancer cells and characterized the ability of this protein to interact with ER α and influence the expression of estrogen-responsive genes.

Materials and Methods

Isolation of RhoGDI α

RhoGDI α was isolated with the ERE-bound ER α using HeLa nuclear extracts, purified ER, ERE-containing oligos, and agarose gel fractionation (Schultz-Norton *et al.* 2007). Five unique peptides (SIQEQELDKDDESLR, VAVSADPNVPNVVVTGLTLVCSSAPGLELDLTGDLESFKK, IDKTDYMVGSYGPR, FTDDDKTDHLSWEWNLTIK, and AEEYEFLTPVEEAPK), which exclusively map to RhoGDI α , were identified by mass spectrometry analysis as described (Loven *et al.* 2003).

Western blots

Nuclear and cytosolic extracts were prepared from MCF-7 and MDA-MB-231 breast cancer cells and U2 osteosarcoma (U2OS) cells as previously described (Wood *et al.* 2001). Ten micrograms of each extract were separated on a 15% SDS polyacrylamide gel and subjected to western analysis with an antibody directed against RhoGDI α , ER α , or lamin A/C (sc-360, sc-8002

and sc-20681 respectively, Santa Cruz Biotechnologies, Santa Cruz, CA, USA). The blots were then developed using a horseradish peroxidase-coupled secondary antibodies and a chemiluminescent detection system. The data included are representative of five different experiments.

Subcloning, expression, and purification of his-tagged RhoGDI α protein for gel shift assays

A *Bam*H1/*Eco*RI fragment of human RhoGDI α from pGST-GDI, kindly provided by M Garabedian (New York University, School of Medicine, New York), was subcloned into a dual-tagged (His and T7) pET-28a (+) vector (Novagen, La Jolla, CA, USA) for the expression of RhoGDI α protein. The plasmid was purified and used to transform *Escherichia coli* BL21-CodonPlus (DE3)-RIL competent cells (Stratagene, La Jolla, CA, USA) which were induced with 1 mM IPTG at 37 °C for 4 h, chilled on ice for 5 min, and pelleted at 4700 g for 10 min at 4 °C. Ni-NTA lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 10 mM imidazole) was added to the cell pellet and the lysate obtained was sonicated on ice and centrifuged at 142 000 g for 30 min at 4 °C. The supernatant was diluted with one-half volume of Ni-NTA lysis buffer and incubated with Ni-NTA agarose beads (Qiagen) with rotation for 1 h at 4 °C. The beads were washed thrice with Ni-NTA wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, and 0.5% Triton X-100). His-RhoGDI α was eluted with Ni-NTA elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 250 mM imidazole). Protein purity was monitored on Coomassie-stained gels. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad) using BSA as a standard.

Pull-down assay using purified proteins

His, T7-tagged RhoGDI α was expressed and purified as described above using Ni-NTA. The purified protein was then immobilized on T7-Tag antibody agarose beads from T7-Tag Affinity purification kit (Novagen) according to the manufacturer's recommendation. Baculovirus expressed and purified ER α was incubated with immobilized RhoGDI α at 4 °C for 1 h without or with 10 nM 17 β -estradiol (E₂). The beads were washed thrice with buffer provided in the purification kit and proteins were eluted with 2X SDS sample buffer (125 mM Tris pH 6.8, 4% SDS, 20% glycerol, 10% β -mercaptoethanol). Lysates from *E. coli* cells transformed with parental plasmid without RhoGDI α were purified in parallel. Western analysis was performed using a monoclonal antibody against ER α (sc-8002, Santa Cruz Biotechnologies). Similar results were obtained in three independent experiments.

Coimmunoprecipitation assays

MCF-7 cells were grown to 80% confluency in 10 cm dishes in phenol red-free minimum essential medium (MEM, Invitrogen) containing 5% charcoal-dextran-treated calf serum (CDCS) then maintained for 6 h in phenol red-free MEM containing 0.4% fetal calf serum (FCS) before being treated for 24 h with 10 nM E₂ or ethanol. The cells were washed thrice in cold 1X PBS and lysed in 500 μ l ice-cold lysis buffer (20 mM Tris pH 7.4, 10 mM EDTA, 100 mM NaCl, 0.5% IGEPAL, 1 mM Na₃VO₄, 50 mM NaF, and 1X protease inhibitor cocktail (PIC, Sigma)). Equal amounts of the cell lysate were incubated with 1 μ g of fluorescein-specific antibody (University of Illinois, Immunological Resource Center, Urbana, IL, USA) or RhoGDI α -specific antibody (sc-360, Santa Cruz Biotechnology) for 12 h at 4 °C under gentle agitation. Forty microlitres of 50% protein A sepharose slurry was added and the samples were rotated for 1 h at 4 °C. The samples were then pelleted, washed thrice with wash buffer (20 mM Tris pH 7.4, 10 mM EDTA, 100 mM NaCl, 0.1% IGEPAL, 1 mM Na₃VO₄, 50 mM NaF, and 1X PIC), and eluted with 2X SDS sample buffer for SDS-PAGE analysis followed by immunoblotting using an antibody against ER α (sc-8002, Santa Cruz Biotechnologies). The data shown are representative of four independent experiments.

Transient transfections

U2OS cells were maintained in phenol red-containing MEM with 10% FCS. Two days before plating, cells were transferred to phenol red-containing MEM supplemented with 5% CDCS. After 24 h, cells were transferred to phenol red-free MEM containing 5% CDCS. Cells were then seeded in 24 well plates at 2.5×10^4 cells/well and transfected with 20 ng CMV5-hER α (Reese & Katzenellenbogen 1991), 10 ng TK-Renilla (Promega), and 1 μ g of a luciferase reporter vector containing two copies of the consensus ERE (2ERE-TK-Luciferase, kindly provided by B Katzenellenbogen, University of Illinois, Urbana, IL, USA) without or with 10–1000 ng of a RhoGDI α expression vector (pCDNA-RhoGDI α , kindly provided by M Garabedian, New York University, School of Medicine, New York). A parental expression vector lacking the RhoGDI α sequence was used to maintain a constant amount of DNA in each well. Cells were transfected using lipofectin (Invitrogen) for 6 h, after which they were treated with ethanol vehicle or 10 nM E₂ for 24 h. Luciferase activity was quantitated using the Dual Luciferase Assay kit (Promega). The data shown were derived from three independent experiments. Significant differences in luciferase activity were calculated by ANOVA using SAS.

Gene silencing with RNA interference

MCF-7 cells were maintained in phenol red-containing MEM supplemented with 5% calf serum and placed on phenol red-free MEM with 5% CDCS 24 h prior to transfection. Cells were then seeded at 4×10^5 cells/well in 12-well plates 24 h prior to transfection using phenol red-free MEM with 5% CDCS without antibiotics and transfected with 50 pmol of control (renilla luciferase) or RhoGDI α -specific siRNA oligos (4630 or 46085 respectively, Ambion, Austin, TX, USA) in the absence of antibiotics using siLenFect (Bio-Rad) for 24 h. Media was replaced with phenol red-free, antibiotic-free MEM with 5% CDCS for an additional 24 h without or with 10 nM E₂. Cells were then lysed in lysis buffer and western blot analysis was performed using antibodies to RhoGDI α , ER α , GAPDH (sc-360, sc-8002, and sc-20357 respectively, Santa Cruz Biotechnology), or PR (RM-9102, LabVision, Fremont, CA, USA). RNA was harvested using Trizol (Invitrogen) and processed according to the manufacturer's directions. cDNA was synthesized using the Reverse Transcription System (Promega). Real-time PCR was performed using iQ SYBR Green Supermix and the iCycler PCR thermocycler (Bio-Rad) according to the manufacturer's directions. Primer sets for: GAPDH (5'-CGC TCT CTG CTC CTC CTG-3' and 5'-TCC GTT GAC TCC GAC CTT-3'), RhoGDI α (5'-ACC CAG CCA GGA ACA AAC-3' and 5'-GCA GAC ACA ACA CGA AGA C-3'), ER α (5'-TGC CCT ACT ACC TGG AGA AC-3' and 5'-CCA TAG CCA TAC TTC CCT TGT TGT C-3'), PR (5'-GTG CCT ATC CTG CCT CTC AAT C-3' and 5'-CCC GCC GTC GTA ACT TTC G-3'), and pS2 (5'-GCT GTT TCG ACG ACA CCG TT-3' and 5'-TTC TGG AGG GAC GTC GAT G-3') were utilized. Standard curves were derived using serial dilutions of cDNA equivalent to 0.025, 0.25, 2.5, and 25 ng input RNA and were run in duplicate for each primer set during each experiment. The relative nanograms of RNA were determined from the standard curve. The average of three replicates from one experiment is shown which is representative of four independent experiments. Significant changes in RNA levels due to specific siRNA or hormone exposure were calculated by ANOVA using SAS 9.1 (SAS Institute Inc., Cary, NC, USA).

Gel mobility shift assays

Gel mobility shift assays were carried out as described previously with the following modifications. ³²P-labeled, 50 bp ERE-containing oligos were incubated for 10 min at 4 °C in binding reaction buffer (15 mM Tris, pH 7.9, 20 mM KCl, 0.2 mM EDTA, 10% glycerol, 50 ng/ μ l poly dI/dC, 4 mM dithiothreitol, and 50 nM E₂) with a constant amount (50 fmoles) of baculovirus expressed, purified ER α , and increasing amounts of purified

RhoGDI α . BSA and Ni-NTA elution buffer were added as needed to maintain constant protein and salt concentrations. For antibody supershift experiments, ER α - and RhoGDI α -specific antibodies (sc-8002 and sc-360 respectively, Santa Cruz Biotechnology) were added to the binding reaction mixture and incubated for an additional 10 min at room temperature. Samples were loaded onto a 6% nondenaturing polyacrylamide gel and fractionated using low ionic strength buffer as described previously (Chodosh & Buratowski 1989). Radioactive bands were visualized by autoradiography. Three independent experiments were performed.

Fluorescence microscopy

MCF-7 cells were grown on poly-L-lysine-treated cover slips in phenol red-free MEM medium containing 5% CDCS and exposed to 10 nM E₂ or ethanol vehicle for the indicated times. Cells were fixed in 1X PBS containing 4% formaldehyde and 4% sucrose for 10 min and permeabilized in 1X PBS with 0.2% Triton X-100 for 20 min. Blocking was performed in blocking buffer (2% BSA, 2% FBS, 0.1% Tween-20, and 0.02% NaN₃ in 1X PBS) for 45 min at room temperature. When digitonin was used, the cells were first washed with transport buffer (20 mM HEPES pH 7.4, 110 mM potassium acetate, 2 mM magnesium acetate, and 0.5 mM EGTA), then treated with 0.005% (wt/vol) digitonin in transport buffer for 6 min at 4 °C. Cover slips were washed thrice in transport buffer and incubated for 20 min at room temperature before being fixed, permeabilized, and blocked as indicated above. Cells were incubated with rabbit polyclonal antibody against RhoGDI α (sc-360, Santa Cruz Biotechnology) alone or combined with either a mouse monoclonal antibody that recognizes a related family of NPC proteins (MMS-120P, Covance, Berkeley, CA, USA) or a mouse monoclonal antibody that recognizes the nucleolar protein fibrillarin (Abcam Ab18380, Cambridge, MA, USA) in blocking buffer for 1 h at room temperature in a humidified box. Cells were washed thrice in 1X PBS with 0.1% Tween-20 and incubated with a fluorescein conjugated, donkey anti-rabbit secondary antibody, alone or combined with donkey anti-mouse, Texas Red conjugated, secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature in a humidified box. The nuclei were counterstained with 4',6-diamidino-2-phenylindole and mounted with Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA, USA). Digital images were captured by a charge-coupled device camera (Hamamatsu ORCA, Bridgewater, NJ, USA) mounted on a Nikon Microphot-SA microscope (Melville, NY, USA) using Openlab software 2.0.6 (Improvision I, Lexington, MA, USA). Montages of digital images were assembled in Adobe Photoshop 7.0.

Results

RhoGDI α has been described as a ubiquitously expressed protein, which is found primarily in the cytoplasm of cells (Koch *et al.* 1997). Thus, we were surprised when we identified endogenously expressed RhoGDI α from HeLa nuclear extracts as a component of a large multiprotein complex associated with the DNA-bound ER α (Schultz-Norton *et al.* 2007).

RhoGDI α is present in the cytoplasm and the nucleus

We undertook a series of experiments to monitor the localization of endogenously expressed RhoGDI α in cultured cell lines that have been used to study estrogen-responsive gene expression. As expected, RhoGDI α was detected in the cytosolic fractions (C) of ER α -positive MCF-7 breast cancer cells in the absence and presence of 10 nM E₂, ER α -negative MDA-MB-231 breast cancer cells, and U2 osteosarcoma (U2OS) cells using western analysis (Fig. 1, lanes 2, 4, 6, and 8). RhoGDI α was also observed in the nuclear fractions (N) of MCF-7 and U2OS cells (Fig. 1, lanes 1, 3, and 7). Although RhoGDI α was not visible in MDA-MB-231 nuclear extracts in the data shown, we were able to detect RhoGDI α in MDA-MB-231 nuclear extracts using higher antibody concentrations (data not shown). As expected, ER α was present only in MCF-7 cells. Lamin A/C was used as a loading control to demonstrate that similar amounts of protein were loaded.

While our western analysis had demonstrated that RhoGDI α was present in MCF-7 and U2OS nuclear extracts, it seemed possible that homogenization of these cells could have resulted in the redistribution of some proteins. To examine the localization of endogenously expressed RhoGDI α in its native cell environment, immunocytochemistry (ICC) was performed in MCF-7 cells using a RhoGDI α -specific antibody.

Figure 1 Western blot analysis of RhoGDI α in cultured cells. Nuclear (N, lanes 1, 3, 5, 7) and cytosolic (C, lanes 2, 4, 6, 8) extracts (10 μ g) from MCF-7, MDA-MB-231, and U2OS cells were tested for the presence of RhoGDI α and ER α using western analysis. Lamin A/C was used as a loading control.

As anticipated, RhoGDI α was found predominantly in the cytoplasm, but was also present in the nucleus (Fig. 2A). The localization of RhoGDI α was largely unaffected when MCF-7 cells were treated with ethanol vehicle or 10 nM E₂ for 20 min or 24 h. No staining was observed when primary antibody was omitted or when primary antibody was preincubated with a tenfold excess of bacterially expressed, purified RhoGDI α (data not shown).

To confirm that RhoGDI α was present in MCF-7 nuclei, cells were treated with digitonin for 6 min to permeabilize the plasma membrane but not the nuclear envelope, so that soluble proteins such as RhoGDI α could diffuse out of the cytoplasm. The boundary of the nuclear compartment was defined by an antibody that recognizes a related family of nuclear pore complex (NPC) proteins (Fig. 2B). Although no RhoGDI α was detected in the cytoplasm after digitonin treatment, it was present in the nuclei and appeared to be more concentrated in the nucleoli in the merged RhoGDI α and NPC images. The presence of RhoGDI α in the nucleoli was confirmed by its colocalization with the nucleolar protein fibrillarin in the merged images (Fig. 2C).

RhoGDI α expression in MCF-7 cells is not affected by E₂ treatment

To assess whether exposure of MCF-7 cells to E₂ affected the level of RhoGDI α , cells were treated with ethanol vehicle or 10 nM E₂ for 0.3, 2, 24, 48, or 72 h and whole

cell extracts were subjected to western analysis. No detectable changes in RhoGDI α protein levels were observed with any of the E₂ treatments examined (Fig. 3). In contrast, ER α levels were decreased after 24 h and remained low as previously reported (Petz *et al.* 2004b). GAPDH, which was used as a loading control, was unaffected by hormone treatment.

RhoGDI α enhances estrogen-mediated transactivation in U2OS cells

Since RhoGDI α was originally identified in a complex with the ERE-bound ER α (Schultz-Norton *et al.* 2007) and we had detected endogenously expressed RhoGDI α in MCF-7 nuclei, we determined whether RhoGDI α could influence ER α -mediated transactivation. Transient transfections were carried out in U2OS cells using an ER α expression vector, a luciferase reporter plasmid containing two copies of the consensus ERE, and a renilla reporter plasmid, which was used as an internal control. As increasing amounts of a RhoGDI α expression vector were included, a dose-dependent increase in E₂-mediated transactivation was observed (Fig. 4). These findings are in agreement with previous transfection experiments carried out in U2OS cells (Su *et al.* 2001).

RhoGDI α alters endogenous expression of estrogen-responsive genes in MCF-7 cells

Our transient transfection studies indicated that overexpression of RhoGDI α increased ER α -mediated transactivation of a reporter gene containing a simple promoter and two tandem EREs. In order to study the effect of endogenously expressed RhoGDI α on transcription of native estrogen-responsive genes, RhoGDI α expression was knocked down in MCF-7 cells using small interfering RNA (siRNA) directed against exon 6 of RhoGDI α . In addition siRNA directed against renilla

Figure 2 Subcellular distribution of endogenous RhoGDI α in MCF-7 cells. (A) MCF-7 cells were treated with ethanol or 10 nM E₂ for the indicated times and subjected to ICC analysis with a RhoGDI α -specific antibody. (B and C) MCF-7 cell plasma membrane was partially permeabilized with digitonin before being subjected to ICC analysis using an antibody directed against RhoGDI α , nuclear pore complex proteins (NPC), or the nucleolar protein fibrillarin. The merged images of RhoGDI α and NPC (B) and RhoGDI α and fibrillarin (C) are shown.

Figure 3 Levels of ER α and RhoGDI α after exposure of MCF-7 cells to E₂. MCF-7 cells were treated with ethanol or 10 nM E₂ for the indicated times. Whole cell extracts were analyzed by western analysis with a RhoGDI α - or ER α -specific antibody. GAPDH was included as a loading control.

Figure 4 Effect of RhoGDI α on the expression of an estrogen-responsive reporter plasmid. U2OS cells were transiently transfected with a constant amount of an ER α expression vector, a reporter plasmid containing two consensus EREs, and increasing amounts of a RhoGDI α expression vector in the absence (open bars) or in the presence (solid bars) of E₂. An asterisk indicates that estrogen responsiveness was significantly greater ($P \leq 0.05$) in the presence than in the absence of the RhoGDI α expression vector.

luciferase was used as a control. RhoGDI α mRNA levels were reduced when RhoGDI α -specific, but not control, siRNA was used regardless of hormone exposure (Fig. 5A). When control siRNA was used, the levels of PR and pS2 mRNA were increased in the presence of E₂ and the level of ER α mRNA was decreased. These findings are consistent with previous reports on the effects of E₂ on PR and pS2 gene expression in MCF-7 cells (Nardulli *et al.* 1988, Kim *et al.* 2000). When RhoGDI α expression was decreased, there was an increase in the level of PR mRNA and a decrease in the level of pS2 mRNA in the presence of E₂ when compared with the control siRNA. Interestingly, ER α mRNA levels were substantially increased in the absence of E₂ when RhoGDI α was knocked down. GAPDH, which was used as an internal control, was unaffected by control or RhoGDI α siRNA. Taken together, these data demonstrate that RhoGDI α differentially influences the expression of endogenous, estrogen-responsive genes in MCF-7 cells.

We also monitored the effect of knocking down RhoGDI α on RhoGDI α , ER α , and PR protein levels using western blot analysis. RhoGDI α levels were dramatically reduced when siRNA targeting RhoGDI α was used (Fig. 5B, compare lanes 1 and 2 with lanes 3 and 4) indicating that the RhoGDI α siRNA was effective in decreasing the levels of RhoGDI α mRNA and protein. When control siRNA was used, the level of ER α protein was decreased and the level of PR protein was increased in the presence of E₂ (lanes 1 and 2) as previously reported (Petz *et al.* 2004b). When RhoGDI α -specific siRNA was used, ER α protein levels were increased in the absence of

hormone (compare lanes 1 and 3) and PR protein levels were increased in the presence of E₂ (compare lanes 2 and 4). These findings are consistent with our RNA analysis. The level of GAPDH, which was used as an internal control, was unaffected by RhoGDI α expression.

RhoGDI α and ER α interact

To this point we had shown that RhoGDI α was present in the nuclei of MCF-7 cells and that altering RhoGDI α expression influenced estrogen-responsive gene expression. However, the mechanism by which RhoGDI α might influence ER α -mediated transactivation remained unclear. It has been hypothesized that RhoGDI α might alter estrogen responsiveness through cytoplasmic RhoGDI α signaling (Su *et al.* 2002). However, the presence of RhoGDI α and ER α in the nucleus of MCF-7 cells and the association of RhoGDI α with the DNA-bound ER α in our agarose gel shift assays suggested that RhoGDI α might interact with ER α and influence its activity. Thus, coimmunoprecipitation experiments were carried out to determine whether endogenously expressed RhoGDI α and ER α interacted in MCF-7 cells. As seen in Fig. 6A, ER α was associated with RhoGDI α in MCF-7 cells in the absence and presence of E₂ when an antibody against RhoGDI α (lanes 5 and 6), but not when a control antibody directed against fluorescein (lanes 3 and 4), was used.

To determine whether the receptor and RhoGDI α could interact directly, RhoGDI α was expressed with dual His and T7-tags and passed through a Ni-NTA column to eliminate background before being immobilized on T7-tag beads. The immobilized protein was then incubated with baculovirus-expressed, purified ER α . As seen in Fig. 6B, RhoGDI α and ER α interacted directly in the absence and in the presence of E₂ (lanes 4 and 5). Control lanes containing lysate from bacteria, which had been transformed with the parent plasmid instead of RhoGDI α plasmid, failed to interact with ER α (lanes 2 and 3).

RhoGDI α enhances the ER α -ERE interaction

Since ER α interacted directly with RhoGDI α , it seemed possible that this interaction might affect the ability of the receptor to bind to its cognate binding site, the ERE. Therefore, gel mobility shift assays were performed using constant amounts of purified ER α and ³²P-labeled, ERE-containing oligos. As increasing amounts of the purified His-tagged RhoGDI α were added, a slight decrease in the intensity of the band corresponding to ER α -ERE binary complex (C1) and an increase in the intensity of another, lower mobility

Figure 5 Effect of RhoGDI α on the expression of endogenous, estrogen-responsive genes. MCF-7 cells were transfected with control or RhoGDI α -specific siRNA for 24 h and then treated with ethanol (open bars) or 10 nM E₂ (solid bars) for 24 h. (A) RNA was harvested, cDNA was synthesized, and quantitative RT-PCR analysis was performed using primers specific to RhoGDI α , PR, pS2, ER α , and GAPDH transcripts. Data are reported as the mean of triplicates $\pm$ S.E.M. and is representative of four independent experiments. Some error bars are too small to be visible. An asterisk indicates that the mRNA level detected in the presence of RhoGDI α siRNA was significantly different from the corresponding ethanol or E₂-treated sample in the presence of control siRNA as determined by ANOVA ($P \leq 0.05$). (B) Whole cell extracts were analyzed by western analysis using antibodies specific to RhoGDI α , ER α , PR, and GAPDH.

Figure 6 Interaction of RhoGDI α with ER α . (A) Whole cell extracts were prepared from MCF-7 cells that had been treated with ethanol or E₂ for 24 h and subjected to immunoprecipitation using antibody directed against fluorescein (control, lanes 3 and 4) or RhoGDI α (lanes 5 and 6). ER α was detected in western blot analysis with an ER α -specific antibody. (B) Purified ER α was incubated with Ni-NTA purified bacterial lysate (lanes 2 and 3) or with Ni-NTA purified His-tagged RhoGDI α (lanes 4 and 5) that had been immobilized on T7-tag beads. Proteins were separated on a denaturing gel and detected with an ER α -specific antibody. Five percent input were included for reference. Results are representative of at least three independent experiments.

band (C2) was observed (Fig. 7A, lanes 2–5). As anticipated, the ER α -specific antibody supershifted both C1 and C2 (lane 6) demonstrating that ER α was present in both complexes. Curiously, the RhoGDI α antibody not only supershifted C2, which presumably contained the trimeric RhoGDI α –ER α –ERE complex (lane 7), but also caused the disappearance of C1, which contained the ER α –ERE complex. These findings suggest that the RhoGDI α -specific antibody fosters the formation of the trimeric RhoGDI α –ER α –ERE complex and the depletion of the ER α –ERE complex. The ability of an antibody to stabilize the ER α –ERE complex has been reported previously and was attributed to the ability of the antibody to enhance receptor dimerization (Fawell *et al.* 1990). It seems possible that the RhoGDI α antibody could likewise stabilize the association of RhoGDI α and ER α with the ERE-containing DNA. When RhoGDI α was incubated with ERE-containing oligos in the absence of ER α , no complex was observed (lane 8) demonstrating that the formation of the trimeric complex requires the receptor. The ability of the RhoGDI α antibody to supershift the trimeric complex required the presence of RhoGDI α protein as demonstrated by the inability of this antibody to supershift the ER α –ERE complex (Fig. 7B, lane 4).

Discussion

We identified RhoGDI α in a large multiprotein complex associated with the DNA-bound ER α and characterized the ability of this protein to function as a regulator of ER α activity. We have shown that RhoGDI α not only increases ER α transcriptional activity in transient transfection assays but also differentially influences the expression of endogenous estrogen-responsive genes in MCF-7 cells. Our studies suggest that RhoGDI α collaborates with other regulatory proteins to modify the expression of ER α target genes.

We were initially surprised to find RhoGDI α among the proteins isolated in the protein–ER α –DNA complex, since it has typically been referred to as a cytoplasmic protein (Fukamoto *et al.* 1990) and we had utilized HeLa nuclear extracts to form our ER α -containing multiprotein complexes. However, western blot analysis and ICC assays confirmed the presence of RhoGDI α in the nuclei of MCF-7 cells. A careful examination of RhoGDI α amino acid sequence failed to identify any nuclear localization signal, suggesting that another protein may assist in the shuttling of RhoGDI α between the cytoplasm and the nucleus. It seems plausible that cdc42 isoform1, a RhoGTPase family member with a polybasic region in its C-terminal end that can function as a nuclear localization signal (Lanning *et al.* 2003, Williams 2003), might perform this function since we identified both isoforms 1 and 2 of cdc42 in our agarose gel purification experiments as ER α -associated proteins (Schultz-Norton *et al.* 2007). Alternatively, RhoGDI α could be accompanied by a protein such as 14-3-3, which binds and helps to redistribute an array of signaling proteins (Fu *et al.* 2000, Kino *et al.* 2003, Diviani *et al.* 2004). It should also be noted that the 28 kDa RhoGDI α is theoretically small enough to traverse the nuclear pores unaccompanied.

The concentration of RhoGDI α in the nucleolus was unexpected since this nuclear compartment has typically been viewed as the site of rRNA synthesis and ribosome assembly. However, there is accumulating evidence to suggest that nucleolar proteins play a dynamic role in regulating a number of cellular processes including cell cycle progression, cell proliferation, and regulation of gene expression (Weber *et al.* 1999, Cerutti & Simanis 2000). It has been suggested that localization of cdc14 in the nucleolus may help to ensure that it is sequestered from its cytoplasmic and nuclear substrates (Bachant & Elledge 1999). Likewise, the localization of RhoGDI α in the nucleolus may help to simultaneously sequester RhoGDI α from cytoplasmic GTPases and from ER α and at the same time serve as a storage depot to maintain a pool of nuclear RhoGDI α protein that could interact with ER α and influence gene expression.

Figure 7 Effect of RhoGDI α on ER α -ERE complex formation. (A) Radiolabeled ERE-containing oligos were combined with a constant amount of baculovirus-expressed, purified ER α (50 fmoles, lanes 2–7) in the absence (lane 2) or in the presence of increasing amounts of RhoGDI α (lane 3, 250 ng; lane 4, 500 ng; lanes 5, 6, and 7, 1 μ g). Antibody (Ab) against ER α (lane 6) or RhoGDI α (lane 7) was added to the binding reaction as indicated. Lane 8 contains the radiolabeled oligo in the presence of RhoGDI α (1 μ g) only. B. Radiolabeled ERE-containing oligos were combined with a constant amount of baculovirus-expressed and purified ER α (50 fmoles, lanes 2–4) in the absence of RhoGDI α . Antibody directed against ER α (lane 3) or RhoGDI α (lane 4) was added to the binding reaction as indicated. Lane 1 contains only radiolabeled, ERE-containing oligos.

The ability of RhoGDI α to alter ER α activity was previously reported by Garabedian and coworkers (Su *et al.* 2001), who showed that RhoGDI α enhances ER α , ER β , glucocorticoid receptor and androgen receptor (AR), but not SRF or Sp1, mediated transactivation in transient transfection assays. A subsequent study by this group suggested that RhoGDI α enhances ER α -mediated transactivation indirectly through its effects on ER α -associated coregulatory proteins (Su *et al.* 2002). In contrast to these studies which proposed that the cytoplasmic RhoGDI α is responsible for altering estrogen-responsive gene expression, our studies provide evidence that nuclear RhoGDI α , through its interaction with ER α , also plays a direct role in regulating ER α -mediated transactivation. The presence of RhoGDI α in MCF-7 nuclear extracts, the localization of endogenously expressed RhoGDI α in the nucleus of MCF-7 cells, the coimmunoprecipitation of endogenously expressed RhoGDI α and ER α , and the direct interaction of purified ER α and RhoGDI α all support the idea that, in addition to its cytoplasmic activity, nuclear RhoGDI α influences ER α transactivation directly by interacting with the receptor.

As indicated in our siRNA assays (Fig. 5), RhoGDI α differentially modulates the expression of the *PR* and

pS2 genes, which have very different cis elements and trans-acting factors involved in conferring their hormone responsiveness. While the *pS2* gene contains an imperfect ERE that interacts directly with ER α (Nunez *et al.* 1989), the *PR* gene contains multiple AP-1 and Sp1 sites through which AP-1 and Sp1 proteins interact with ER α (Jeltsch *et al.* 1987, Petz & Nardulli 2000, Petz *et al.* 2002, 2004a,b, Schultz *et al.* 2003). Thus, we believe that the ability of RhoGDI α to differentially alter estrogen-responsive gene expression may be due to differences in the population of cis elements and the trans-acting factors associated with various target genes.

As might be expected from the effects of RhoGDI α on estrogen-responsive gene expression, this protein is required for fertility and reproductive competence in mice. RhoGDI α –/– males have spermatogenesis defects and are infertile and RhoGDI α –/– females have implantation defects (Togawa *et al.* 1999). Thus, RhoGDI α not only influences estrogen-responsive gene expression in MCF-7 breast cancer cells, but also has profound effects on the reproductive tract and reproduction.

Interestingly, there is evidence for the involvement of other GTPase regulatory proteins in

influencing nuclear receptor activity. For example, the GEF protein Brx interacts directly with and enhances the transcriptional activity of ER α (Rubino *et al.* 1998) and the glucocorticoid receptor (Kino *et al.* 2006). Vav3, another GEF protein, increases AR-mediated transactivation, but does not appear to interact with the receptor (Lyons & Burnstein 2006). It is thought that the ability of Vav3 to enhance AR responsiveness may contribute to the relapse of prostate cancer in patients undergoing androgen deprivation therapy (Lyons & Burnstein 2006). Interestingly, RhoGDI α , which opposes the actions of GEFs on RhoGTPases, increases the resistance of cancer cells to chemotherapeutic agents (Zhang *et al.* 2005).

The ability of RhoGDI α to act as a nuclear receptor coregulatory protein challenges the classical paradigm in which this protein is solely involved in the propagation of signals initiated at the plasma membrane. Our studies suggest that the nuclear actions of RhoGDI α supplement its effects in the cytoplasm and that RhoGDI α functions in both membrane and nuclear signaling pathways.

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