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Polo-like kinase 2 gene expression is regulated by the orphan nuclear receptor estrogen receptor-related receptor gamma (ERRγ)

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Abstract

Estrogen receptor-related receptor gamma (ERRγ) is a member of the nuclear receptor family of transcriptional activators. To date, the target genes and physiological functions of ERRγ are not well understood. In the current study, we identify that Plk2 is a novel target of ERRγ. Northern blot analysis showed that overexpression of ERRγ induced *Plk2* expression in cancer cell lines. ERRγ activated the *Plk2* gene promoter, and deletion and mutational analysis of the *Plk2* promoter revealed that the ERRγ-response region is located between nucleotides (nt) −2327 and −2229 and −441 and −432 (relative to the transcriptional start site at +1). Electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) analysis demonstrated that ERRγ binds directly to the *Plk2* promoter. Overexpression of ERRγ in the presence of the mitotic inhibitor nocodazole significantly decreased apoptosis, and induced S-phase cell cycle progression through the induction of *Plk2* expression. Taken together, these results demonstrated that *Plk2* is a novel target of ERRγ, and suggest that this interaction is crucial for cancer cell proliferation.

Keywords: ERRγ; Plk2; Gene regulation

The estrogen-related receptors (ERRs) α, β, and γ are orphan nuclear receptors that are closely related to estrogen receptors (ERs), and have identical target DNA response elements as ERs [1,2]. However, ERRs do not respond to the ER ligand or target the same genes as ERs [1,2]. ERRγ is the most recently identified member of the ERR subfamily, and is a constitutively active nuclear receptor [3,4]. To date, little is known about its *in vivo* functions.

Several studies have demonstrated that the ER antagonist 4-hydroxytamoxifen (4-OHT) directly binds to and inactivates ERRγ, which suggests that 4-OHT is an inverse agonist of ERRγ [5]. However, the underlying mechanism and physiological relevance of the action of 4-OHT toward ERRγ has yet to be elucidated. Recently, GSK4716 was identified as a synthetic agonist of ERRγ. Since ERRγ is a transcription factor that binds to specific target promoter sequences, the identification of ERRγ target genes is key to understanding the role of ERRγ *in vivo*. Previously, we and others reported that ERRγ regulates *SHP*, *DAX-1*, *PGC-1*, *ERRα*, *MAO*, and *PDK4* gene expression [6–11].

The Plk proteins are members of a family of highly conserved, multi-functional serine/threonine kinases that participate in cell cycle regulation and the cellular response to stresses, such as DNA damage [12–14]. There are four Plk family members in mammalian cells: Plk1, Plk2 (Snk), Plk3 (Fnk/Prk), and Plk4 (Sak). Each Plk family member has an N-terminal catalytic domain and a C-terminal Polo box, which mediates the intracellular localization

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of the kinase. Both Plk2 and Plk3 were originally identified as immediate early-transcripts induced by serum stimulation of quiescent mouse fibroblasts [14]. Several lines of evidence indicate that Plk2 mRNA is induced by the tumor suppressor p53 in cancer cells, and that Plk2 functions as a tumor suppressor in B-lymphocytes (BL) [15,16]. Plk2 is required for centriole duplication near the G_1-S transition, which suggests that it plays a critical role in cellular proliferation [17]. Plk2−/− mice are viable, which implies that other Plk family members or cell cycle kinases can compensate for Plk2 during replication [17]. Silencing of *Snk*/*Plk2* by small interfering RNA (siRNA) gene targeting in the presence of the mitotic inhibitors paclitaxel (Taxol) or nocodazole significantly increased apoptosis, similar to the effect of p53 mutations that confer Taxol sensitivity [15].

In the current study, we performed DNA microarray analysis following overexpression of ERRγ in 293T and INS-1 cells to identify putative target genes of ERRγ. Among the genes whose expression was altered by ERRγ, *Plk2* expression was significantly increased. The *Plk2* promoter was activated by ERRγ. Overexpression of ERRγ decreased apoptosis in the presence of nocodazole, and induced S-phase cell cycle progression through the induction of *Plk2*. We conclude that ERRγ regulates cellular apoptosis by activating Plk2, and suggest that the functional interaction of ERRγ and Plk2 is a potential target for the development of cancer therapeutics.

Materials and methods

Plasmids and DNA constructs. pcDNA3/HA-ERR α, β, and γ, p53, and V5, pCDNA3 Plk2, and pGL3 containing the *Plk2* promoter were described previously [6,7,15]. The *Plk2* promoter deletion constructs −1847 *Plk2* and −448 *Plk2* were amplified by PCR using appropriate primers and cloned into pGL3 using the MluI and XhoI restriction enzyme sites. The mutant gene reporter constructs mtERRE1/Luc, mtERRE2/Luc, and mtERRE1&2/Luc were generated with the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). All clones were verified by sequencing analysis.

Cell culture and transient transfection assay. The rat insulinoma cell line INS-1 was cultured in media containing 11.2 mM glucose and 2 mM L-glutamine. Human embryonic kidney 293T, monkey kidney COS-1, and human cervical cancer Hela cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (Gibco) in humidified air containing 5% CO_2 at 37 °C. Transfections were carried out using Superfect reagents (Qiagen Inc., Germany), according to the manufacturer's instructions. Approximately 40–48 h post-transfection, cells were harvested and luciferase activity was measured and normalized to β-galactosidase activity. Experiments were performed three times in duplicate.

Gel mobility shift assay. Double-stranded oligonucleotides containing the estrogen-related receptors response element (ERRE) were radiolabeled with [α-^{32}P]dCTP using the Klenow fragment of DNA polymerase I. The sequences of the oligonucleotides used in these studies are depicted in Fig. 2C. *In vitro* translation reactions were carried out using the TNT T7 quick-coupled transcription–translation system (Promega). Radiolabeled probe (15,000 cpm) was incubated with *in vitro* translated ERRγ, or pcDNA3, as a control in binding buffer containing 10 mM Tris (pH 8.0), 40 mM KCl, 0.05% Nonidet P-40, 6% glycerol, 1 mM dithiothreitol, and 1 μg of poly(dI–dC). Binding reactions were incubated at room temperature for 20 min and then a 50–100-fold molar excess of cold competitor oligonucleotide was added, and the reactions were incubated for an additional 15 min. DNA-protein complexes were analyzed by 5% polyacrylamide gel electrophoresis in 1× TBE. Gels were dried and then exposed to Kodak RX film (Eastman Kodak Co., Rochester, NY) for 12 h at −80 °C.

ChIP assay. ChIP assays were carried out using a ChIP Assay Kit (Upstate, USA) according to the manufacturer's instructions. Hela cells were transfected with 1 μg of pcDNA3 HA-ERRγ or pcDNA3 HA using Superfect reagents (Qiagen Inc., Germany). Forty-eight hours after transfection, cells were fixed with formaldehyde, and then subjected to immunoprecipitation using an anti-HA antibody (Sigma). DNA was extracted and then amplified by PCR (32 cycles) using primers specific for the ERR response region within the human *Plk2* promoter, as depicted in Fig. 3B. The sequences of the primers used for PCR were as follows: ERRE1, 5′-AGC CTT CTA CAT CTT AGA GAG TAG G-3′ (Forward) and 5′-GAG GAG AAA GGG AAA GGC TTT GCC A-3′ (Reverse); ERRE2, 5′-TGC CCC AGC TAG ACA CCA GCG AGC T-3′ (Forward) and 5′-ACA AAA CAG AAT CGC GAG GGC CGA C-3′ (Reverse).

Recombinant adenovirus and viral infection. The generation of the recombinant adenovirus for the expression of ERRγ (Ad ERRγ) and the control adenovirus for mock-infection (AdGFP) were described previously [7,18]. Hela and U2OS cells were infected with adenovirus in a volume of 2 ml DMEM at a multiplicity of infection (MOI) of 25 or 50 for 3 h at 37 °C. Culture medium was replaced with fresh DMEM supplemented with 10% fetal calf serum and the infection was allowed to proceed for 24 h. Cells were then harvested for RNA isolation.

Reverse transcriptase PCR (RT-PCR) analysis. Hela cells were seeded in 6-well plates, allowed to incubate for 24 h, then treated with siRNAs for ERRγ (siERRγ) or GFP (siGFP) as a control. Total RNA was isolated using the TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions, and ERRγ and Plk2 mRNAs were analyzed by RT-PCR. First-strand cDNA was synthesized from 1 μg of total RNA using anchored oligo(dT) primers and reverse transcriptase. The mRNAs of ERRγ and Plk2 were then amplified by PCR using 23 and 31 cycles, respectively, and the following primers: ERRγ forward, 5′-GAC TTG ACT CGC CAC CTC TC-3′ and ERRγ reverse, 5′-GTG GTA CCC AGA AGC GAT GT-3′; Plk2 forward, 5′-TCA GCA ACC CAG CAA ACA CAG G-3′ and Plk2 reverse, 5′-TTT CCA GAC ATC CCC GAA GAA CC-3′.

Northern blot analysis. Total RNA from cultured cells was isolated using the Tri-Reagent solution (Molecular Research Center, Inc., Cincinnati, OH). Twenty micrograms of total RNA was fractionated by electrophoresis on a 1.2% agarose gel containing formaldehyde, and then transferred to a nylon membrane by capillary transfer in 20× sodium citrate-sodium chloride (SSC). Membranes were subjected to UV cross-linking and prehybridization, then hybridized overnight at 42 °C in a solution containing 50% formamide, 5× SSC, 50 g/L dextran sulfate, 1 mM EDTA, 250 μg/ml denatured herring sperm DNA, and 2–4×10^6 cpm of a ^{32}P-labeled cDNA probe specific for human *Plk2* [15]. After hybridization, membranes were washed twice for 5 min each time at room temperature in 2× SSC and 0.1% SDS, followed by 20 min at 55 °C in 0.5× SSC and 0.1% SDS. Membranes were then exposed to Kodak RX film (Eastman Kodak Co., Rochester, NY) for 2 days at −80 °C. To normalize the data, membranes were stripped by boiling in 0.1× SSC and 0.5% SDS twice for 20 min each time and then re-probed using a full-length cDNA probe specific for glyceraldehyde-3-phosphate-dehydrogenase (GAPDH).

siRNA gene targeting. si-ERRγ was chemically synthesized (Shamchully Pharm. Co., Ltd), deprotected, annealed, and then transfected into the indicated cells (Fig. 1D), as previously described [7]. Transfections were carried out using Oligofectamine reagent (QIAGEN), and 48 h after transfection, total RNA was isolated from the cells and subjected to RT-PCR analysis.

FACS analysis. The indicated cells (approximately 1×10^6 cells) were collected, washed with 1× PBS, and then incubated with monoclonal antibodies against Annexin 5 (PE conjugation) and 7-AAD (FITC conjugation). Cells were also stained with propidium iodide for analysis of cell cycle distribution. FACS analysis was carried out using BECKMAN COULTER XL system I.

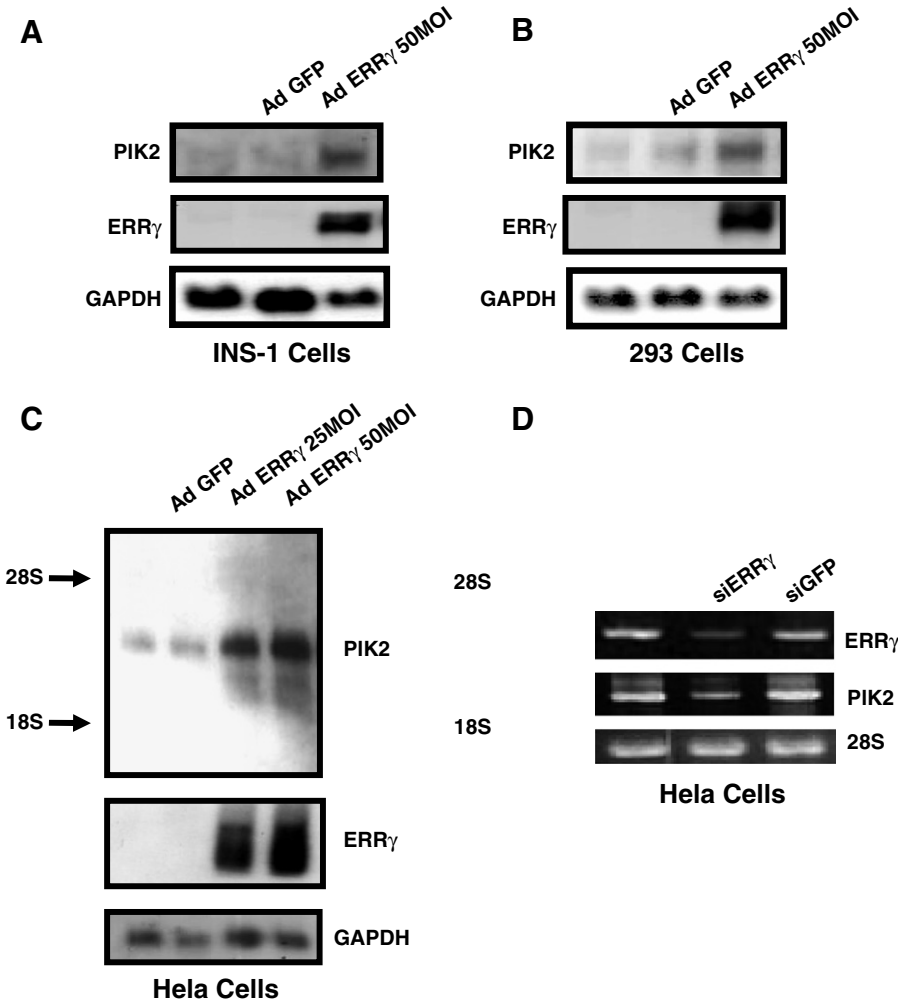

Fig. 1. ERRγ regulates *Plk2* expression. (A–C) Cells were infected with an adenoviral expression vector for ERRγ (AdERRγ) (25 or 50 MOI), or mock-infected. Total RNA was isolated and analyzed for Plk2 and ERRγ mRNA levels by Northern blot using a human *Plk2* and mouse *ERRγ* cDNA, respectively, as a probe. The expression of *GAPDH* was used as an internal control. (D) Endogenous ERRγ gene expression is inhibited by siERRγ in Hela cells. Expression of *ERRγ*, *Plk2* and *GAPDH* (as a control) were analyzed by RT-PCR.

Results

Plk2 gene expression is regulated by ERRγ in cancer cells

To identify the target of ERRγ, we performed DNA microarray analysis of gene expression in cells that overexpressed ERRγ. In 293T cells, 1405 genes were up-regulated and 904 genes were down-regulated by ERRγ. In INS-1 cells, 408 genes were up-regulated and 233 genes were down-regulated by ERRγ (data not shown). The expression of *Plk2* was significantly increased in both 293T and INS-1 cells. We then examined ERRγ-induced activation of *Plk2* expression in INS-1 and 293T cells by Northern blot analysis. INS-1 and 293T cells were infected with an adenoviral expression vector for ERRγ (Ad ERRγ), and the mRNA level of *Plk2* was examined. As shown in Fig. 1A and B, overexpression of ERRγ activated *Plk2* expression in both INS-1 and 293T cells. Previously, it was reported that Plk2 is expressed in several cancer cell lines [15]. As shown in Fig. 1C, overexpression of ERRγ induced *Plk2* expression in the Hela and U2OS (data not shown) cancer cell lines, but not the breast cancer cell line MCF-7 (data not shown). ERRγ is constitutively expressed in Hela cells [9]. We were interested in whether inhibition of ERRγ expression in Hela cells effected *Plk2* expression. As shown in Fig. 1D, *Plk2* expression was inhibited in HeLa cells that were pre-treated with an siRNA that targeted ERRγ siERRγ). These results demonstrated that *Plk2* is up-regulated by ERRγ in certain cancer cell lines.

ERRγ directly regulates the Plk2 promoter via ERREs

Previously, it was reported that *Plk2* is a novel transcriptional target of p53, and there are several p53 response elements in the human *Plk2* promoter [15]. The results of Northern blot analysis indicated that *Plk2* expression is induced by ERRγ (Fig. 1). To determine whether ERRγ regulated *Plk2* expression via transcriptional activation of the *Plk2* promoter, we performed a series of transient transfection experiments using various *Plk2* promoter con-

structs and an expression vector for ERRγ. As shown in Fig. 2A and B, expression of ERRγ activated a reporter gene driven by the full-length *Plk2* promoter (−2534 *Plk2*) in Hela and COS-1 cells. ERRβ also activated the *Plk2* promoter, but ERRα had no effect on the transcriptional activity of *Plk2*. These results suggested that ERRγ regulates *Plk2* expression by recognizing specific DNA response elements in the *Plk2* gene promoter. Previously, we showed that ERRγ recognizes the sequence T(N)AA GGTCA or AGGTCA (half sites) [7]. Sequence alignment of Plk2 promoter showed that there are two putative ERRγ response elements (ERRE1 and ERRE2) in the *Plk2* promoter (Fig. 2C). To determine whether these sequences were necessary for ERRγ-mediated activation of the *Plk2*, a series of 5′ deletion mutants of the *Plk2* promoter were constructed (see Fig. 2C). Since the *Plk2* promoter contained two putative ERREs, we designed deletion constructs that contained one, both, or neither ERRE. As shown in Fig. 2D, deletion of the promoter sequence to nt −1847 decreased activation of the *Plk2* promoter by ERRγ. Further deletion of the promoter sequence to nt −428 gradually decreased activation of *Plk2* promoter by ERRγ. In addition, mutation of ERRE2 (mtERRE2/Luc) decreased ERRγ-induced *Plk2* promoter activity,

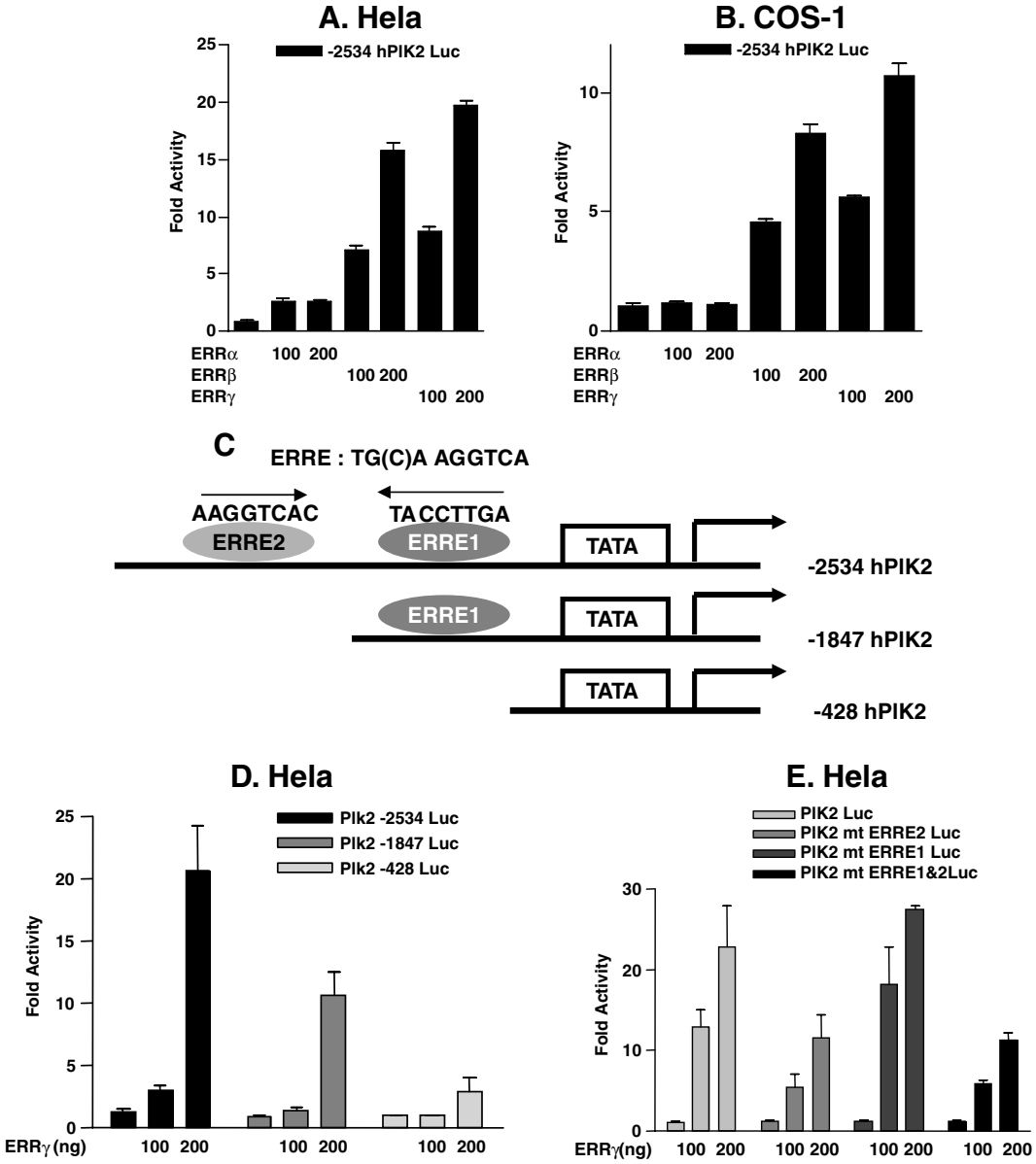

Fig. 2. ERRγ activates the *Plk2* gene promoter. (A, B, D, and E) Cells were transiently transfected with 200 ng of the indicated reporter gene construct, together with the indicated concentrations (ng) of HA-pcDNA3-ERR α, β, and γ. Approximately 40 h after transfection, the cells were harvested and luciferase activity was measured and normalized against β-galactosidase activity. Data represent the means and standard error of duplicate assays, and are representative of at least three independent experiments. The results are expressed as *n*-fold activation over cells that were transfected with a control empty vector, and are representative of three independent experiments. Data represent the means and standard error of duplicate assays. (C) Schematic representation of the *Plk2* promoter.

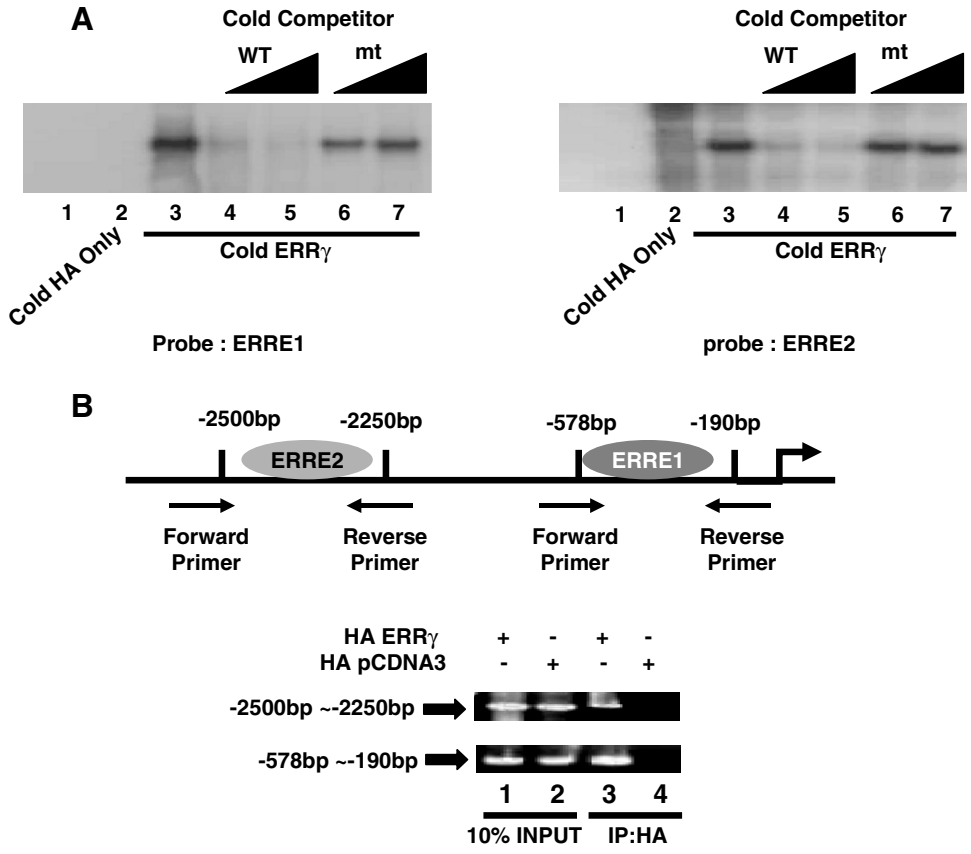

Fig. 3. ERRγ binds to ERREs in the *Plk2* promoter. (A) Gel mobility shift assay performed with the indicated ^{32}P-labeled probe and *in vitro* translated ERRγ (2 μl). Where indicated, a 50- to 100-fold molar excess of unlabeled competitor oligonucleotide was added. (B) Hela cells were transfected with an expression vector for HA alone, or HA-ERRγ. Soluble chromatin was prepared from cells, and subjected to immunoprecipitation using a monoclonal antibody against HA (lanes 3 and 4). Lane 1, a 250-bp fragment that contains the ERRγ binding site; lane 2, 10% of the soluble chromatin used in the reaction (input).

whereas mutation of ERRE1 (mtERRE1/Luc) had no effect (Fig. 2E). These results indicated that ERRE2 is necessary for the activation of the *Plk2* promoter by ERRγ, and suggested that ERRE2 plays a major role in the activation of *Plk2* expression by ERRγ.

ERRγ directly binds to the Plk2 promoter in vitro and in vivo

We next performed a gel mobility shift assay and ChIP analysis to determine whether ERRγ interacted directly with the ERREs of the *Plk2* promoter. As shown in Fig. 3A, ERRγ formed specific complexes with ERRE1 and ERRE2, and addition of a 50- or 100-fold molar excess of unlabeled ERRE1 and ERRE2 competed strongly for the formation of DNA-protein complexes. To determine whether ERRγ binds to the *Plk2* promoter *in vivo*, we performed a ChIP assay, using primers designed to amplify ERRE1 or ERRE2. Expression vectors for a hemagglutinin (HA) fusion protein of ERRγ (HA-ERRγ), or HA alone, were transfected into Hela cells. As shown in Fig. 3B, a 250-bp fragment (corresponding to ERRE1 and ERRE2) was amplified from cells that expressed HA-ERRγ, but not HA alone. This indicated that ERRγ forms a specific complex with the *Plk2* promoter *in vivo*. Taken together, these results suggested that ERRγ regulates *Plk2* expression by binding directly to the ERREs in the *Plk2* promoter.

ERRγ modulates sensitization of cancer cell by nocodazole

Silencing of *Plk2* sensitizes cells to antimicrotubule agents, such as nocodazole, or to Taxol-induced apoptosis [15]. We next examined whether overexpression of ERRγ could decrease the sensitivity of cancer cells to nocodazole by inducing *Plk2*. Hela cells were infected with AdERRγ, or mock-infected with AdGFP for 24 h, then treated with nocodazole for 24 h. As shown in Fig. 4A, nocodozole induced apoptosis of Hela cells (20.8%). Hela cells that overexpressed ERRγ had a significantly reduced level of apoptosis (6.2%) compared to mock-infected cells, which indicated that they were less sensitive to nocodazole. *Plk2* expression was also induced by ERRγ in Hela cells (data not shown). These results indicated that ERRγ is involved in nocodazole sensitization. Warnke and colleagues showed that Plk2 is activated near the G_1-to-S transition of the cell cycle and is required for centriole duplication, which suggests that Plk2 is crucial for the G_1-to-S transi-

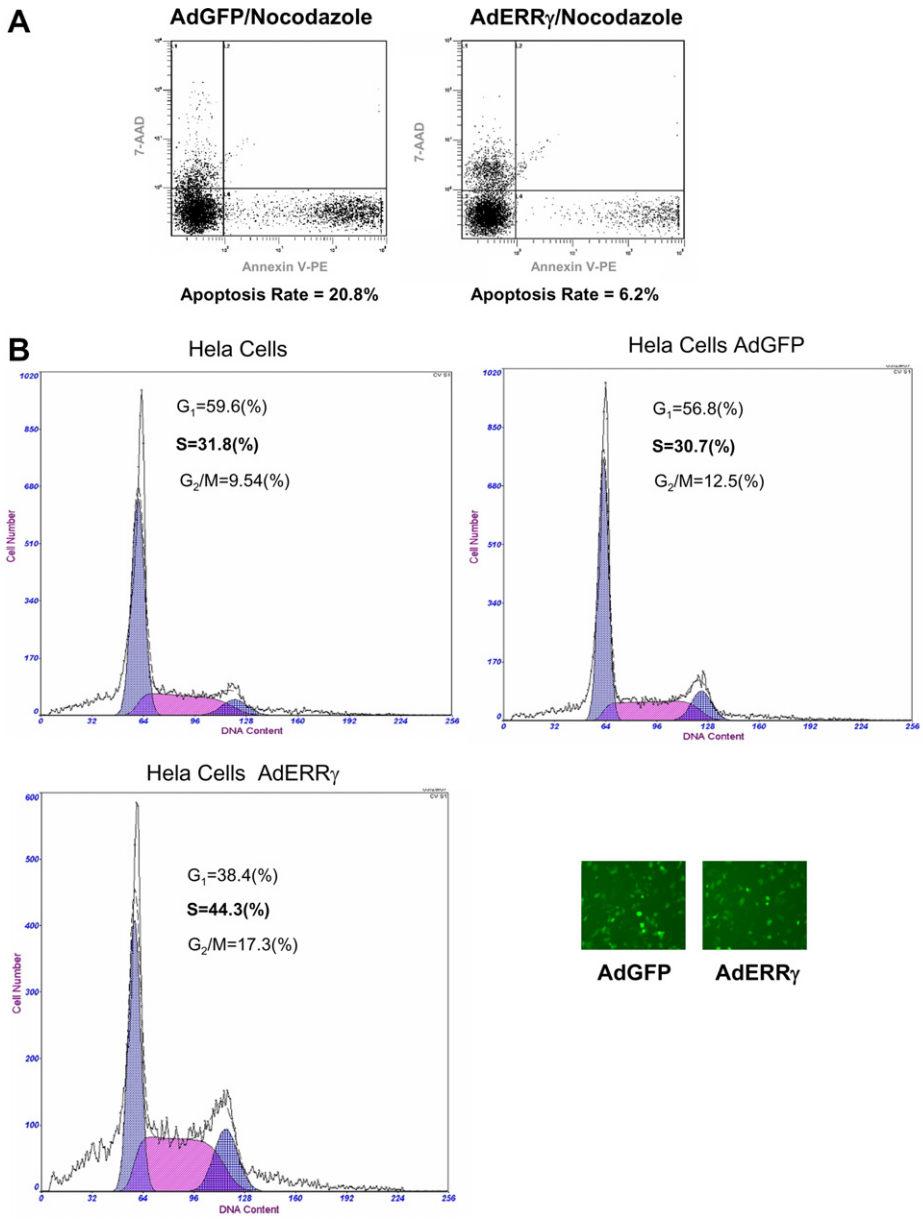

Fig. 4. ERRγ inhibits apoptosis in the presence of nocodazole. (A) Hela cells were infected with AdGFP (MOI of 50) or AdERRγ (MOI of 50). Twenty-four hours after infection, cells were treated with 1 μM nocodazole for an additional 24 h, then harvested and stained for Annexin V and 7-AAD. Cell apoptosis was measured by FACS analysis. (B) Hela cells were synchronized by incubation with nocodazole for 24 h. Cells were then mock-infected with AdGFP (MOI of 50) or infected with AdERRγ (MOI of 50) for 24 h. Cell cycle analysis was performed by FACS. The results are representative of three independent experiments. AdGFP or AdERRγ indicates virus infection efficiency (right, bottom panel).

tion during the cell cycle [17]. In their study, overexpression of a kinase-deficient mutant of Plk2 during S-phase arrest blocked centriole duplication [17]. To determine whether ERRγ affected the cell cycle, cells were pre-treated with nocodazole, infected with AdERRγ for 24 h, and then cells were analyzed by FACS. Overexpression of ERRγ significantly increased the percentage of cells in S-phase compared to mock-infected cells (Fig. 4B, 30.7% vs. 44.3%). Thus, ERRγ appeared to effect cell cycle progression via Plk2-induced S-phase progression. These results suggested that ERRγ regulates cell cycle progression and desensitizes cells to nocodazole through the activation of *Plk2*.

Discussion

ERRγ is a recently identified orphan nuclear receptor, and its function is not well understood. In the current study, we performed DNA microarray analysis of 293T and INS-1 cells that overexpressed ERRγ and identified the Plk2 is a target of ERRγ.

As shown in Figs. 1 and 2, ERRγ significantly activated *Plk2* gene expression and *Plk2* promoter activity. Our results showed that ERRγ regulates *Plk2* expression and the transcriptional activity of the *Plk2* promoter by binding directly to ERREs in the *Plk2* promoter (Fig. 3). However, mutation

or deletion of the ERREs of the *Plk2* promoter region did not completely abolish promoter activity, possibly because of the binding of ERRγ-interacting proteins to the *Plk2* promoter via as-yet unidentified regulatory regions.

In contrast to the function of Plk1, the physiological role of Plk2 is poorly understood. Recently, it was suggested that Plk2 plays a role in B-cell malignancies, and it was shown that the expression of *Plk2* is down-regulated in lymphomas [16]. The expression of *Plk2* induces apoptosis in BLs, which indicates that Plk2 functions as a tumor suppressor in B-cells [16]. Silencing of Plk2 induces apoptosis in the presence of nocodazole; thus, Plk2 appears to have a positive regulatory role in cell survival, although underlying mechanism is not clear. Since *Plk2* was shown to be a target of ERRγ, we were interested in whether ERRγ also affected apoptosis. As shown in Fig. 4, overexpression of ERRγ in Hela cells significantly reduced apoptosis in the presence of nocodazole, which indicates that ERRγ regulates Plk2 function in the inhibition of apoptosis in the presence of nocodazole.

ERRγ has also been shown to be involved in cell growth in prostatic cells, which indicates that the function of ERRγ is cell type specific.

We showed that overexpression of ERRγ induces S-phase progression. The expression of cyclin family genes is regulated by the cell cycle, and we found that overexpression of ERRγ affected cyclin gene expression as well (data not shown). Thus, ERRγ may regulate cell cycle gene expression via Plk2 directly, or via an indirect pathway. Additional studies are needed to clarify the functional relationship between Plk2, ERRγ, and the cell cycle.

In summary, we have shown that *Plk2* is a novel target of ERRγ. Furthermore, ERRγ regulates cell apoptosis and cell cycle progression in the presence of nocodazole. These results will help in gaining a better understanding of the intrinsic functions of ERRγ.

Acknowledgments

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