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

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Abstract

Estrogen receptor α (ERα) is a ligand-activated transcription factor that regulates expression of estrogen-responsive genes. Upon binding of the ligand-occupied ERα 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 ERα. One of these proteins, Rho guanosine diphosphate (GDP) dissociation inhibitor α (RhoGDIα), is a negative regulator of the Rho family of GTP-binding proteins. In this study, we demonstrate that endogenously expressed RhoGDIα is present in the nucleus as well as the cytoplasm of MCF-7 breast cancer cells, and that RhoGDIα binds directly to ERα, alters the ERα–ERE interaction, and influences the ability of ERα 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 RhoGDIα in the cytoplasm, it also influences ERα 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

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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 (ERα; Schultz-Norton et al. 2007). One of these ERα-associated proteins was the 28 kDa protein Rho GDP dissociation inhibitor α (RhoGDIα), 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 RhoGDIα 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 RhoGDIα (Su et al. 2001, 2002). Because we found RhoGDIα associated with the DNA-bound ERα, we investigated the localization of endogenously expressed RhoGDIα in MCF-7 breast cancer cells and characterized the ability of this protein to interact with ERα and influence the expression of estrogen-responsive genes.

Materials and Methods

Isolation of RhoGDIα

RhoGDIα was isolated with the ERE-bound ERα using HeLa nuclear extracts, purified ERα, ERE-containing oligos, and agarose gel fractionation (Schultz-Norton et al. 2007). Five unique peptides (SIQEQLDKDESRL, VAVSADPNVPNVTGILTVCSSAPGPLEDLTLGDLESFKK, IDKTDYMVGYGPR, FTDDDKTDLHLSWEWNTIK, and AEEYFLTPVEAPK), which exclusively map to RhoGDIα, 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 RhoGDIα, ERα, 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 RhoGDIα protein for gel shift assays

A BamH1/EcoRI fragment of human RhoGDIα from pGST-GDI1, kindly provided by M Garabedian (New York University, School of Medicine, New York), was subcloned into a dual-tagged (His and T7) pET-28α (+) vector (Novagen, La Jolla, CA, USA) for the expression of RhoGDIα 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–RhoGDIα 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 RhoGDIα 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 ERα was incubated with immobilized RhoGDIα at 4 °C for 1 h without or with 10 nM 17β-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 RhoGDIα were purified in parallel. Western analysis was performed using a monoclonal antibody against ERα (sc-8002, Santa Cruz Biotechnologies). Similar results were obtained in three independent experiments.
**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 $4 \times 10^5$ 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 RhoGDIz-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 E$_2$. Cells were then lysed in lysis buffer and western blot analysis was performed using antibodies to RhoGDIz, ERz, 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'-GGG TCT CTC CTC CTG-3' and 5'-CTG GCC TTC TGG AGG GTC-3'), RhoGDIz (5'-ACC CAG CCA GGA ACA AAC-3' and 5'-GCA GAC ACA ACA CGA AGA C-3'), ERz (5'-TTC CCT ACT ACC TGG AGA AC-3' and 5'-CAG ACA TAC TTC CCT TGT C-3'), PR (5'-GTG CCT ATC CTT CCT CTT CTA AAT C-3' and 5'-CCC GCC GTT GAC TCC GAT G-3') were utilized. Standard curves were derived using serial dilutions of cDNA equivalent to 0-25, 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. $^{32}$P-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/µl poly dI/dC, 4 mM dithiothreitol, and 50 nM E$_2$) with a constant amount (50 fmoles) of baculovirus expressed, purified ERz, and increasing amounts of purified...
RhoGDIα. BSA and Ni-NTA elution buffer were added as needed to maintain constant protein and salt concentrations. For antibody supershift experiments, ERα- and RhoGDIα-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% non-denaturing 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 RhoGDIα (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-mouse secondary antibody, alone or combined with a mouse anti-rabbit secondary antibody, then washed thrice in 1X PBS with 0.1% Tween-20 and mounted with Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA, USA). Digital images were captured with 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

RhoGDIα 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 RhoGDIα from HeLa nuclear extracts as a component of a large multiprotein complex associated with the DNA-bound ERα (Schultz-Norton et al. 2007).

RhoGDIα is present in the cytoplasm and the nucleus

We undertook a series of experiments to monitor the localization of endogenously expressed RhoGDIα in cultured cell lines that have been used to study estrogen-responsive gene expression. As expected, RhoGDIα was detected in the cytosolic fractions (C) of ERα-positive MCF-7 breast cancer cells in the absence and presence of 10 nM E2, ERα-negative MDA-MB-231 breast cancer cells, and U2 osteosarcoma (U2OS) cells using western analysis (Fig. 1, lanes 2, 4, 6, and 8). RhoGDIα was also observed in the nuclear fractions (N) of MCF-7 and U2OS cells (Fig. 1, lanes 1, 3, and 7). Although RhoGDIα was not visible in MDA-MB-231 nuclear extracts in the data shown, we were able to detect RhoGDIα in MDA-MB-231 nuclear extracts using higher antibody concentrations (data not shown). As expected, ERα 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 RhoGDIα 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 RhoGDIα in its native cell environment, immunocytochemistry (ICC) was performed in MCF-7 cells using a RhoGDIα-specific antibody.

Figure 1 Western blot analysis of RhoGDIα in cultured cells. Nuclear (N, lanes 1, 3, 5, 7) and cytosolic (C, lanes 2, 4, 6, 8) extracts (10 μg) from MCF-7, MDA-MB-231, and U2OS cells were tested for the presence of RhoGDIα and ERα using western analysis. Lamin A/C was used as a loading control.
As anticipated, RhoGDI<sub>α</sub> was found predominantly in the cytoplasm, but was also present in the nucleus (Fig. 2A). The localization of RhoGDI<sub>α</sub> was largely unaffected when MCF-7 cells were treated with ethanol vehicle or 10 nM E<sub>2</sub> 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<sub>α</sub> (data not shown).

To confirm that RhoGDI<sub>α</sub> 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<sub>α</sub> 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<sub>α</sub> 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<sub>α</sub> and NPC images. The presence of RhoGDI<sub>α</sub> in the nucleoli was confirmed by its colocalization with the nucleolar protein fibrillarin in the merged images (Fig. 2C).

**RhoGDI<sub>α</sub> expression in MCF-7 cells is not affected by E<sub>2</sub> treatment**

To assess whether exposure of MCF-7 cells to E<sub>2</sub> affected the level of RhoGDI<sub>α</sub>, cells were treated with ethanol vehicle or 10 nM E<sub>2</sub> for 0·3, 2, 24, 48, or 72 h and whole cell extracts were subjected to western analysis. No detectable changes in RhoGDI<sub>α</sub> protein levels were observed with any of the E<sub>2</sub> treatments examined (Fig. 3). In contrast, ER<sub>α</sub> 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<sub>α</sub> enhances estrogen-mediated transactivation in U2OS cells**

Since RhoGDI<sub>α</sub> was originally identified in a complex with the ERE-bound ER<sub>α</sub> (Schultz-Norton et al. 2007) and we had detected endogenously expressed RhoGDI<sub>α</sub> in MCF-7 nuclei, we determined whether RhoGDI<sub>α</sub> could influence ER<sub>α</sub>-mediated transactivation. Transient transfections were carried out in U2OS cells using an ER<sub>α</sub> 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<sub>α</sub> expression vector were included, a dose-dependent increase in E<sub>2</sub>-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<sub>α</sub> alters endogenous expression of estrogen-responsive genes in MCF-7 cells**

Our transient transfection studies indicated that overexpression of RhoGDI<sub>α</sub> increased ER<sub>α</sub>-mediated transactivation of a reporter gene containing a simple promoter and two tandem EREs. In order to study the effect of endogenously expressed RhoGDI<sub>α</sub> on transcription of native estrogen-responsive genes, RhoGDI<sub>α</sub> expression was knocked down in MCF-7 cells using small interfering RNA (siRNA) directed against exon 6 of RhoGDI<sub>α</sub>. In addition siRNA directed against renilla
luciferase was used as a control. RhoGDIα mRNA levels were reduced when RhoGDIα-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 ERα 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 RhoGDIα 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, ERα mRNA levels were substantially increased in the absence of E2 when RhoGDIα was knocked down. GAPDH, which was used as an internal control, was unaffected by control or RhoGDIα siRNA. Taken together, these data demonstrate that RhoGDIα differentially influences the expression of endogenous, estrogen-responsive genes in MCF-7 cells.

We also monitored the effect of knocking down RhoGDIα on RhoGDIα, ERα, and PR protein levels using western blot analysis. RhoGDIα levels were dramatically reduced when siRNA targeting RhoGDIα was used (Fig. 5B, compare lanes 1 and 2 with lanes 3 and 4) indicating that the RhoGDIα siRNA was effective in decreasing the levels of RhoGDIα mRNA and protein. When control siRNA was used, the level of ERα 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 RhoGDIα-specific siRNA was used, ERα 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 RhoGDIα expression.

**RhoGDIα and ERα interact**

To this point we had shown that RhoGDIα was present in the nuclei of MCF-7 cells and that altering RhoGDIα expression influenced estrogen-responsive gene expression. However, the mechanism by which RhoGDIα might influence ERα-mediated transactivation remained unclear. It has been hypothesized that RhoGDIα might alter estrogen responsiveness through cytoplasmic RhoGDIα signaling (Su et al. 2002). However, the presence of RhoGDIα and ERα in the nucleus of MCF-7 cells and the association of RhoGDIα with the DNA-bound ERα in our agarose gel shift assays suggested that RhoGDIα might interact with ERα and influence its activity. Thus, coimmunoprecipitation experiments were carried out to determine whether endogenously expressed RhoGDIα and ERα interacted in MCF-7 cells. As seen in Fig. 6A, ERα was associated with RhoGDIα in MCF-7 cells in the absence and presence of E2 when an antibody against RhoGDIα (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 RhoGDIα could interact directly, RhoGDIα 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 ERα. As seen in Fig. 6B, RhoGDIα and ERα 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 RhoGDIα plasmid, failed to interact with ERα (lanes 2 and 3).

**RhoGDIα enhances the ERα–ERE interaction**

Since ERα interacted directly with RhoGDIα, 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 ERα and 32P-labeled, ERE-containing oligos. As increasing amounts of the purified His-tagged RhoGDIα were added, a slight decrease in the intensity of the band corresponding to ERα–ERE binary complex (C1) and an increase in the intensity of another, lower mobility
Figure 5 Effect of RhoGDIα on the expression of endogenous, estrogen-responsive genes. MCF-7 cells were transfected with control or RhoGDIα-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 RhoGDIα, PR, pS2, ERα, and GAPDH transcripts. Data are reported as the mean of triplicates ± 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 RhoGDIα siRNA was significantly different from the corresponding ethanol or E2-treated sample in the presence of control siRNA as determined by ANOVA (P ≤ 0.05). (B) Whole cell extracts were analyzed by western analysis using antibodies specific to RhoGDIα, ERα, PR, and GAPDH.
Figure 6 Interaction of RhoGDIα with ERα. (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 fluoresein (control, lanes 3 and 4) or RhoGDIα (lanes 5 and 6). ERα was detected in western blot analysis with an ERα-specific antibody. (B) Purified ERα was incubated with Ni-NTA purified bacterial lysate (lanes 2 and 3) or with Ni-NTA purified His-tagged RhoGDIα (lanes 4 and 5) that had been immobilized on T7-tag beads. Proteins were separated on a denaturing gel and detected with an ERα-specific antibody. Five percent input were included for reference. Results are representative of at least three independent experiments.

Discussion

We identified RhoGDIα in a large multiprotein complex associated with the DNA-bound ERα and characterized the ability of this protein to function as a regulator of ERα activity. We have shown that RhoGDIα not only increases ERα 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 RhoGDIα collaborates with other regulatory proteins to modify the expression of ERα target genes.

We were initially surprised to find RhoGDIα among the proteins isolated in the protein–ERα–DNA complex, since it has typically been referred to as a cytoplasmic protein (Fukumoto et al. 1990) and we had utilized HeLa nuclear extracts to form our ERα-containing multiprotein complexes. However, western blot analysis and ICC assays confirmed the presence of RhoGDIα in the nuclei of MCF-7 cells. A careful examination of RhoGDIα amino acid sequence failed to identify any nuclear localization signal, suggesting that another protein may assist in the shuttling of RhoGDIα between the cytoplasm and the nucleus. It seems plausible that cdc42 isozyme1, 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 ERα-associated proteins (Schultz-Norton et al. 2007). Alternatively, RhoGDIα 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 RhoGDIα is theoretically small enough to traverse the nuclear pores unaccompanied.

The concentration of RhoGDIα 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 RhoGDIα in the nucleolus may help to simultaneously sequester RhoGDIα from cytoplasmic GTPases and from ERα and at the same time serve as a storage depot to maintain a pool of nuclear RhoGDIα protein that could interact with ERα and influence gene expression.
The ability of RhoGDIα to alter ERα activity was previously reported by Garabedian and coworkers (Su et al. 2001), who showed that RhoGDIα enhances ERα, ERβ, 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 RhoGDIα enhances ERα-mediated transactivation indirectly through its effects on ERα-associated coregulatory proteins (Su et al. 2002). In contrast to these studies which proposed that the cytoplasmic RhoGDIα is responsible for altering estrogen-responsive gene expression, our studies provide evidence that nuclear RhoGDIα, through its interaction with ERα, also plays a direct role in regulating ERα-mediated transactivation. The presence of RhoGDIα in MCF-7 nuclear extracts, the localization of endogenously expressed RhoGDIα in the nucleus of MCF-7 cells, the coimmunoprecipitation of endogenously expressed RhoGDIα and ERα, and the direct interaction of purified ERα and RhoGDIα all support the idea that, in addition to its cytoplasmic activity, nuclear RhoGDIα influences ERα transactivation directly by interacting with the receptor.

As indicated in our siRNA assays (Fig. 5), RhoGDIα 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 ERα (Nunez et al. 1989), the PR gene contains multiple AP-1 and Sp1 sites through which AP-1 and Sp1 proteins interact with ERα (Jeltsch et al. 1987, Petz & Nardulli 2000, Petz et al. 2002, 2004a,b, Schultz et al. 2003). Thus, we believe that the ability of RhoGDIα 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 RhoGDIα on estrogen-responsive gene expression, this protein is required for fertility and reproductive competence in mice. RhoGDIα−/− males have spermatogenesis defects and are infertile and RhoGDIα−/− females have implantation defects (Togawa et al. 1999). Thus, RhoGDIα 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 ERz (Rubino et al. 1998) and the glucocorticoid receptor (Kino et al. 2006). Vav3, another GEF protein, increases AR-mediated transcriptional activation, 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, RhoGDIz, which opposes the actions of GEFs on RhoGTPases, increases the resistance of cancer cells to chemotherapeutic agents (Zhang et al. 2005).

The ability of RhoGDIz 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 RhoGDIz supplement its effects in the cytoplasm and that RhoGDIz functions in both membrane and nuclear signaling pathways.

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