Disulfide isomerase glucose-regulated protein 58 is required for the nuclear localization and degradation of retinoic acid receptor α

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Abstract

Retinoic acid receptor α (RARA) is critical for spermatogenesis, as shown by a sterility phenotype observed in Rara knockout mice. RARA is important in both Sertoli and germ cells of the testis. Here, we demonstrate that a disulfide isomerase glucose-regulated protein 58 (GRp58) participates in the nuclear import and degradation of RARA in Sertoli cells. GRp58 interacted with RARA in the presence of all-trans retinoic acid (ATRA) ligand and, as a complex, it was translocated from the cytoplasm to the nucleus and, then with time, GRp58 dissociated from RARA and was found in the cytoplasm. The GRp58 RNAi treatment disrupted ATRA-dependent RARA nuclear localization, indicating the requirement of GRp58 for RARA nuclear localization. Moreover, treatment with sulfhydryl-modifying agents that oxidize SH-groups of cysteine residues to disulfide bonds abolished ATRA-mediated RARA nuclear localization, suggesting that the thiol oxidoreductase activity of GRp58 may be required for RARA nuclear import. Additionally, the proteasome inhibitor treatment resulted in the co-localization of GRp58 and RARA at the endoplasmic reticulum (ER), suggesting that GRp58 may bring RARA to the ER for the ER-associated degradation (ERAD) of RARA before it is de-coupled from RARA for recycling. In this regard, proteasome inhibitor treatment also increased the interaction of RARA with UBE2J2, an ERAD-associated ubiquitin E2 enzyme. Collectively, the results indicate that GRp58 may act as a molecular chaperone that alters the protein conformation of RARA for its delivery to the nucleus and, then with time, accompanies RARA to the ER for RARA ubiquitination and proteasome-mediated ERAD.


Introduction

Retinoic acid is a potent trigger of many physiological processes that include growth, differentiation of somatic and germ cells, and meiosis in germ cells. Its action is mediated by the ligand-dependent transcriptional factors, retinoid receptors, which belong to the super-family of nuclear steroid/thyroid hormone receptors. The ligand binding induces a conformational change in the receptors that favors co-activator tethering to the receptors, switching on the transcription of genes containing a retinoic acid response element (RARE; Chambon 1996). This assembled protein complex at the promoter induces chromatin remodeling and increases the affinity of RNA polymerase II to these promoters, thereby stabilizing the transcriptional initiation complex and the transcriptional activation of target genes.

Genetic studies have shown that one of the retinoid receptors, retinoic acid receptor α (RARA; the gene and protein nomenclatures approved by the International Committee on Standardized Genetic Nomenclature for mice (http://www.informatics.jax.org/mgihome/nomen/gene.shtml) were used.) is critical for spermatogenesis, as shown by a sterility phenotype observed in Rara knockout mice (Lufkin et al. 1993). Further characterization has revealed that RARA plays an important role in the synchronization of the spermatogenic cycle (Chung et al. 2004, 2005). In Rara knockout mice, a normal proportion of the spermatogenic stages of the seminiferous epithelial cycle could not be maintained, instead, a major disorganization of germ cell associations was observed (Chung et al. 2004, 2005, Doyle et al. 2007). Moreover, it has been reported that RARA might function in the somatic nurse cells, Sertoli cells, to promote the survival and development of early meiotic prophase spermatocytes, whereas in germ cells, RARA might function to enhance the proliferation and differentiation of spermatogonia before meiotic prophase (Doyle et al. 2007). Furthermore, the ablation of Rara specifically in Sertoli cells indicates that RARA controls paracrine pathways required for spermatogonia differentiation and germ cell survival (Vernet et al. 2006). Thus, unraveling...
the regulatory processes by which the RARA transcriptional activity changes in the germ cells and Sertoli cells is an important task. To better understand the molecular elements regulating the RARA transcriptional activity in Sertoli cells, we performed a yeast two-hybrid screen using RARA as the bait and the protein products of a primary Sertoli cell cDNA expression library as the prey. One of the proteins that strongly interacted with RARA was glucose-regulated protein 58 (GRp58).

GRp58, also known as ERp57 and PDIA3, is a thiol oxidoreductase and an isomerase of protein disulfide isomerase (PDI). It is composed of an endoplasmic reticulum (ER) recognition signal sequence at the N-terminal end, two thioredoxin-like domains and a nuclear localization signal in the middle, and an ER retention motif QDEL at the C-terminal end (Khanal & Nemere 2007a). The redox activity of the thioredoxin-like domains is provided by two cysteine residues present in a characteristic CXXC sequence (Khanal & Nemere 2007a). GRp58 is considered as an ER chaperone protein, and the majority of the protein is in the lumen of the ER. However, different from other ER chaperone proteins, it is also present in non-ER compartments, one-third of GRp58 is in the cytoplasm (Lewis et al. 1986) and some is in the nuclear matrix (Altieri et al. 1993). GRp58 has an extensive role in protein maturation, post-translational modification, and protein folding processes, defects of which have been associated with many diseases such as Alzheimer’s, prion neurotoxicity, cancer, and hepatotoxicity (Khanal & Nemere 2007b, Ni & Lee 2007). In vivo, homozygous gene knockout of GRp58 resulted in embryonic lethality, indicating its requirement during mammalian development (Ni & Lee 2007). Catalytically, GRp58 isomerizes both native and non-native disulfide bonds, facilitating the eventual acquisition of the native conformation of a protein (Jessop et al. 2004).

In the ER, GRp58 functions as a chaperone protein, a subunit of complexes formed with the other ER-resident proteins, calreticulin and calnexin that require Ca\(^{2+}\) for their activities (Oliver et al. 1999). Calreticulin and calnexin are molecular lectins that recognize a glycoprotein with a single terminal glucose on the N-linked oligosaccharide as an incompletely folded glycoprotein, and subject it to a quality-control protein folding cycle. If the glycoprotein has attained its native conformation, it no longer has a single glucose on the N-linked oligosaccharide, it is not recognized by calnexin or calreticulin, and thus it exits the ER to the Golgi (Vembar & Brodsky 2008). If not, the substrate is either subjected to additional protein folding cycles or selected for degradation by ER-associated degradation (ERAD) machinery. ERAD involves ubiquitination by ER-bound ubiquitin-conjugating enzymes such as the UBC6E E2 enzyme and E3 RMA1 ubiquitin ligase, and degradation by proteasomes (Younger et al. 2006, Vembar & Brodsky 2008).

In addition, GRp58 is also known as the 1,25D\(_3\)-MARRS receptor, a membrane-associated rapid response receptor that binds the vitamin D active metabolite, 1,25(OH)\(_2\)D\(_3\). It is able to translocate to the nucleus of intestinal cells, upon 1,25(OH)\(_2\)D\(_3\) binding, where it may interact with transcription factors and/or DNA and activate protein kinase C activity, which is important for the regulation of calcium channels in the kidney or intestinal cells (Khanal & Nemere 2007b). This effect does not seem to involve the classical vitamin D receptor (Khanal & Nemere 2007b).

Moreover, GRp58 has been demonstrated to be important in sperm function. It is found to undergo post-translational modification during sperm capacitation (Zhang et al. 2007). Blocking GRp58 with antibodies significantly inhibited human sperm from penetrating zona-free hamster oocytes in a dose-dependent manner. Different inhibitors of PDI activity were able to inhibit sperm–egg fusion in vitro (Ellerman et al. 2006). However, little is known regarding the protein substrates of GRp58 or the mechanism by which it can regulate these substrates in the testis. Here, we evaluated the role of GRp58 in regulating the transport of RARA, and demonstrated that GRp58 may act as a novel molecular chaperone, and that its function is important for RARA nuclear import and subsequent proteasome-mediated degradation of RARA at the ER.

**Results**

**GRp58 interacted with RARA in yeast two-hybrid screening**

To investigate RARA-interacting proteins, a yeast two-hybrid screen was conducted. Full-length RARA was cloned into a GAL4 DNA-binding domain yeast expression vector (pGAL4-DBD RARA), and it was used as the bait to screen a rat Sertoli cell cDNA library. The cDNA library from 20-day-old rat Sertoli cells was constructed and introduced as a translational fusion with the GAL4 DNA-binding domain yeast expression vector (pGAL4-DBD). RARA-interacting clones were identified by their ability to activate reporter constructs with the UAS\(_{\text{GAL}4}\) when cotransformed with GAL(DBD)–RARA (Table 1). Of particular interest, we isolated a 1.7 kb cDNA clone that was sequenced and compared to the GenBank database using the BLAST search program. This clone contained an open reading frame of 1515 bp (505 amino acids), and showed 100% identity to rat GRp58 (NM_017319; Fig. 1A). GRp58 is a 58 kDa protein, and it has a N-terminal ER recognition signal, two thioredoxin active sites, and a nuclear localization signal next to an ER retention signal QEDL sequence at the C-terminal (Fig. 1A).
RARA interacted with GRp58

To confirm the interaction between RARA and GRp58 in mammalian cells, COS-7 cells, which have a low detectable endogenous RARA, were cotransfected with either pHis empty vector or pHis-hRARA and pFLAG-GRp58 cDNA expression constructs. Proteins interacting with histidine (HIS)-RARA were captured using nickel–nitrilotriacetic acid (Ni–NTA) magnetic agarose beads, and were identified by immunoblotting with anti-FLAG M2 antibody. GRp58 co-purified with HIS-RARA in the extracts from the cells transfected with pHis-RARA, but not in the extracts from the cells bearing the empty vector, indicating that GRp58 was associated with RARA in COS-7 cells (Fig. 1B). Furthermore, in reverse, proteins interacting with FLAG-GRp58 were captured using anti-FLAG M2 affinity gel, and were identified by immunoblotting with anti-HIS antibody. RARA was co-purified with FLAG-GRp58 from the extracts of the cells transfected with pFLAG-GRp58 cDNA expression construct, but not from the extracts of the cells bearing the empty vector (Fig. 1C), suggesting again that RARA interacted with GRp58 in COS-7 cells.

GRp58 mRNA and protein were highly present in rat testes

To determine the location of GRp58 mRNA expression in male rats, RNA extracted from a panel of different tissues, germ cells, and Sertoli cells was used in semi-quantitative RT-PCR. Of all rat tissues examined, GRp58 mRNA was highly expressed in prostate and testis, and weakly present in small intestine and heart (Fig. 2A). Additionally, we found that GRp58 mRNA (Fig. 2B) and protein (Fig. 2C) were highly expressed in both Sertoli cells and germ cells in rat testes.

Immunohistochemical analysis of GRp58 expression in developing and adult rat testes

To characterize the cellular localization of GRp58 in testes, immunohistochemical analysis was performed on testicular sections collected from adult rats or rats at the ages of 5, 10, 15, 20, 25, 30, 35, and 60 days during the first wave of spermatogenesis. GRp58 was present throughout the development in rat testes (Fig. 2D–K). GRp58 was expressed in Sertoli cells throughout the development (Fig. 2D–K). The staining was mainly localized on the membrane and/or in the cytoplasm, and it seemed to be higher in Sertoli cells from younger animals. Similarly, the subcellular localization of GRp58 was primarily on the membrane and/or the cytoplasm early in development in germ cells. However, starting P20, GRp58 was observed in the nucleus of the early pre-meiotic germ cells, the same cells that express a high level of RARA as published previously (Dufour & Kim 1999), pachytene spermatocytes, and the elongated spermatids, as well as in the acrosomal structure of round spermatids (Fig. 2G–K). GRp58 immunostaining was also observed in interstitial Leydig cells.

RARA co-localized with GRp58 in the presence or absence of ATRA

To determine whether RARA co-localizes with GRp58 in primary Sertoli cells, time-dependent immunofluorescence studies were conducted using anti-RARA and anti-GRp58 antibodies. As controls, western blot analyses were conducted to validate the specificity of anti-RARA antibody (Fig. 3T). Anti-RARA antibody was able to recognize endogenous RARA protein in mouse Sertoli cells (MSC-1) and primary rat Sertoli cells (1SC), but not in COS-7 cells, which are known to express low or no RARA. However, when COS-7 cells or MSC-1 were

Table 1 Potential retinoic acid receptor α-interacting proteins.

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aHighest β-gal activity. bMedium β-gal activity. cLowest β-gal activity.
transfected with pFLAG-hRARA, the western blot analysis showed that a same-sized band, representing the expressed RARA, was detected by both anti-RARA and anti-FLAG antibodies (Fig. 3T). Additionally, this anti-RARA antibody has been used to show the developmental pattern of RARA (Dufour & Kim 1999), and it was used to distinguish the wild-type germ cells, expressing endogenous RARA, from germ cells from the Rara knockout mice previously (Doyle et al. 2007).

Using antibodies to RARA and GRp58, RARA and GRp58 were found to co-localize at the periphery of the nucleus and in the cytoplasm of Sertoli cells (Fig. 3A–C). This is consistent with previous results that demonstrated that RARA is primarily located in the cytoplasm of Sertoli cells in the absence of ligand (Braun et al. 2000), and that GRp58 is partially in the ER near the nucleus and partially in the cytoplasm (Lewis et al. 1986). To determine whether GRp58 co-localizes with RARA after ATRA treatment, primary Sertoli cells were treated with ATRA for 2, 4, or 8 h, fixed, and immunostained with anti-RARA and anti-GRp58 antibodies. ATRA treatment changed the shape of Sertoli cells to a more spherical shape, with occasional long extensions of the cytoplasm. After 2 h of ATRA treatment, RARA was primarily in the nucleus (Fig. 3D) as reported previously (Braun et al. 2000). As indicated by the overlap (Fig. 3F), an appreciable amount of GRp58 was in the nucleus, co-localized with RARA, although some of GRp58 was also in the cytoplasm (Fig. 3E and F). Interestingly, upon 4 h of ATRA treatment, RARA was near the periphery of the nucleus, while GRp58 was mainly in the cytoplasm (Fig. 3G–I), indicating that RARA and GRp58 may have dissociated from each other by 4 h. After 8 h of ATRA treatment, the majority of GRp58 was found in the cytoplasm (Fig. 3K and L), while a lower amount of RARA was still located near the periphery of the nucleus (Fig. 3J and L).

To determine whether RARA, located at the periphery of the nucleus, is at the ER, primary Sertoli cells were treated with ATRA for 2 or 4 h, fixed, and immunostained with anti-RARA and anti-calreticulin antibodies. Calreticulin is a soluble ER-resident protein, often used as an ER marker (Desai et al. 1996), and is shown to be associated with GRp58 in the ER (Oliver et al. 1999). Within 2 h of ATRA treatment, RARA was primarily translocated into the nucleus, although some of RARA was overlapped with calreticulin in the ER (Fig. 3M and O).
Figure 2 GRp58 mRNA and protein are expressed in rat testes. (A and B) Total RNA was isolated from each indicated tissue in A, adult testes (TT), isolated germ cells (GC) from adult rat testes, and cultured primary Sertoli cells (SC) from 20-day-old rats, and was analyzed by semi-quantitative RT-PCR with primers specific for rat GRp58. Loading can be gauged by the amount of amplicon obtained for a housekeeping mRNA, ribosomal protein S2 RNA. (C) Proteins extracted from TT, GC, and SC were subjected to immunoblotting with anti-GRp58 or anti-actin antibody. Loading was determined by western blot analysis with anti-actin antibody. (D–K) Immunohistochemical analysis was conducted using anti-GRp58 on testicular sections from rats at the ages of 5 (D), 10 (E), 15 (F), 20 (G), 25 (H), 30 (I), 35 (J), and 60 (K) days. Bar in D = 50 μm for D, E, F, and G; bar in H = 100 μm for H, I, J, and K. s, Sertoli cell; g, germ cell; a, acrosome; e, elongated spermatid. All the experiments were repeated three times.
In contrast, upon 4 h of ATRA treatment, the majority of RARA was co-localized with calreticulin residing in the ER (Fig. 3P–R), indicating that RARA was located at the ER within 4 h. Since ATRA seemed to increase the intensity of GRp58 immunostaining with time, we conducted western blot analysis on cell extracts isolated from Sertoli cells treated with ATRA for 2, 4, and 8 h. Indeed, it showed that the amount of GRp58 in Sertoli cells increased with ATRA treatment (Fig. 3S).

**Disruption of GRp58 by RNAi interfered with the nuclear localization of RARA**

To determine the role of GRp58 in the nuclear localization of RARA, we disrupted the expression of endogenous GRp58 using RNAi technology in primary Sertoli cells. Primary Sertoli cells transfected with GRp58-specific siRNA or control siRNA were collected after 48 h, and were lysed for RNA or protein extraction. GRp58-specific siRNA was able to efficiently knock down more than 70% of the endogenous GRp58 RNA (Fig. 4A) and protein (Fig. 4B). Then, primary Sertoli cells transfected with either control siRNA or siRNA specific to GRp58 were treated with either vehicle or ATRA for 2 h, fixed, and immunostained with anti-RARA and anti-GRp58 antibodies. Strikingly, in the cells transfected with GRp58-specific siRNA, RARA staining was observed only in the cytoplasm in the presence of ATRA (Fig. 4F and H), indicating that the disruption of endogenous GRp58 interfered with the nuclear import of RARA in Sertoli cells. In contrast, in the cells transfected with control siRNA, both RARA and GRp58 were overlapped in the cytoplasm of Sertoli cells in the absence of ATRA (Fig. 4I–K) and, upon 2 h of ATRA treatment, co-localized to the nucleus of Sertoli cells (Fig. 4L–N). These results suggest that GRp58 is required for the nuclear localization of RARA in Sertoli cells.

**Disruption of ATRA binding to RARA by sulfhydryl-modifying agents influenced the nuclear localization of RARA**

To determine whether GRp58 works as a disulfide isomerase with the thiol oxidoreductase activity, sulfhydryl-modifying agents, methyl methanethiosulfonate (MMTS) and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), were used to block thiol–disulfide interchange...
reaction of the disulfide isomerase. Previously, these sulfhydryl-modifying agents were shown to modify cysteine residues in the ligand-binding domain of RARA and block ATRA binding to RARA (Dallery et al. 1993, Wolfgang et al. 1997). Primary Sertoli cells were pretreated with MMTS (1 mM) for 30 min, and were then treated with ATRA and MMTS, or treated with MMTS alone for 2 h, fixed, and immunostained with anti-RARA and anti-Grp58 antibodies. Interestingly, in the presence of ATRA and MMTS, both RARA and Grp58 were found in the cytoplasm of Sertoli cells (Fig. 4O–Q), which was opposite of the localization pattern observed in the presence of ATRA (Fig. 3D–F). As expected, RARA and Grp58 were in the cytoplasm of Sertoli cells in the presence of MMTS alone (Fig. 4R–T). Additionally, experiments with a lower concentration of MMTS (0.5 mM; Fig. 4U–W) and another sulfhydryl-modifying reagent DTNB (100 μM; Fig. 4X–Z) showed similar

Figure 4 GRp58 is required for the nuclear localization of RARA. (A–N) Primary Sertoli cells transfected with GRp58-specific siRNA (C–H) or control siRNA (I–N) were treated with ATRA (1 μM) for 2 h. Isolated RNA was analyzed by semi-quantitative RT-PCR (A), and isolated protein extracts were analyzed by western blot analysis with anti-GRp58 or anti-actin antibody (B). Additionally, cells were fixed for immunofluorescence analysis with anti-RARA (C, F, I, and L) and anti-GRp58 antibodies (D, G, J, and M). Merged images are presented in E, H, K, and N. The intensities of mRNA (A) of GRp58 and ribosomal protein S2 and proteins (B) of GRp58 and actin were scanned and graphed with means ± S.E.M. from n = 3 independent experiments. RD, relative density. Different letters denote a significant difference from each other at P<0.05. (O–Z) Primary Sertoli cells were pretreated with MMTS (1 mM) (O–T), MMTS (0.5 mM) (U–W), or DTNB (100 μM) (X–Z) for 30 min, and were then treated with ATRA (1 μM) (O–Q, U–Z) or without ATRA (R–T) for 2 h, fixed, and subjected to immunofluorescence analysis with anti-RARA (O, R, U, and X) and anti-GRp58 (P, S, V, and Y) antibodies. Merged images are presented in yellow color in Q, T, W, and Z. DAPI-stained nucleus is presented in blue color. Bar in C, I, O, R, U, and X=100 μm for C–Z. All the experiments were repeated at least three times.
results. Together, these results indicate that the thiol oxidoreductase activity of GRp58 was required for the ATRA-mediated nuclear co-localization of RARA.

**MG132 changed the subcellular localization of RARA and GRp58 at 4 h of ATRA treatment**

Previously, we have shown that ATRA treatment increased RARA protein expression level, reaching the maximum level at 3 h, and then decreased afterwards (Braun et al. 2000). It is known that ATRA triggers RARA to undergo degradation by the ubiquitin-mediated proteasome pathway immediately after the transcription of its target gene (Bour et al. 2007). In fact, it is known that RARA is still attached to the response elements of the target gene (Bour et al. 2007). To determine if the ubiquitin-mediated proteasome pathway has a role in the dissociation of RARA with GRp58 at 4 h of ATRA treatment, primary Sertoli cells were pretreated for 30 min with MG132, a reversible inhibitor of proteasome, and were then treated with ATRA and MG132, or MG132 alone for 4 h, fixed, and immunostained with anti-RARA and anti-GRp58 or anti-calreticulin antibodies. RARA (Fig. 5A and D) and GRp58 (Fig. 5B) were co-localized near the periphery of the nucleus, probably at the ER of Sertoli cells, as indicated by co-localization with an ER-resident calreticulin (Fig. 5E), in the presence of ATRA and MG132 at 4 h. The co-localization result for RARA and GRp58 was distinctly different from the predominantly cytoplasmic localization of GRp58 in the absence of MG132 and in the presence of ATRA at 4 h (Fig. 3H and I). As expected, MG132 treatment alone did not change the localization patterns of RARA or GRp58 (Fig. 5G–I). Together, these results suggest that GRp58 may accompany RARA to the ER for RARA degradation, and that when RARA degradation is blocked by MG132, RARA remains associated with GRp58. The implication is that only when RARA is degraded, GRp58 is recycled back to the cytoplasm.

**MG132 restored the amount of GRp58 associated with RARA**

To determine whether ATRA affected the amount of GRp58 that interacted with RARA, COS-7 cells were treated with either vehicle or ATRA for 4 h after transfection with pHis-hRARA cDNA and pFLAG-GRp58 cDNA expression constructs. Additionally, COS-7 cells were transfected with increasing amounts of pFLAG-GRp58 to make sure that GRp58 was not limiting. Proteins interacting with HIS-RARA were captured using Ni-NTA magnetic agarose beads. The amount of GRp58 co-purified with HIS-RARA upon 4 h of ATRA treatment was significantly less than that of GRp58 in the untreated sample (Fig. 6 A and B). The decrease in GRp58 with time was not due to the amount of GRp58 present in the cell, since ATRA was actually shown to increase the steady-state level of GRp58 in primary Sertoli cells and COS-7 cells (Figs 3S and 6D). Furthermore, similar transfection experiments were performed with the pretreatment of MG132, a reversible inhibitor of proteasomes. Interestingly, upon MG132 treatment, the amount of GRp58 co-purified with HIS-RARA was significantly higher than that of GRp58 in 4-h of ATRA-treated samples (compare Fig. 6C and B), indicating that MG132 blocks the ATRA-induced degradation of RARA at 4 h.

**RARA is associated with UBE2J2 in Sertoli cells**

To investigate whether RARA is degraded by ERAD, we sought to identify potential factors that may function in ERAD machinery. In yeast, the tail-anchored ubiquitin-conjugating enzyme UBC6, localized to the cytoplasmic surface of the ER, is involved in ERAD of misfolded proteins (Sommers & Jentsch 1993, Biederer et al. 1996). Two mammalian UBC6 homologues UBE2J1 and UBE2J2 are also located in the ER membrane, and are critical for ERAD (Lenk et al. 2002). To determine whether these two mammalian Ubc6 homologues are...
expressed in Sertoli cells, real-time RT-PCR was performed with RNA that was isolated from primary Sertoli cells. In Sertoli cells, the relative mRNA expression level of \( Ube2j2 \) was significantly higher than that of \( Ube2j1 \) (Fig. 7A). The UBE2J2 protein was also present in both germ cells and Sertoli cells (Fig. 7B). To determine whether RARA interacts with UBE2J2, primary Sertoli cells were treated with ATRA with or without MG132 for 2 or 4 h, and the lysates were immunoprecipitated with anti-RARA antibody, followed by immunoblotting with anti-UBE2J2 antibody. RARA was able to immunoprecipitate UBE2J2 (Fig. 7C). Upon ATRA treatment, association of UBE2J2 with RARA decreased with time (Fig. 7C, compare lane 4 with lane 5). In contrast, in the presence of both ATRA and MG132, the level of RARA did not decrease with time (Fig. 7C, lanes 6 and 7). This result indicates that RARA association with UBE2J2 decreases with time, after ATRA treatment, as RARA is degraded by proteasome-mediated degradation.

To determine whether RARA and UBE2J2 were co-localized in primary Sertoli cells, the cells were treated with either vehicle or ATRA (1 \( \mu M \)) for 2 or 4 h, and were analyzed by immunofluorescence with anti-RARA and anti-UBE2J2 antibodies. Localization of UBE2J2 was cytoplasmic, regardless of ATRA treatment (Fig. 7E, H, and K), whereas RARA localization and the amount of RARA changed with ATRA treatment (Fig. 7D, G, and J) as observed previously (Fig. 3). Altogether, these results indicate that, unlike Grp58, UBE2J2 does not associate with RARA during its nuclear import, but RARA and Grp58 probably migrate to UBE2J2 in the ER for UBE2J2-mediated ubiquitination and proteasome-mediated ERAD.

**Discussion**

Recently, Grp58 (Erp57), a molecular chaperone, has been reported to be a part of the quality control system that directs proteins for either correct folding or degradation, as well as for other functions such as binding the vitamin D active metabolite, 1,25(OH)\(_2\)D\(_3\), the regulation of calcium homeostasis, and activation of specific transcription factors (Bedard et al. 2005, Khanal & Nemere 2007b). Here, we report that Grp58 was strongly associated with RARA from Sertoli cells using a full-length RARA as the bait and protein products expressed from a Sertoli cell cDNA library as the prey in a yeast two-hybrid screen. This interaction was confirmed in transfected COS-7 cells (Figs 1B and C and 6), indicating that RARA and Grp58 interacted in other mammalian cells. Thus, this interaction could be a general mechanism in mammalian cells that co-express RARA and Grp58.

Grp58 mRNA was highly expressed in prostate and testis (Fig. 2A–C). At the protein level, Grp58 was located in the membrane and/or the cytoplasm of germ...
cells and Sertoli cells in early testis development (Fig. 2D–K). We report a novel finding that GRp58 is in the nucleus of pre-meiotic germ cells, (Fig. 2H–K), the same cells which contain RARA in the nucleus. Additionally, it was mainly found in the acrosome of the round spermatids (Fig. 2J) and continued its expression in developing spermatids (Fig. 2K), which is consistent with previous results (Ohtani et al. 1993, Ellerman et al. 2006).

In Sertoli cells, we found that both RARA and GRp58 were co-translocated from the cytoplasm to the nucleus upon ATRA treatment (Fig. 3D–F). More convincingly, when GRp58 was knocked down by RNAi technique, RARA was observed in the cytoplasm of Sertoli cells, even in the presence of ATRA (Fig. 4F and H), indicating that GRp58 is required for the nuclear localization of RARA. In addition, the nuclear co-localization of RARA and GRp58 was disrupted by pretreatment with sulfhydryl-modifying agents, MMTS (Fig. 4O–Q) and DTNB (Fig. 4X–Z), that could modify cysteine residues on RARA. Previous study has shown that cysteines in the ligand-binding domain of RARA are involved in ligand–receptor interactions, and that the ligand-binding activity of RARA is markedly decreased by treatment with sulfhydryl-modifying agents such as MMTS and DTNB (Dallery et al. 1993, Wollang et al. 1997). Thus, GRp58 may be the chaperone protein that is involved in enhancing ATRA binding to RARA by changing RARA conformation to a nuclear import-competent state. Thus, taken together, it appears that cysteine residues are important for the ATRA-dependent nuclear localization of RARA, and that the thiol oxidoreductase activity of GRp58 may be required for thiol exchange reactions with the cysteine residues of RARA for enhancing the ATRA binding to RARA.

Our findings are consistent with previous results that nuclear receptors, such as glucocorticoid receptor (Smith 1993, Pratt & Toft 1997), progesterone receptor (Carson-Jurica et al. 1989, Schowalter et al. 1991), and estrogen receptor (Chambraud et al. 1990, Czar et al. 1994), form a multiprotein heterocomplex that contains heat shock protein 90 (HSP90), HSP70, and immunophilin, such as FKBP51 or CYP40, in the cytoplasm. In particular, HSP70, HSP90, and immunophilin are molecular chaperones that maintain appropriate ligand-binding conformation for nuclear receptors, and participate in the nuclear–cytoplasmic shuttling and decay of nuclear receptors (Kang et al. 1994, Czar et al. 1997, Pratt & Toft 1997, Grad & Picard 2007). HSP90 is an established drug target, and its inhibitor geldanamycin effectively blocked the ligand-binding ability of progesterone receptor, glucocorticoid receptor, and mineralocorticoid receptor, and also promoted the degradation of nuclear receptors (Smith et al. 1995, Whitesell & Cook 1996, Bamberger et al. 1997). Similarly, retinoid receptors, retinoic acid receptor (RAR) and retinoid X receptor (RXR),
have been shown to interact with HSP90, which is required for ligand binding and transcriptional activation of RAR or RXR–RAR heterodimers (Holley & Yamamoto 1995). Hence, it is clear that molecular chaperone proteins are a group of key molecules that may be critical to ensure proper biological responses of nuclear receptors, including RARs, and now, GRp58 joins this list of molecular chaperone proteins.

Although it is known that ATRA triggers RARA to undergo degradation by the ubiquitin-mediated proteasome pathway immediately after the transcription of its target gene (Gaillard et al. 2006, Bour et al. 2007), the subcellular location of RARA degradation has not been studied. It was assumed that degradation occurs in the nucleus. However, our results indicate that RARA degradation may take place at the ER. With ATRA treatment, GRp58 and RARA were found to be co-localized to the nucleus in 2 h (Fig. 3D–F), but at 4 and 8 h, the majority of RARA was localized to the ER (Fig. 3G, I, J, and L), while GRp58 was localized to the cytoplasm (Fig. 3H, I, K, and L). More interestingly, in the presence of MG132 and ATRA, RARA, GRp58, and calreticulin were co-localized to the ER (Fig. 5A–F), suggesting that inhibition of the proteasome-mediated degradation pathway caused the retention of GRp58 and RARA association at the ER. This is consistent with the decreased level of GRp58 associated with HIS-RARA in the cells treated with ATRA at 4 h, which is significantly less than that in the untreated cells (Fig. 6A). Moreover, the amount of GRp58 co-purified with HIS-RARA was significantly higher at 4 h in the presence of both ATRA and MG132 than the amount of GRp58 in ATRA-alone-treated samples (Fig. 6B and C). Furthermore, upon ATRA treatment, RARA interacted (Fig. 7C) and co-localized with UBE2J2 (Fig. 7D–L), which is known to be located on the cytoplasmic side of the ER and critical for the target protein degradation via ERAD system (Sommer & Jentsch 1993, Biederer et al. 1996, Lenk et al. 2002). Collectively, these results suggest that ATRA induced the degradation of RARA via UBE2J2-mediated proteasome of the ERAD system, and that this process also involves GRp58.

Whether calreticulin or calnexin serves as a conduit for RARA degradation is not known. However, it should be noted that previous reports indicate that calreticulin can physically interact with RARA, as shown by immunoprecipitation (Desai et al. 1996) and as shown by the presence of an amino acid sequence motif, that is known to bind calreticulin in the DNA-binding region of RARA (Dedhar et al. 1994). Similarly intriguing is a previous result that a corepressor, N-CoR, associated with a fusion protein of promyelocytic leukemia (PML) and RARA (PML–RARA) that causes acute PML, was shown to undergo ERAD, including ubiquitination of N-CoR by an ubiquitin-conjugating enzyme, UBC6, causing a decrease in the soluble N-CoR protein levels in the nucleus (Khan et al. 2004). This report suggests that a reduction in N-CoR protein levels in the nucleus could be partially responsible for uncontrolled growth in PML–RARA-mediated leukemogenesis.

In conclusion, we have identified GRp58 as a molecular chaperone for RARA, and showed its participation in enhancing the nuclear localization of RARA. RNAi and proteasome inhibitor studies have elucidated that GRp58 is essential for the nuclear localization of RARA, and that it accompanies RARA to the ER, where degradation of RARA seems to occur. Sulfhydryl-modifying agent studies allow us to speculate that GRp58 may work as a thiol oxidoreductase to enhance the ligand binding to RARA and change the conformation of RARA to a nuclear import-competent state. Hence, we propose a model (Fig. 8) that GRp58 interacts with RARA to form a RARA–GRp58 complex in the cytoplasm of Sertoli cells. In the presence of ATRA, the complex is imported into the nucleus. During the process, GRp58 may work as a thiol oxidoreductase to enhance the ATRA binding to RARA. Within the nucleus, the RARA–GRp58 complex binds to the RARE and initiates the transcription of target genes. Afterwards, GRp58 accompanies the used RARA to the UBE2J2-mediated proteasome ERAD machinery for degradation before GRp58 is recycled back into the cytoplasm for the next cycle. Thus, we demonstrate that manipulation of GRp58 could be an alternative mechanism to modulate the biological functions of RARA in Sertoli cells.

**Materials and Methods**

**Antibodies and reagents**

Goat anti-GRp58 antibody, goat anti-calreticulin antibody, rabbit anti-RARA antibody, and goat anti-UBE2J2 antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). FITC-conjugated anti-rabbit antibody, anti-FLAG M2, and anti-HIS MABs were obtained from Sigma–Aldrich, Inc. Texas red-coupled anti-goat antibody was purchased from Vector Laboratories, Inc. (Burlingame, CA, USA). Sulfhydryl-modifying agents, MMTS and DTNB, were purchased from Sigma–Aldrich, Inc.

**Plasmid constructs**

A human RARA (hRARA) cDNA from LRARaSN (Collins et al. 1990) was subcloned into pFLAG-CMV2 vector (Sigma–Aldrich Co.), pBD-GAL4 (Stratagene, La Jolla, CA, USA) and pQE-Tri-system (Qiagen, Inc). Total RNA that was isolated from 20-day-old rat Sertoli cells using TRizol reagent (Invitrogen) was used to generate cDNA using iScript cDNA synthesis kit (Bio-Rad). The synthesized cDNA was used to conduct PCR to generate GRp58 cDNA using specific primers (5’-TCGGCCGGCTACGCTAGCTGGC-3’ and 5’-TGTCAAGATTAGGATCTTGTGC-3’). The GRp58 cDNA was digested with NolI and Clal, and was subcloned into pFLAG-CMV2 vector (Sigma–Aldrich).
Yeast two-hybrid screening

Yeast strain AH109 (Clontech Laboratories, Inc.) containing pBD-GAL-hRARA was mated with Y187 (Clontech Laboratories) with the rat Sertoli cell cDNA expression library in pAD-GAL4, and was plated on a medium lacking HIS, adenine-2 (ADE2), leucine (LEU), and tryptophan (TRP) and supplemented with 3-amino-triazole (Sigma–Aldrich) to decrease HIS background. HIS+, ADE2+, LEU+, and TRP+ colonies were measured for β-galactosidase activity using the filter lift assay (Wrenn & Katzenellenbogen 1990). HIS+, ADE2+, LEU+, and TRP+ colonies exhibiting high β-galactosidase activity (LacZ+) colonies were further characterized. To recover cDNA library plasmids, total DNA from HIS+, ADE2+, LEU+, and TRP+ colonies was isolated and used to transform Escherichia coli DH5α (Invitrogen). To ensure that the correct cDNAs were identified, cDNA library plasmids that were isolated were transformed into AH109-containing pBD-GAL-hRARA, and plated on a medium lacking HIS, ADE2, LEU, and TRP and supplemented with 3-amino-triazole. β-galactosidase activity was determined from HIS+, ADE2+, LEU+, and TRP+ colonies using the filter lift assay.

Cell culture, transfection and RNA interference

COS-7 cells were maintained in DMEM containing 10% fetal bovine serum supplemented with penicillin (100 IU/ml) and streptomycin (100 μg/ml) at 37 °C in a saturated atmosphere of 5% CO₂. Primary Sertoli cells were isolated from the testes of 20-day-old rats by sequential enzymatic digestions (Dufour et al. 2003). Decapsulated testis fragments were digested first with 0.25% (w/v) trypsin (Gibco BRL) to remove the interstitial cells, and then with collagenase (0.7 mg/ml, Sigma–Aldrich), and they were maintained in Ham's F-12 medium (Invitrogen Laboratories) in a 32 °C 5% CO₂ atmosphere.

Germ cells were isolated from the testes from P70 SD rats by sequential enzymatic digestion (Dufour & Kim 1999). Decapsulated testis fragments were digested first with collagenase (0.7 mg/ml) and then with 0.25% trypsin (w/v). Cells were smeared onto slides, fixed with Bouin's solution for 1 h, and stained with hematoxylin. More than 200 cells per preparation were counted to determine the number of germ cells. The average percentage of germ cells in three separate preparations was 84.2 ± 0.4% (mean ± s.d.). Animal protocols, following the NIH guidelines, were approved by the Institutional Animal Care and Use Committee of Washington State University.

COS-7 cells or primary Sertoli cells grown in 24-well plates or 60-mm plates were transiently transfected with 6 μg/60-mm plate or 1 μg/well (24-well plate) of GRp58 and RARA cDNA expression constructs at a ratio of 1:1, or 20 μM of GRp58 siRNA or control siRNA using Lipofectamine 2000 reagent (Invitrogen). Forty-eight hours post transfection, cells were either untreated or treated with each indicated reagents, and were harvested for pull-down assay, immunoblotting, or immunofluorescence.

Immunohistochemistry

Testes taken from rats at the ages of 5, 10, 15, 20, 25, 30, 35, 40, and 60 days were fixed in Bouin's solution for 4 h, embedded in paraffin, cut into 4-μm-thick sections, and mounted onto slides coated with polylysine. The tissue sections were deparaffinized, rehydrated, and microwaved in 50 mM glycine (pH 3.5) for 15 min, cooled to room temperature, and rinsed in 3% hydrogen peroxide for 10 min. Then, tissue sections were blocked with 10% normal rabbit serum (Vector Laboratories) in PBS for 30 min at room temperature, and incubated with anti-GRp58 antibody (1:100), followed by incubation with the biotinylated rabbit anti-goat secondary antibody (1:300) (Vector Laboratories). As a negative control, serial sections were put through the same procedure without the primary antibody.

Semi-quantitative RT-PCR and real-time RT-PCR

PCR primers were designed using Primer Express software version 2.0 (Applied Biosystems, Foster City, CA, USA), and are listed in Table 2. RNA was isolated from different tissues, germ cells, or primary Sertoli cells using TRIzol reagent. cDNA was synthesized from 500 ng of RNA samples using iScript cDNA synthesis kit (Bio-Rad Laboratories). For the semi-quantitative RT-PCR analysis of GRp58, the amount of housekeeping ribosomal protein S2 product was used as a loading control. The number of PCR cycles was determined by performing a series of RT-PCR with different PCR cycles to make sure PCR amplification was in the log linear phase.
For the real-time PCR for Ube2j1 and Ube2j2, a Gene Amp 7000 thermocycler (Applied Biosystems) was used. Threshold (Ct) values for Ube2j1, Ube2j2, and S2 were determined using the 2^{-ΔΔCt} method (Livak & Schmittgen 2001). The Ct value of each gene real-time PCR product was normalized to that for S2 real-time PCR product in each sample. The real-time PCR was conducted on three samples of cDNAs in triplicate.

**Western blotting**

Western blot analyses were performed as described previously (Braun et al. 2002). Proteins were subjected to SDS-PAGE and were transferred to Immobilon-P membranes (Millipore Co., Bedford, MA, USA). After blocking with 5% non-fat milk in Tris-buffered saline with 0.5% Tween (0.5% TBST) for 1 h, membranes were incubated with primary antibody and then with HRP-conjugated secondary antibody in 0.5% TBST for 1 h at room temperature. Equal loading was determined using western blot analysis with anti-actin antibody. Blots were processed with enhanced chemiluminescence western blotting system (Amersham Biosciences).

**His and FLAG pull-down assay**

For His pull-down assay, cells were lysed in an ice-cold lysis buffer (50 mM HEPES–KOH (pH 7.5), 200 mM KCl, 10 mM MgCl₂, 0.1 mM EDTA, and 10% (v/v) Triton X-100) supplemented with a protease inhibitor (Roche Diagnostics). After centrifugation, a part of the supernatant was saved as the input. The remaining supernatant was diluted to 1 ml using the same lysis buffer containing protease inhibitors, mixed with 50 μl of Ni–NTA magnetic beads (Qiagen Inc.), and incubated with anti-RARA antibody overnight at 4 °C. The beads were washed with 1.5 ml of Ni–NTA magnetic beads (Qiagen Inc.) three times, followed by 1.5 ml of the same buffer supplemented with a protease inhibitor. After centrifugation, a part of the supernatant was saved as the input.

The remainder was mixed with 40 μl of anti-FLAG M2 affinity gel (Sigma–Aldrich), and pre-cleaned by mixing with the lysate, followed by incubation on an end-over-end shaker overnight at 4 °C. The resin was centrifuged for 30 s at 5000 g, washed with TE, and mixed with SDS-PAGE loading buffer to elute the complex for western blot analysis.

**Immunoprecipitation**

Primary Sertoli cells plated in a 100-mm plate were treated with ATRA (1 μM) with or without MG132 (10 μM) for 0, 2, or 4 h, harvested, and lysed with RIPA buffer (50 mM Tris–HCl, pH 7.4, containing 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 1 mM dithiothreitol, and a protease inhibitor). The lysates were pre-cleaned by incubation with True Blot Protein G Sepharose IP beads (eBiosciences, Inc., San Diego, CA, USA) for 1 h at 4 °C. After centrifugation at 10 000 g at 4 °C, the supernatant was incubated with anti-RARA antibody or normal rabbit serum as a negative control for 1 h, and then with True Blot Protein G Sepharose IP beads at 4 °C overnight. Immunoprecipitates were washed extensively and were analyzed by western blotting with an anti-Ube2j2 antibody.

**Indirect immunofluorescence and confocal microscopy**

Primary Sertoli cells seeded in 24-well plates were fixed with −20 °C methanol for 10 min, and were blocked with 10% normal goat serum (Vector Laboratories, Inc.) for 1 h before incubation with anti-RARA and anti-GRp58 or anti-calreticulin antibodies overnight at 4 °C. After washing, the RARA antibody was visualized by a FITC-conjugated secondary antibody (Sigma–Aldrich), whereas GRp58 and calreticulin were detected with Texas red-coupled secondary antibody (Vector Laboratories). All digital images were obtained using a laser confocal system (Zeiss LSM 510, Hitachi).

**Statistical analysis**

Statistical analysis consisted of one-way ANOVA, followed by a pairwise comparison of the means at α = 0.05 (Tukey–Kramer method, JMP; SAS Institute Inc., Cary, NC, USA).

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Table 2 Primers used in real-time RT-PCR.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primers</th>
<th>Reverse primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRp58</td>
<td>TATGATGGGCCCTAGGACTGC</td>
<td>GATTCAACGTTGGTGTGTGC</td>
</tr>
<tr>
<td>Ube2j1</td>
<td>AATCCATCAGGAGCACCTT</td>
<td>GAGGATTCTTATGGAGGTAC</td>
</tr>
<tr>
<td>Ube2j2</td>
<td>TTAGTGGTTTTGCGGCC</td>
<td>TTAGTGGTTTTGCGGCC</td>
</tr>
<tr>
<td>S2</td>
<td>CTGCTCCTGTCACCAAAGAG</td>
<td>AAGGTGGCCTTGGCAAAGTT</td>
</tr>
</tbody>
</table>

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For the real-time PCR for Ube2j1 and Ube2j2, a Gene Amp 7000 thermocycler (Applied Biosystems) was used. Threshold (Ct) values for Ube2j1, Ube2j2, and S2 were determined using the 2^{-ΔΔCt} method (Livak & Schmittgen 2001). The Ct value of each gene real-time PCR product was normalized to that for S2 real-time PCR product in each sample. The real-time PCR was conducted on three samples of cDNAs in triplicate.
Funding
This work was supported by a NIH grant HD44569 (to K H Kim).

Acknowledgements
We thank Dr Michael Skinner (Washington State University) for generously providing a Sertoli cell cDNA expression library. We acknowledge Dr Steven Collins (Fred Hutchinson Cancer Institute, Seattle, WA, USA) for LRARASN. We are grateful to Dr Jannette Dufour (Texas Tech University Health Sciences Center, Lubbock, TX, USA) and the members of the Kim laboratory for helpful discussions and critical reading of this manuscript.

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Received 25 November 2009
First decision 22 December 2009
Accepted 3 February 2010