RXRα acts as a carrier for TR3 nuclear export in a 9-cis retinoic acid-dependent manner in gastric cancer cells

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Summary

Retinoid X receptor (RXR) plays a crucial role in the cross talk between retinoid receptors and other hormone receptors including the orphan receptor TR3, forming different heterodimers that transduce diverse steroid/thyroid hormone signaling. Here we show that RXRα exhibits nucleocytoplasmic shuttling in MGC80-3 gastric cancer cells and that RXRα shuttling is energy-dependent through a nuclear pore complex (NPC)-mediated pathway for its import and an intact DNA binding domain-mediated pathway for its export. In the presence of its ligand 9-cis retinoic acid, RXRα was almost exclusively located in the cytoplasm. More importantly, we also show that RXRα acts as a carrier to assist translocation of TR3, which plays an important role in apoptosis. Both RXRα and TR3 colocalized in the nucleus; however, upon stimulation by 9-cis retinoic acid they cotranslocated to the cytoplasm and then localized in the mitochondria. TR3 export depends on RXRα, as in living cells GFP-TR3 alone did not result in export from the nucleus even in the presence of 9-cis retinoic acid, whereas GFP-TR3 cotransfected with RXRα was exported out of the nucleus in response to 9-cis retinoic acid. Moreover, specific reduction of RXRα levels caused by anti-sense RXRα abolished TR3 nuclear export. In contrast, specific knockdown of TR3 by antisense-TR3 or TR3-siRNA did not affect RXRα shuttling. These results indicate that RXRα is responsible for TR3 nucleocytoplasmic translocation, which is facilitated by the RXRα ligand 9-cis retinoic acid. In addition, mitochondrial TR3, but not RXRα, was critical for apoptosis, as TR3 mutants that were distributed in the mitochondria induced apoptosis in the presence or absence of 9-cis retinoic acid. These data reveal a novel aspect of RXRα function, in which it acts as a carrier for nucleocytoplasmic translocation of orphan receptors.

Key words: Retinoid X receptor α (RXRα), Orphan receptor TR3, 9-cis retinoic acid, Nucleocytoplasmic translocation, Gastric cancer cells

Introduction

Nuclear receptors comprise a superfamily of structurally related transcription factors, such as receptors of hormones and vitamins, as well as orphan receptors, which regulate a variety of biological processes including proliferation, differentiation and apoptosis. Retinoid receptors, retinoic acid receptor (RAR) and retinoid X receptor (RXR) are members of the steroid/thyroid hormone receptor superfamily, which function as ligand-activated transcription factors, and play an important role in mediating retinoid effects (Kastner et al., 1995; Mangelsdorf and Evans, 1995; Zhang and Pfahl, 1993). RXR forms a homodimer that binds to retinoic acid response elements to control the expression of retinoic acid response genes in the presence of retinoids (Bugge et al., 1992; Kliewer et al., 1992; Zhang et al., 1992). In addition, RXR also plays a central role in mediating many nuclear hormone receptors through heterodimerization, including the retinoic acid receptor (Leid et al., 1992; Mader et al., 1993), thyroid hormone receptor (Yu et al., 1991), vitamin D3 receptor (Thompson et al., 1999), peroxisome proliferator-activated receptor (Nunez et al., 1997) and orphan receptor (Tran et al., 1992; Perlmann and Jansson, 1995). Therefore, RXR is a crucial protein in the cross talk between retinoid receptors and other hormone receptors.

TR3, also termed nerve growth factor-induced clone B (NGFI-B) and Nur77, is an orphan receptor (Chang and Kokontis, 1988; Hazel et al., 1988; Milbrandt, 1988). Although its ligand is unknown, TR3 can, through its heterodimerization with RXR, bind to retinoic acid response elements (Forman et al., 1995; Perlmann and Jansson, 1995; Wu et al., 1997b) to regulate their transcriptional activity and mediate diverse signals relating to cell proliferation, differentiation and apoptosis (Kang et al., 2000; Woronicz et al., 1995; Wu et al., 1997a; Wu et al., 1997b). Involvement of TR3 mRNA in apoptosis was first demonstrated by showing that TR3 mRNA was rapidly induced by T-cell antigen receptor signaling in immature thymocytes and T-cell hybridomas (Liu et al., 1994; Woronicz et al., 1994). In addition, treatment of lung cancer

cells with AHPN/CD437 (a new class of synthetic retinoids) strongly induces TR3 mRNA expression, which ultimately contributes to apoptosis induction (Li et al., 1998). Rapid induction of TR3 mRNA was also found in other carcinoma cells after stimulation with a variety of apoptosis-inducing agents, such as phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA), calcium ionophore A23187 and etoposide VP-16 (Cheng et al., 1997; Li et al., 2000; Uemura and Chang, 1998; Weih et al., 1996). However, recent studies have highlighted that expression of TR3 mRNA is not sufficient to promote apoptosis (Li et al., 2000; Wu et al., 2002), suggesting that TR3 functions in apoptosis induction through distinct mechanisms at both transcriptional and post-transcriptional levels.

It has been documented that nuclear receptors, including steroid and thyroid hormone receptors, shuttle between the cytoplasm and the nucleus (DeFranco, 1997; Guiochon-Mantel et al., 1996; Kaffman and O'Shea, 1999). Protein export from the nucleus is often mediated by nuclear export receptors that bind to specific nuclear export signal (NES) sequences. Several export receptors have been identified, such as CRM1 (Fornerod et al., 1997; Stade et al., 1997), calreticulin (Burns et al., 1994; Michalak et al., 1996), KNS and HNS sequences (Black et al., 2001; Henderson and Eleftheriou, 2000). CRM1 binds to leucine-rich NES, and this binding is specifically inhibited by leptomycin B (LMB); other export receptors are not sensitive to LMB. Nuclear import, like nuclear export, takes place via multiple pathways (Adachi et al., 1999; Liao et al., 1999; Pruschy et al., 1994; Schmalz et al., 1998). After synthesis in the cytoplasm, proteins are imported into the nucleus exclusively through the large nuclear pore complex (NPC) (Gorlich and Kutay, 1999; Rout et al., 2000). In addition to serving as a gated entry point for large proteins, NPC also provides a passive diffusion channel for proteins smaller than ~50-60 KDa (Gorlich and Kutay, 1999). However, in most cases, small proteins, like larger proteins, enter the nucleus by an energy-dependent and receptor-mediated process (Gorlich and Kutay, 1999; Jakel et al., 1999). Many diverse import receptors exist that recognize different classes of nuclear localization signal (NLS) sequences present in the majority of nuclear proteins (Gorlich and Kutay, 1999).

We have demonstrated that RXR heterodimerizes with TR3 and binds to the promoter of RARβ in regulating RARβ mRNA expression, which is critical in the induction of apoptosis by RXR-selective retinoids in breast cancer cell lines (Wu et al., 1997a). In addition, although TR3 is thought to exert its pro-apoptotic effect by acting as a transcription factor to regulate gene expression (Cheng et al., 1997; Woronicz et al., 1994), repression of TR3 transactivational activity is also caused by its relocalization from the nucleus to the cytoplasm (Li et al., 2000). Consistent with this notion, we found that in gastric cancer cells, the mechanism of TR3 in apoptosis induction mainly relies on its translocation, rather than its transactivation activity (Wu et al., 2002). Recently, we showed that retinoic acid-induced TR3 and RXRα cotranslocation is associated with the regulation of Bcl-2, Bcl-xl and Bax expression, and ultimately initiates apoptosis in breast cancer cells (Ye et al., 2004). These results reinforce the fact that relocalization of cellular proteins is an important part in the regulation of diverse signaling and physiological processes.

Recently, Katagiri et al. (Katagiri et al., 2000) showed that upon stimulation by nerve growth factor (NGF), phosphorylation of TR3 at certain specific residues reduces its DNA-binding activity and increases its nuclear export along with RXR into the cytoplasm in PC12 cells, suggesting an important role for TR3 in RXR translocation by NGF. Interestingly, in the present study, we found that RXRα shuttled between the nucleus and the cytoplasm and that its ligand 9-cis retinoic acid could significantly induce its nuclear export. We also showed that when stimulated by 9-cis retinoic acid, RXRα could, through heterodimerization with TR3, cause translocation of TR3 from the nucleus to the cytoplasm and on to its ultimate location in the mitochondria. These apparently paradoxical results prompted us to examine further the respective as well as coordinate roles of TR3 and RXRα in their translocation processes. We found that during nuclear export, RXRα acts as a carrier for TR3 to transport it to the mitochondria where TR3, but not RXRα, contributed to apoptosis induction in MGC80-3 gastric cancer cells. Therefore, our data reveal a novel aspect of the mechanism underlying the coordinated and yet distinct roles of TR3 and RXRα nucleocytoplasmic translocation.

Materials and Methods

Cell lines

The human gastric cancer cell line MGC80-3 was established by the Cancer Research Center in Xiamen University. Human cervix epitheloid carcinoma cell line HeLa and murine fibroblast cell line NIH3T3 were obtained from the ATCC. MGC80-3 cells were maintained in RPMI-1640 medium; HeLa and NIH3T3 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 1 mM glutamine and 100 U/ml penicillin.

Heterokaryon nucleocytoplasmic shuttling assay

The modified interspecies heterokaryon analysis was performed essentially according to the procedures previously described (Black et al., 2001; Connor et al., 2003). NIH3T3 cells were transfected with different vectors using the calcium phosphate precipitation method as previously described (Wu et al., 1997a). 24 hours post transfection, an equal number of HeLa cells were seeded onto the same coverslip as NIH3T3 cells (before mixing, NIH3T3 cells were washed twice with PBS without Ca^{2+} or Mg^{2+} to avoid expression vector transfer into HeLa cells). After a ~3-6 hour incubation, cycloheximide (CHX, 100 μg/ml) was added to inhibit protein synthesis. The co-cultured cells were washed twice with PBS after 2 hours and were added with polyethylene glycol-1500 (PEG) (50%, Sigma) to serum-free medium for 2 minutes to allow cell fusion, followed by washing twice with medium containing CHX. Cells were then incubated in DMEM medium (plus CHX) for 1 hour. After fixation in 4% paraformaldehyde, the cells were counterstained with Hoechst 33258 (Sigma) to distinguish human HeLa nuclei from those of murine NIH3T3 cells and were then immunostained with tubulin antibody (with Cy3-tagged secondary antibody).

Treatment of cells with wheat germ agglutinin

As wheat germ agglutinin (WGA) is unable to move across the cytoplasmic membrane, we used the Chariot protein delivery system to transfect WGA into cells (Chipuk et al., 2004). Chariot (Active Motif, CA) is a non-cytotoxic agent and efficiently delivers proteins into a wide range of cell lines. Briefly, WGA was mixed with Chariot at room temperature for 30 minutes and then added to the cells. After incubation for less than 1 hour at 37°C and 5% CO_2, cells were

maintained in complete medium (containing serum) for another 2 hours and then harvested for further use.

Immunofluorescence staining and confocal microscopy

Cells were cultured on glass coverslips overnight and then treated with 9-cis retinoic acid. After they were washed with phosphate buffered saline (PBS), cells were fixed in 4% paraformaldehyde. To display endogenous TR3, RXRα or Hsp60 protein, cells were first incubated with anti-TR3 (Santa Cruz), anti-RXRα (Santa Cruz) or anti-Hsp60 antibody (Santa Cruz) respectively, and then separately reacted with corresponding FITC-conjugated anti-IgG (Pharmingen), Cy3-conjugated anti-IgG (Sigma) or Cy5-congugated anti-IgG (Sigma) as secondary antibodies. TR3, RXRα and Hsp60 proteins were visualized under a confocal microscope (Bio-Rad MRC-1024ES).

Preparation of mitochondrial, cytosolic and nuclear fractions

To obtain separate mitochondrial, cytosolic and nuclear fractions, cells were suspended in 2 ml MS buffer (210 mM mannitol, 70 mM sucrose, 5 mM Tris-HCl, pH 7.5 and 1 mM EDTA) containing 1% protease inhibitor cocktail, then homogenized using a Dounce homogenizer. The homogenate was spun at 1300 ***g*** for 10 minutes at 4°C to pellet nuclei and unbroken cells. The pellet was washed with ice-cold PBS and resuspended in MS buffer. The nuclei were purified by a second round of centrifugation at 1300 ***g***. The supernatant containing the heavy-membrane (HM) fraction enriched with mitochondria was subjected to centrifugation at 17,000 ***g*** for 30 minutes at 4°C. After centrifugation, the supernatant was collected as the cytosolic fraction and the pellet was further resuspended in 3 ml MS buffer and layered on a sucrose gradient: 2 ml of 1 M sucrose buffer and 5 ml of 1.5 M sucrose buffer (10 mM Tris-HCl at pH 7.5 and 1 mM EDTA), which was then centrifuged at 60,000 ***g*** for 30 minutes at 4°C. Gradient-purified mitochondria were collected at the interface of the sucrose gradients and dissolved in dilution buffer (5 mM Tris-HCl, pH 7.5 and 1 mM EDTA).

Western blot analysis

Extracts of cellular protein were essentially prepared according to the method previously described (Wu et al., 2003). 50 μg protein extract were electrophoresed on a 10% denaturing gel and electroblotted onto a nitrocellulose membrane. The membrane was incubated with different antibodies at 4°C overnight, followed by corresponding secondary antibodies at room temperature for 3-4 hours. A Pierce ECL (enhanced chemiluminescence) kit was used to detect the antibody reactivity according to the manufacturer's instructions.

Co-immunoprecipitation

Cell extracts were prepared as described previously (Wu et al., 2003). The supernatant obtained was mixed with protein-A beads, incubated for 30 minutes and centrifuged for 5 minutes at 4000 ***g*** for pre-clearing, and then incubated with the primary antibody for 1 hour. Protein-A beads were added again, incubated overnight, and centrifuged for 5 seconds at 15,000 ***g***. The immunoprecipitates were collected and washed three times with RIPA buffer and finally subjected to western blot analysis as required.

Transient transfection of GFP-TR3 into living cells

MGC80-3 cells were transiently transfected with GFP-TR3 alone or with RXRα, using a modified calcium phosphate precipitation method (Wu et al., 1997a). Transfected cells were maintained in medium containing CHX and were treated with 9-cis retinoic acid for different time points as indicated. Fluorescent imaging of living cells was visualized under a fluorescent microscope. Medium was maintained at 37°C.

Stable transfection

The antisense RXRα and antisense TR3 expression vectors were stably transfected into cells using Cellfectin (Gibco/BRI Life Technologies), as described (Wu et al., 1997a; Ye et al., 2004). Briefly, cells were seeded on six-well plates and transfection was carried out when cells were approximately 70% confluent: 6 μl Cellfectin in 1 ml standard medium was added to each well containing 1 ml standard medium supplemented with the desired expression vector (20 ng). Cells were then screened with 400-600 μg/ml G418 (Sigma) and expression levels of endogenous RXRα and TR3 protein were determined by western blotting.

Preparation and transfection of siRNA

Short interfering TR3-siRNA was designed according to published data (Kolluri et al., 2003). The oligonucleotide sequence for TR3 was: 5′-CAGUCCAGCCAUGCUCCUC dTdT-3′; and scrambled sequence used was: 5′-GCGCGCTTTGTAGGATTCG dTdT-3′. TR3-siRNA was prepared according to the manufacturer's protocol (Silencer™ siRNA Labeling Kit, Invitrogen). Cells were seeded in a six-well plate supplemented with medium containing 10% FBS to give 50-70% confluence and TR3-siRNA transfection was performed using Oligofectamine (Invitrogen, Carlsbad, CA) in a final RNA concentration of 50 nM. The transfected cells were harvested the next day for further experiments.

Apoptosis analysis

Cells were transfected with various expression vectors fused with GFP, and then treated with or without 9-cis retinoic acid. After fixation in 4% paraformaldehyde, cells were stained with DAPI (50 μg/ml) to visualize nuclei. Apoptotic cells showing typical morphology of nuclear condensation and fragmentation were counted in 300 randomly selected transfected cells. The results represent the mean values of three independent experiments.

Results

RXRα shuttles between the nucleus and the cytoplasm

It has been well established that many nuclear receptors, such as PR, ER, GR, AR and TR, undergo nucleocytoplasmic shuttling (Baumann et al., 2001; Bunn et al., 2001; Georget et al., 1997; Guiochon-Mantel et al., 1996; Madan and DeFranco, 1993; Dauvois et al., 1993). We first investigated if RXRα shares this specific shuttling property using a heterokaryon assay. Before fusing murine NIH3T3 cells with human HeLa cells by PEG, both cells were treated with cycloheximide (CHX) to repress protein synthesis. To distinguish the human and murine nuclei, the cells were stained with Hoechst 33258 dye that gave a characteristic staining of intranuclear bodies (speckles) in NIH3T3 nuclei and a diffuse pattern throughout the HeLa nuclei. As shown in Fig. 1A, when GFP-RXRα-transfected NIH3T3 cells were fused with HeLa cells, GFP-RXRα transferred from the NIH3T3 nucleus to the HeLa nucleus (marked with arrow), demonstrating that RXRα shuttled between nuclear and cytoplasmic compartments. Furthermore, this RXRα nucleocytoplasmic shuttling is independent of its ligand, 9-cis retinoic acid. Thus, nucleocytoplasmic shuttling of RXRα occurs in the absence and presence of 9-cis retinoic acid.

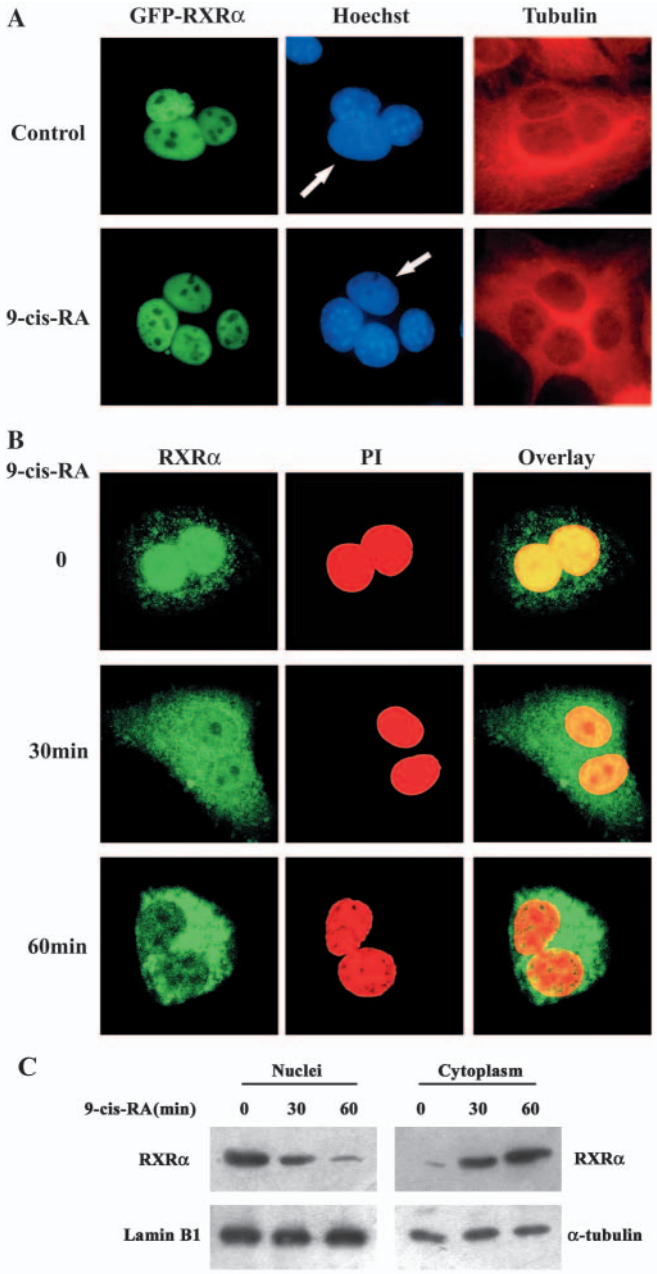


Fig. 1. RXRα shuttles between the nucleus and the cytoplasm. (A) NIH3T3 cells were transfected with GFP-RXRα expression vector and HeLa cells were then seeded onto the same coverslip. Before being fused by PEG to form heterokaryons, cells were cultured in medium containing cycloheximide (CHX) to inhibit new protein synthesis. Fused cells were treated with or without 9-cis retinoic acid (9-cis-RA, 1 μM) for 1 hour, then immunostained with Hoechst 33258 and tubulin antibody followed by Cy3-conjugated secondary antibody to display the nuclei and the whole cells simultaneously. The murine NIH3T3 nuclei gave a characteristic staining of intranuclear bodies (speckles) and the human HeLa nuclei displayed a diffuse pattern indicated by the arrows. (B) Subcellular localization of RXRα in response to 9-cis retinoic acid. MGC80-3 cells were treated with or without 9-cis retinoic acid for the indicated times, fixed and immunostained with anti-RXRα antibody followed by FITC-conjugated secondary antibody. Cells were also stained with propidium iodide (PI, Sigma, 50 μg/ml) to visualize nuclei. Images were visualized using a confocal microscope and the two images were merged (Overlay). (C) 9-cis retinoic acid-induced redistribution of RXRα shown by western blotting in MGC80-3 cells. Cells were treated with 9-cis retinoic acid for the times indicated with nuclear and cytoplasmic fractions prepared as described in Materials and Methods. Different protein portions were then subjected to western blotting with anti-RXRα antibody. Lamin B1 and α-tubulin were used to quantify the amount of protein loaded.

The cellular distribution pattern of RXRα at different time points in the presence or absence of 9-cis retinoic acid was examined by confocal microscopy. Endogenous RXRα was largely present in the nucleus of MGC80-3 cells (Fig. 1B). Surprisingly, 9-cis retinoic acid induced the majority of RXRα to export from the nucleus into the cytoplasm (Fig. 1B), and it remained in the cytoplasm even after prolonged treatment with 9-cis retinoic acid (up to 48 hours, data not shown). To verify this result, cytoplasmic and nuclear protein fractions were prepared and distribution of RXRα was determined by western blotting. As shown in Fig. 1C, in MGC803 cells, RXRα appeared in the nuclear moiety and 9-cis retinoic acid treatment resulted in a time-dependent shift of RXRα protein from the nuclear to the cytoplasmic fraction, consistent with the result shown in Fig. 1B. Thus, these data demonstrated that RXRα nuclear export is drastically facilitated by 9-cis retinoic acid.

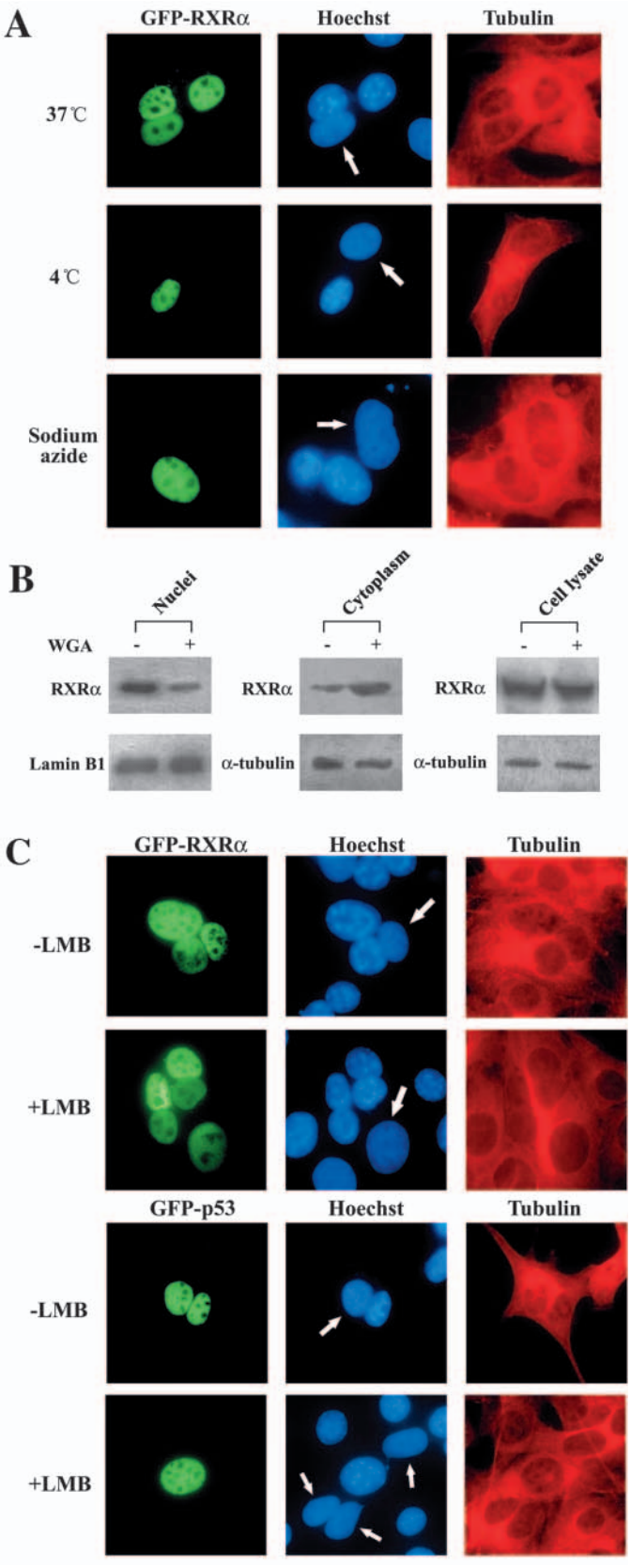
Import and export pathways of RXRα

To investigate the nucleocytoplasmic shuttling mechanism of RXRα, we performed heterokaryon assays and western blotting under different conditions that permit passive diffusion but inhibit active transport processes. Firstly, cells were incubated at different temperatures with or without sodium azide, which depletes intracellular stores of ATP and consequently blocks the ATP-dependent inward and outward passage of nuclear proteins (Guiochon-Mantel et al., 1991). In the NIH3T3-HeLa heterokaryon system, GFP-RXRα shuttled from NIH3T3 nuclei into HeLa nuclei (marked with arrow) at 37°C, but not at 4°C or with sodium azide treatment (Fig. 2A), indicating that GFP-RXRα shuttling was not only temperature-dependent, but also energy-dependent.

Next, we assessed whether specific interaction with the nuclear pore complex (NPC) was required for nuclear import of RXRα. Wheat germ agglutinin (WGA) is a lectin that binds to NPC proteins and inhibits nuclear import without affecting passive diffusion (Bunn et al., 2001). As WGA does not cross the cell membrane, it was transfected into cells by means of the Chariot protein delivery system (Chipuk et al., 2004). WGA-transfected total cell lysate, nuclear and cytoplasmic fractions were prepared and were then subjected to western blotting with anti-RXRα antibody. As shown in Fig. 2B, although WGA did not affect expression levels of total RXRα protein, it increased cytoplasmic RXRα from 21.4% to 78.6% as quantified by densitometry. RXRα import, therefore, is an NPC-mediated event.

Export of nuclear protein is often mediated by nuclear export receptor CRM1, which binds to nuclear export signal (NES) sequences, facilitating the export of nuclear proteins into the cytoplasmic compartment; such binding is specifically inhibited by leptomucin B (LMB) (Kudo et al., 1999; Wolff et al., 1997). NIH3T3-HeLa heterokaryons were treated with LMB for 2 hours. It was found that GFP-RXRα shuttled from the NIH3T3 nuclei to the HeLa nuclei (marked with arrow)

regardless of the presence or absence of LMB (Fig. 2C). As a positive control, GFP-p53, a transcription factor that follows the CRM1-mediated export pathway (Stommel et al., 1999), was detected in the HeLa nuclei (marked with arrow) in the absence of LMB, but remained in the NIH3T3 nuclei in the presence of LMB (Fig. 2C). Therefore, these data suggested that the exit of nuclear RXRα did not rely on the CRM1 pathway.



It has been reported that the DNA binding domain (DBD) of nuclear receptors can act as a new type of NES (Black et al., 2001). We generated several GFP fusion proteins containing the RXRα DBD alone [GFP-RXRα (DBD)], or RXRα lacking different segments of DBD (GFP-RXRαΔ134-155, GFP-RXRαΔ156-165, GFP-RXRαΔ172-191), as illustrated in Fig. 3A. When transfected into NIH3T3 cells, GFP-RXRα (DBD), GFP-RXRαΔ134-155 or GFP-RXRαΔ172-191 could be seen in the nucleus, as could wild-type RXRα (Fig. 3B), indicating that these mutants retained their ability to enter into the nucleus. However, removal of amino acids 155-166 (GFP-RXRαΔ156-165) failed to translocate RXRα into the nucleus and it remained in the cytoplasm, in agreement with a previous report that an NLS exists within amino acid region 160-165 in RXRα (Prufer and Barsony, 2002). We then carried out a heterokaryon assay, showing that GFP-RXRα and GFP-RXRα (DBD), but not GFP-RXRαΔ134-155 or GFP-RXRαΔ172-191, shuttled from the NIH3T3 nucleus to the HeLa nucleus (Fig. 3C). These results clearly indicate that an intact DBD in RXRα is crucial and sufficient for NES function.

RXRα acts as a carrier for TR3 nuclear export

We previously reported that RXRα could heterodimerize with TR3 and cotranslocate to the cytoplasm when induced by retinoic acid in breast cancer cells (Ye et al., 2004). As observed previously, RXR and TR3 could cotranslocate from nucleus to cytoplasm in the presence of 9-cis retinoic acid when observed by confocal microscopy (Fig. 4A, overlay of TR3/RXR). Furthermore, when the staining of endogenous RXRα and TR3 was superimposed with that of Hsp60, a specific mitochondrial protein, a white color was observed, indicating that TR3 and RXRα are colocalized with Hsp60 in the mitochondria in response to 9-cis retinoic acid (Fig. 4A, overlay of TR3/RXRα/Hsp60). However, in the absence of 9-cis retinoic acid, no RXRα-TR3 mitochondrial colocalization could be seen, with TR3 remaining in the nucleus (Fig. 4A). Co-immunoprecipitation assay showed that RXRα-TR3 complex could be induced by 9-cis retinoic acid within 1 hour

Fig. 2. Import and export pathways of RXRα. (A) Effects of temperature and sodium azide on shuttling of RXRα. Heterokaryons were prepared and visualized as described in Fig. 1A. Fused cells were separately incubated at 37°C or 4°C or with sodium azide (1 mM) for 2 hours. Arrows indicates the HeLa nuclei. (B) Effect of wheat germ agglutinin (WGA) on RXRα import. WGA (100 μg/ml) was transfected into MGC80-3 cells by means of the Chariot protein delivery system for 2 hours and then harvested. Cell lysate, nuclear and cytoplasmic fractions were prepared as described in Materials and Methods, and were then subjected to western blotting with anti-RXRα antibody. Lamin B1 and α-tubulin were used as loading controls. (C) Effects of leptomycin (LMB) on shuttling of RXRα. NIH3T3 cells were transfected with GFP-RXRα, then fused with HeLa cells. The heterokaryons were treated with or without LMB (100 μg/ml) for 2 hours. GFP-p53 was used as a positive control. The HeLa nuclei are indicated by arrows.

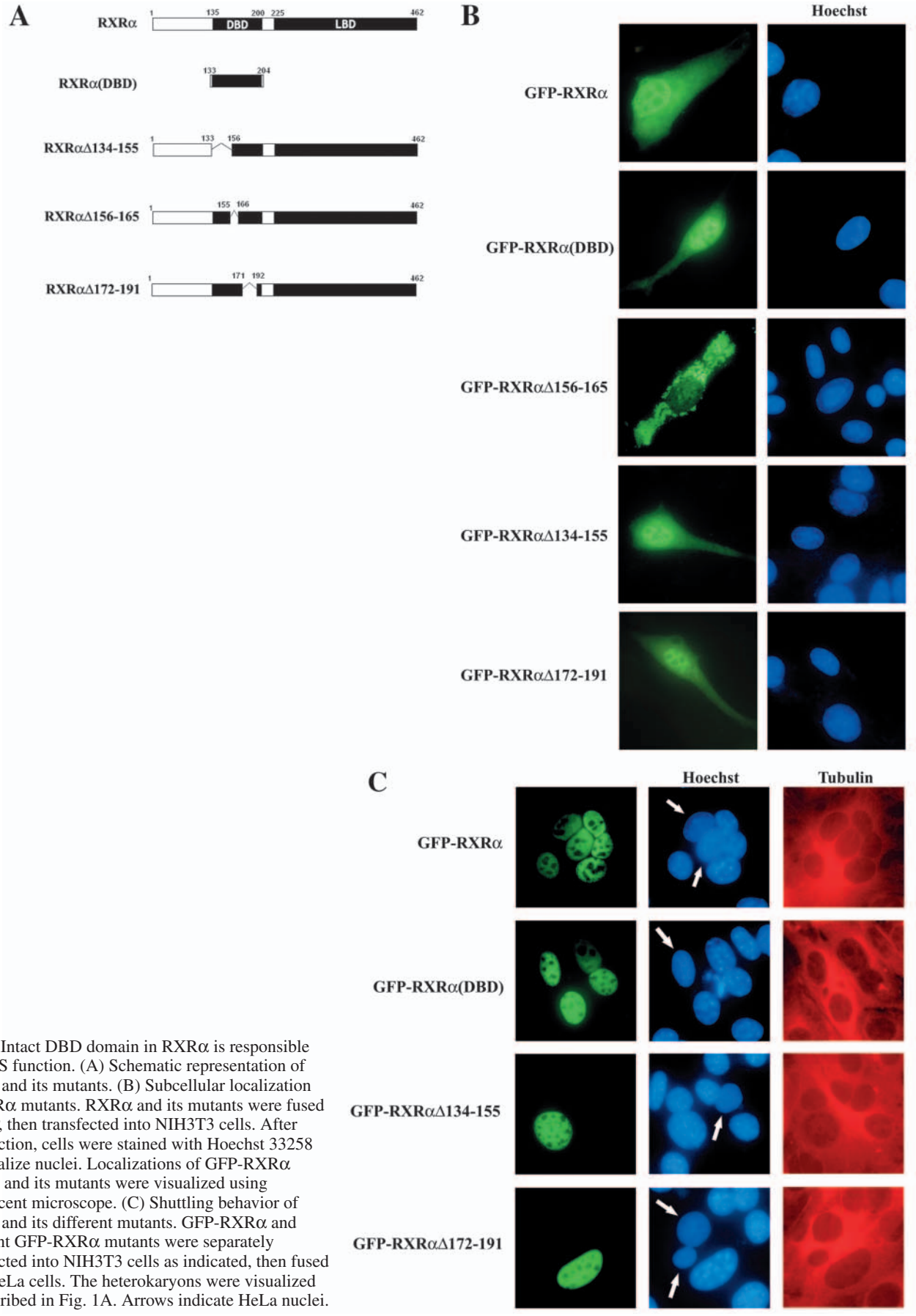


Fig. 3. Intact DBD domain in RXRα is responsible for NES function. (A) Schematic representation of RXRα and its mutants. (B) Subcellular localization of RXRα mutants. RXRα and its mutants were fused to GFP, then transfected into NIH3T3 cells. After transfection, cells were stained with Hoechst 33258 to visualize nuclei. Localizations of GFP-RXRα protein and its mutants were visualized using fluorescent microscope. (C) Shuttling behavior of RXRα and its different mutants. GFP-RXRα and different GFP-RXRα mutants were separately transfected into NIH3T3 cells as indicated, then fused with HeLa cells. The heterokaryons were visualized as described in Fig. 1A. Arrows indicate HeLa nuclei.

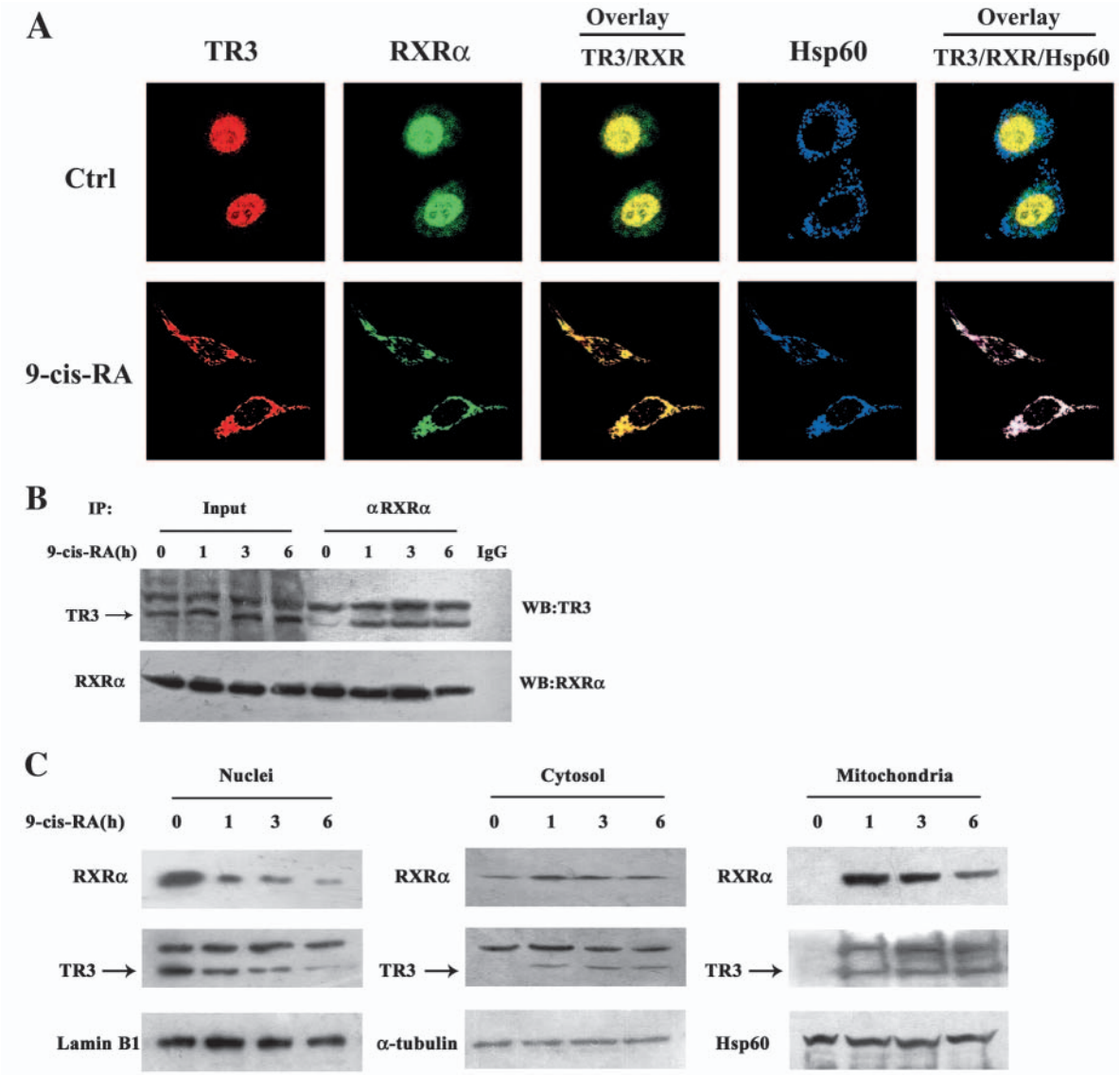


Fig. 4. Translocation of TR3 and RXRα in response to 9-cis retinoic acid. (A) Colocalization of endogenous RXRα and TR3 in the mitochondria. MGC80-3 cells were treated with or without 9-cis retinoic acid (1 μM) for 12 hours and then immunostained with anti-TR3, anti-RXRα and anti-Hsp60 antibodies followed with corresponding FITC-, Cy3- and Cy5-conjugated secondary antibodies to show endogenous TR3, RXRα and Hsp60 proteins simultaneously. The fluorescent images were visualized with a confocal microscope, and the images were merged as indicated (Overlay). (B) MGC80-3 cells were treated with or without 9-cis retinoic acid (1 μM) for the indicated times. Cell extracts were prepared and immunoprecipitated with anti-RXRα antibody, then subjected to SDS-PAGE, blotted, and probed with anti-TR3 antibody. The immunoprecipitated RXRα used in each lane was quantified by western blotting with anti-RXRα antibody. The same extract was applied to ascertain the position and expression of TR3 by western blotting with antibodies against TR3 (Input). IgG was used as negative control where no signal band was detected. (C) Distribution of TR3 and RXRα in MGC80-3 cells. Nuclear, cytosolic and mitochondrial fractions were prepared as described in Materials and Methods, and the expression of RXRα and TR3 in response to 9-cis retinoic acid at different time points was determined by western blotting. Mitochondrial Hsp60, nuclear protein Lamin B1 and cytosolic α-tubulin were detected as protein loading controls.

and remained constant with prolonged 9-cis retinoic acid treatment (Fig. 4B). Western blot analysis of mitochondrial, cytosolic and nuclear fractions confirmed that mitochondrial RXRα and TR3 were detected only in 9-cis retinoic acid-treated MGC80-3 cells, and that their highest levels of mitochondrial localization were reached within 1 hour of treatment. The mitochondrial RXRα leveled off afterwards, whereas TR3 remained constant for up to 6 hours (Fig. 4C). As expected, levels of nuclear RXRα and TR3 decreased over time with treatment with 9-cis retinoic acid (Fig. 4C). Therefore, all these data suggested that a carrier role of RXRα and stimulation by 9-cis retinoic acid are both required for the export of TR3, although RXRα itself could shuttle between the nucleus and the cytoplasm in the absence of 9-cis retinoic acid.

Based on these results, it is likely that TR3 translocation into the cytoplasm might be in the form of a TR3/RXRα heterodimer, in which RXRα acts as a carrier. To test this, living MGC80-3 cells transfected with GFP-TR3 alone or

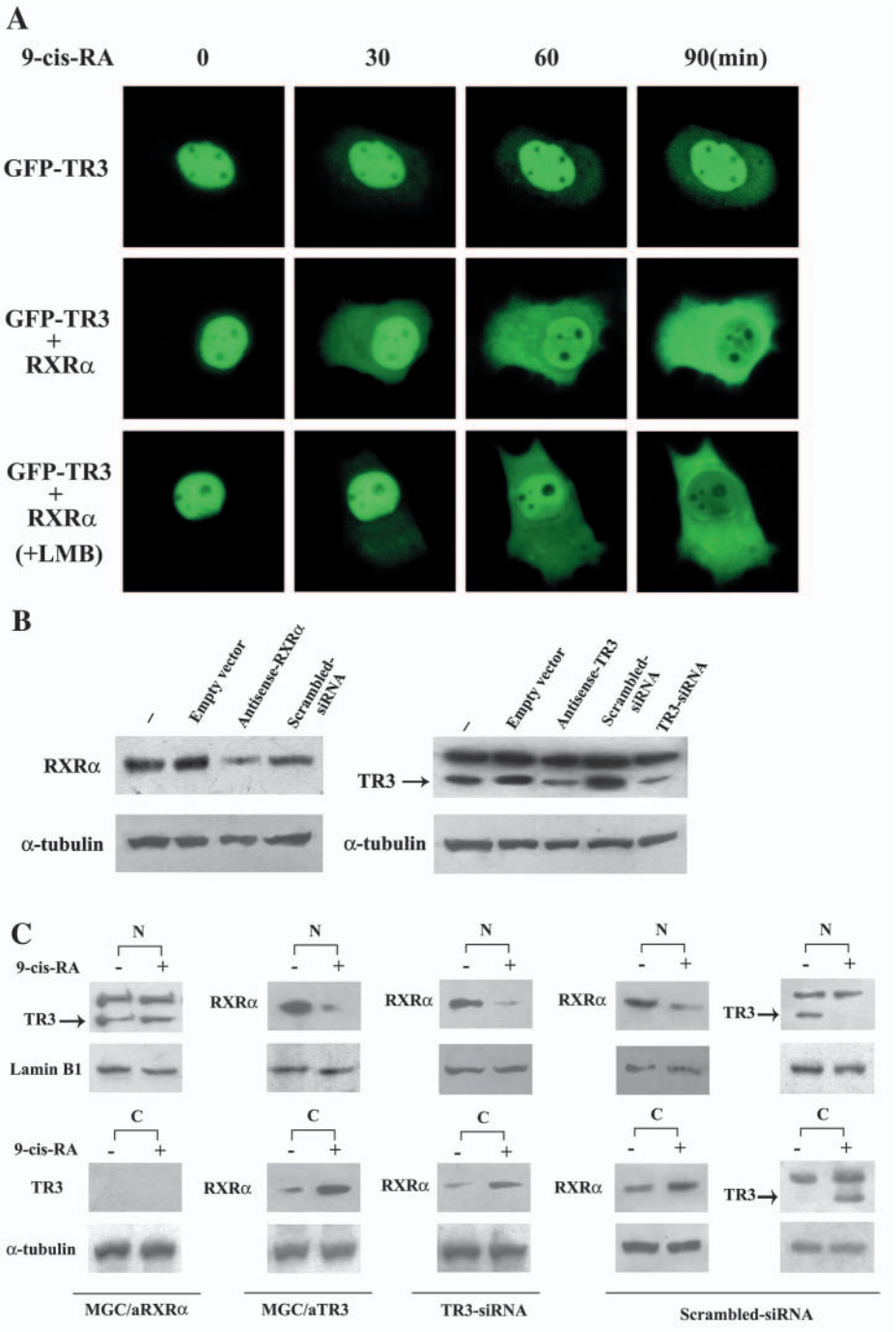


Fig. 5. RXRa serves as a carrier for TR3 translocation initiated by 9-cis retinoic acid. (A) Detection of TR3 translocation mediated by RXRα in living MGC80-3 cells. Living cells transfected with GFP-TR3 alone or together with RXRα were treated with 9-cis retinoic acid at different time points as indicated. The GFP-TR3 translocation from the nucleus to the cytoplasm was visualized under a fluorescent microscope. To detect the effect of LMB on TR3 translocation, transfected cells were pre-treated with LMB for 2 hours, followed by treatment of 9-cis retinoic acid for the indicated times. (B) Repression of endogenous RXRα and TR3 by antisense RXRα, antisense TR3 or TR3-siRNA. Cells were stably transfected with different expression vectors or transiently transfected with siRNA as described in Materials and Methods. Expression level of endogenous RXRα or TR3 was detected by western blotting. Empty vector and scrambled-siRNA were used as controls. α-tubulin was used to quantify the amount of protein loaded in each lane. (C) Effects of antisense RXRα, antisense TR3 and TR3-siRNA on the translocation of RXRα and TR3. MGC80-3 cells were transfected with different expression vectors or siRNAs as described in B and then treated with 9-cis retinoic acid (1 μM) for 6 hours. Nuclear (N) and cytoplasmic (C) fractions were subjected to western blotting, probed with anti-TR3 or anti-RXRα antibody as indicated. α-tubulin and lamin B1 were used as loading controls. Scrambled-siRNA was used as positive control.

together with RXRα were examined at different time points under a fluorescent microscope, in the absence or presence of 9-cis retinoic acid. In cells transfected with GFP-TR3 alone, TR3 was mainly concentrated in the nucleus; its export was only slightly promoted by 9-cis retinoic acid, possibly due to the existence of endogenous RXRα (Fig. 5A). However, when GFP-TR3 and RXRα were coexpressed, TR3 export was significantly enhanced in response to 9-cis retinoic acid (Fig. 5A), indicating again that RXRα and 9-cis retinoic acid stimulation are both critical for nucleocytoplasmic transport of TR3. Furthermore, this RXRα-mediated TR3 translocation by 9-cis retinoic acid was insensitive to LMB (Fig. 5A), consistent with the result from the heterokaryon assay shown in Fig. 2C. Therefore, these data demonstrated that RXRα is required for relocalization of TR3 from the nucleus to the cytoplasm, and that this translocation is 9-cis retinoic acid dependent.

To address this RXRα function further, two modified MGC80-3 cell lines that stably express antisense-TR3 (MGC/aTR3) and antisense-RXRα (MGC/aRXR), respectively, were generated. Cytoplasmic and nuclear fractions from these two cells were prepared and analyzed by western blotting separately with antibodies against RXRa and TR3. In MGC/aRXR cells, RXRα expression was reduced (Fig. 5B), TR3 resided in the nucleus and no cytoplasmic TR3 could be detected even in the presence of 9-cis retinoic acid (Fig. 5C), indicating that specific knockdown of RXRa diminished the

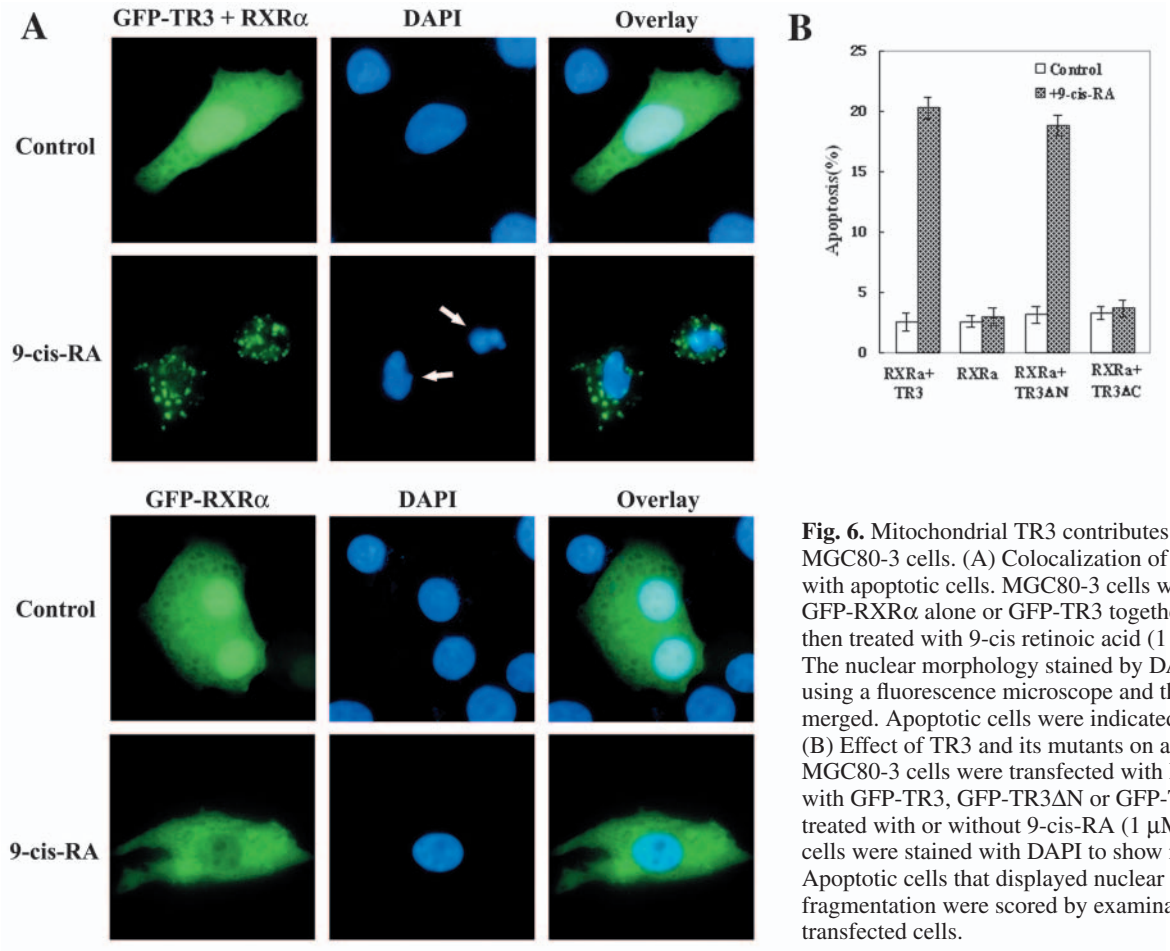


Fig. 6. Mitochondrial TR3 contributes to apoptosis of MGC80-3 cells. (A) Colocalization of mitochondrial TR3 with apoptotic cells. MGC80-3 cells were transfected with GFP-RXRα alone or GFP-TR3 together with RXRα, and then treated with 9-cis retinoic acid (1 μM) for 48 hours. The nuclear morphology stained by DAPI was visualized using a fluorescence microscope and the two images were merged. Apoptotic cells were indicated by arrows. (B) Effect of TR3 and its mutants on apoptosis induction. MGC80-3 cells were transfected with RXRα either alone or with GFP-TR3, GFP-TR3ΔN or GFP-TR3ΔC, and then treated with or without 9-cis-RA (1 μM) for 48 hours. The cells were stained with DAPI to show nuclear morphology. Apoptotic cells that displayed nuclear condensation and fragmentation were scored by examination of 300 transfected cells.

export of nuclear TR3. By contrast, in MGC/aTR3 cells with little TR3 (Fig. 5B), RXRα fully retained its ability to shuttle between the nucleus and the cytoplasm in either the presence or absence of 9-cis retinoic acid (Fig. 5C). In parallel, we also performed knockdown experiments with small interfering RNA specifically against TR3 (TR3-siRNA). Similar to that seen in antisense-TR3 transfected cells, endogenous expression of TR3 was reduced in TR3-siRNA transiently transfected MGC80-3 cells (Fig. 5B), with the expression and distribution of RXRα being unchanged (Fig. 5C). As a positive control, in scrambled-siRNA transfected cells that contained unaffected levels of TR3 and RXRα (Fig. 5B), RXRα still transported out of the nucleus in the absence of 9-cis retinoic acid and accumulated in parallel with TR3 in the cytoplasm when stimulated by 9-cis retinoic acid (Fig. 5C). Taken together, these results all point to the possibility that RXRα serves as a carrier for TR3 translocation initiated by 9-cis retinoic acid.

Mitochondrial TR3, but not RXRα, contributes to apoptosis induction

The mitochondrion is an important organelle for apoptosis initiation. We found that in GFP-TR3 and RXRα cotransfected MGC80-3 cells, mitochondrial localization of TR3 induced by 9-cis retinoic acid correlated well with extensive nuclear fragmentation, a typical apoptotic morphology revealed by DAPI staining (marked with arrow in Fig. 6A). However, such a phenomenon could hardly be seen in cells transfected with GFP-RXRα alone, where RXRα was evenly distributed all around the cytoplasm in the presence of 9-cis retinoic acid (Fig. 6A). Consistent with this result, a higher apoptotic rate induced by 9-cis retinoic acid was seen in GFP-TR3 and RXRα cotransfected MGC80-3 cells than that in RXRα transfected cells (Fig. 6B), suggesting that apoptosis of MGC80-3 cells might be initiated by mitochondrial TR3, but not RXRα. It has been reported that deletion of DBD in TR3 not only resulted in mitochondrial TR3 distribution but also retained TR3 apoptotic activity in lung cancer cells (Kolluri et al., 2003). The same TR3 deletion mutant (Fig. 7A, GFP-TR3ΔDBD) was constructed and used in this study. We found it overlapped with mitochondrial Hsp60 when transfected into MGC80-3 cells (Fig. 7B). Under these conditions, 19.26% cells underwent apoptosis, a higher figure than found in cells transfected with GFP alone (3.48%) (Fig. 7C) and in cells transfected with GFP-TR3 alone (3.86%), because of its nuclear localization (Fig. 7B). Therefore, localization of TR3 in the mitochondria was critical to induce apoptosis in MGC80-3 cells even in the absence of 9-cis retinoic acid.

Two more TR3 deletion mutants were generated, one lacking the N-terminal segment (amino acids 1-106) and the other lacking the C-terminal 25 amino acids (574-598), as indicated in Fig. 7A (TR3ΔN and TR3ΔC, respectively). These mutants displayed nuclear localization when cotransfected with RXRα into MGC80-3 cells (Fig. 8A). In contrast, TR3ΔC completely

abolished RXRα-mediated mitochondrial translocation of TR3 induced by 9-cis retinoic acid, whereas TR3ΔN was clearly localized in the mitochondria in the presence of 9-cis retinoic acid (Fig. 8A). In parallel, the two deletion mutants TR3ΔN and TR3ΔC were assayed for their ability to induce apoptosis when cotransfected with RXRα. Results showed that apoptosis occurred in TR3ΔN transfected cells, but not in TR3ΔC transfected cells in the presence of 9-cis retinoic acid (Fig. 6B), indicating a good correlation between mitochondrial TR3 and apoptosis induction. Furthermore, we also determined if TR3ΔN and TR3ΔC could form a dimer with RXRα by co-immunoprecipitation assays. TR3ΔN, but not TR3ΔC, could form a complex with RXRα (Fig. 8B). These results clearly demonstrated that RXRα-TR3 interaction correlates well with their ability to target to the mitochondria, which is a prerequisite for TR3 to cause apoptosis.

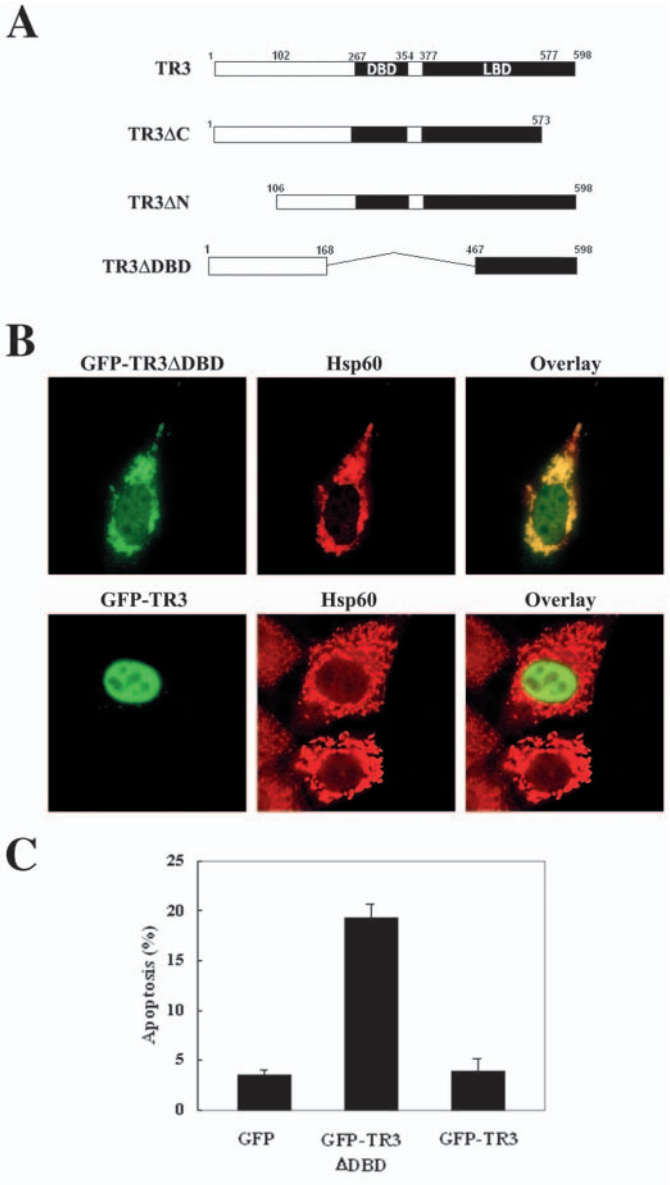


Fig. 7. TR3 with a deletion of its DBD is able to induce apoptosis. (A) Schematic representation of TR3 and its mutants. (B) Cellular localization of GFP-TR3ΔDBD and GFP-TR3 in MGC80-3 cells. Cells were transfected with GFP-TR3ΔDBD or GFP-TR3 respectively and immunostained with anti-Hsp60 antibody followed by Cy3-conjugated secondary antibody to visualize mitochondria. GFP-TR3ΔDBD or GFP-TR3 and Hsp60 were visualized with a confocal microscope. (C) Effect of TR3ΔDBD on apoptosis induction. MGC80-3 cells were transfected with GFP-TR3ΔDBD or GFP-TR3, and then stained with DAPI to show nuclear morphology. Apoptotic cells were determined as described in Fig. 6B. GFP was used as a control.

Discussion

RXR can function as a heterodimer with many members of the nuclear receptor superfamily, facilitating their DNA binding ability and nuclear localization (Zhang et al., 1992; Zhang and Pfahl, 1993; Mangelsdorf and Evans, 1995). In this report, we observed that RXRα shuttled between the nucleus and the cytoplasm, and its ligand 9-cis retinoic acid facilitated the export of nuclear RXRα. Furthermore, we found that RXRα forms complexes with TR3 in the nucleus. Treatment with 9-cis retinoic acid enhanced TR3 nuclear export only when functional RXRα was present, indicating that RXRα is required for cytoplasmic localization of TR3. These data suggest that RXRα is not statically associated with chromatin, but rather is dynamically modulated by its ligand 9-cis retinoic acid, in agreement with recent studies in which nuclear hormone receptors are dynamic transcription factors capable of shuttling between the nucleus and the cytoplasm (Htun et al., 1996; Lim et al., 1999; Hache et al., 1999; Tyagi et al., 2000; Baumann et al., 2001; Bunn et al., 2001; Prufer and Barsony, 2002; Walther et al., 2003; Maruvada et al., 2003).

Nucleocytoplasmic shuttling has been recognized as an important cellular process that regulates many proteins (Komeili et al., 2000). This process may be achieved by a group of proteins acting as carriers for cargo delivery between the nucleus and the cytoplasm, coordinating the activation and inactivation of regulatory factors involved in cell cycle and apoptosis. The proteins that undergo nucleocytoplasmic shuttling change their subcellular compartments in response to diverse signals. RXRα shuttling may play a carrier role in the regulation of TR3 function. In 9-cis retinoic acid-treated living MGC80-3 cells, transfection of GFP-TR3 alone into cells displayed a nuclear distribution of TR3, whereas cotransfection of GFP-TR3 with RXRα significantly induced TR3 translocation into the cytoplasm. Moreover, repression of RXRα by antisense-RXRα affected nuclear export of TR3. By contrast, repression of TR3 by TR3-siRNA or antisense-TR3 did not show any inhibitory effect on RXRα shuttling even in the presence of 9-cis retinoic acid. Therefore, the subcellular trafficking of RXRα suggested, for the first time, a new mechanism, with RXRα serving as a carrier for nucleocytoplasmic translocation of TR3. In addition, the experimental result that RXRα was not sufficient to carry TR3 out of nuclear membrane in the absence of 9-cis retinoic acid indicated a critical role of 9-cis retinoic acid in regulating cotranslocation of RXRα and TR3. Accordingly, both transport of RXRα and stimulation of 9-cis retinoic acid were required for TR3 translocation.

Recently, Katagiri and colleagues convincingly showed that TR3 contains a hydrophobic nuclear export signal (NES) that directs itself together with RXR out of the nucleus in PC12 cells after NGF treatment (Katagiri et al., 2000). It was also

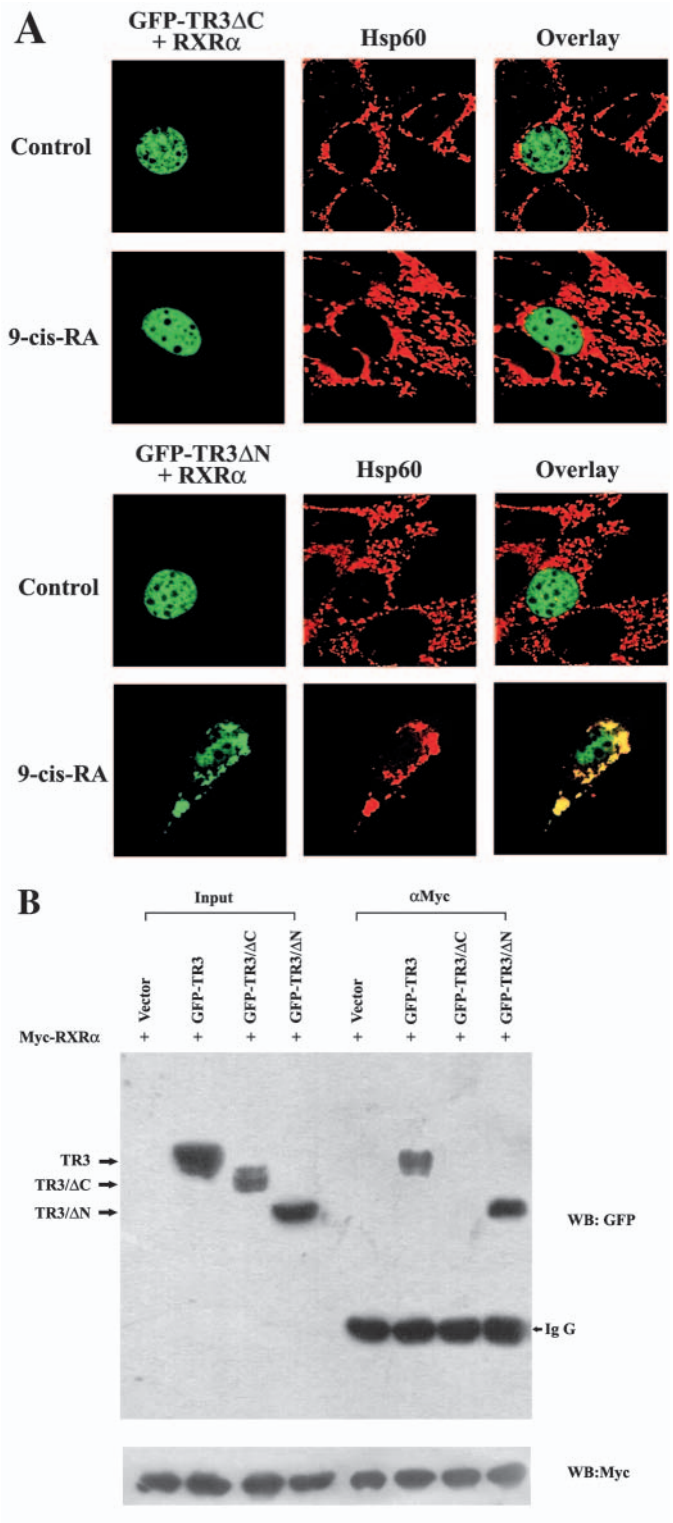


Fig. 8. RXRα-TR3 interaction correlates well with their ability to target to the mitochondria. (A) Cellular localization of GFP-TR3ΔN and GFP-TR3ΔC in response to 9-cis-RA. GFP-TR3ΔN or GFP-TR3ΔC together with RXRα was transfected into MGC80 cells. Cells were treated with or without 9-cis-RA (1 μM) for 12 hours, then immunostained with anti-Hsp60 antibody. GFP-TR3ΔN, GFP-TR3ΔC and mitochondria (Hsp60) were visualized using a confocal microscope. (B) Interaction of RXRα with different TR3 mutants. GFP-TR3ΔN or GFP-TR3ΔC together with Myc-RXRα was cotransfected into HEK293 cells. Cell extracts were prepared and immunoprecipitated with anti-Myc antibody. The immunoprecipitate was subjected to SDS-PAGE, blotted and probed with anti-GFP antibody. The same membrane was also blotted with anti-Myc antibody to determine immunoprecipitation (IP) specificity and efficiency. Input represents 5% of cell lysates used in the IP western blot assay. The empty vector was used as a control.

demonstrated that TR3 nuclear export depends on the CRM1 pathway and thus is sensitive to LMB (Katagiri et al., 2000). In our present study, we found that 9-cis retinoic acid stimulated nuclear TR3 export in the form of a RXRα/TR3 heterodimer, pointing to the possibility that there exist multiple mechanisms for TR3 export that appear to be determined by distinct stimuli and/or different cell types. In support of the conclusions drawn by Katagiri et al. (Katagiri et al., 2000), we also found that the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA) induces the mitochondrial localization of endogenous TR3 in gastric cancer cells BGC-823 in an LMB-sensitive manner and presumably through the CRM1 pathway (Wu et al., 2002). However, in the current model, RXRα nuclear export was insensitive to LMB, consistent with the fact that there is no classical NES in RXRα (Black et al., 2001). Instead, a novel type of NES within the DBD of RXRα was identified, as an intact DBD was required for RXRα export from the nucleus, although RXRα deletion mutants that lacked different segments of DBD maintained their ability to enter the nucleus. In addition, RXRα shuttling might be associated with ATP energy supply. Nuclear pore complex and nuclear localization signal (NLS) sequences were responsible for RXRα nuclear import. Thus, distinct stimuli do indeed promote different receptor imports and exports.

TR3, through its ability to bind to a variety of response elements, can regulate their transcriptional activities and mediate diverse signaling to affect cell proliferation and apoptosis (Kang et al., 2000; Nigg, 1997; Woronicz et al., 1995). For example, binding of TR3/RXRα heterodimer to the retinoic acid receptor β (RARβ) promoter in regulating RARβ expression is critical in apoptosis induction (Wu et al., 1997a). However, recently we and other groups have reported that TR3-dependent apoptosis in gastric and prostate cancer cells does not require its DNA binding and transactivation, but is associated with translocation of TR3 from the nucleus to the mitochondria (Li et al., 2000; Wu et al., 2002), revealing a novel linkage between the translocation of TR3 and its role in apoptosis initiation. The fact that TR3ΔDBD directly localized in the mitochondria and induced apoptosis regardless of 9-cis retinoic acid treatment, and also that TR3ΔN effectively targeted to mitochondria through RXRα under 9-cis retinoic acid stimulation and induced apoptosis of MGC80-3 cells, strongly suggested that mitochondrial TR3 is critical for apoptosis induction. These results were consistent with a recent report showing that TR3 functions in the nucleus to induce cell proliferation, whereas it acts in mitochondria to induce apoptosis in lung cancer cells (Kolluri et al., 2003). In addition, mitochondrial targeting of TR3 was also shown to mediate the apoptotic effect of the Sindbis virus in NIH3T3 cells (Lee et al., 2002). In PC12 cells, NGF-induced TR3 nucleocytoplasmic translocation potentially regulates cell differentiation (Katagiri et al., 2000). Thus, all of these findings point to the possibility that the cellular redistribution of TR3

plays an important role in determining its distinct biological functions.

In summary, we found that RXRα can undergo nucleocytoplasmic shuttling, by which it plays an important role in assisting TR3 translocation from the nucleus to the cytoplasm through heterodimerization with TR3. However, such TR3 transportation cannot be performed by RXRα alone: only under the stimulation of 9-cis retinoic acid can the RXRα/TR3 heterodimer enter the mitochondria, where TR3, rather than RXRα, exerts its function on apoptosis initiation. Therefore, our data demonstrate the distinct and coordinated biological functions of the nucleocytoplasmic translocation of TR3 and RXRα in gastric cancer cells, with the former initiating apoptosis and the latter facilitating TR3 translocation as a carrier.

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