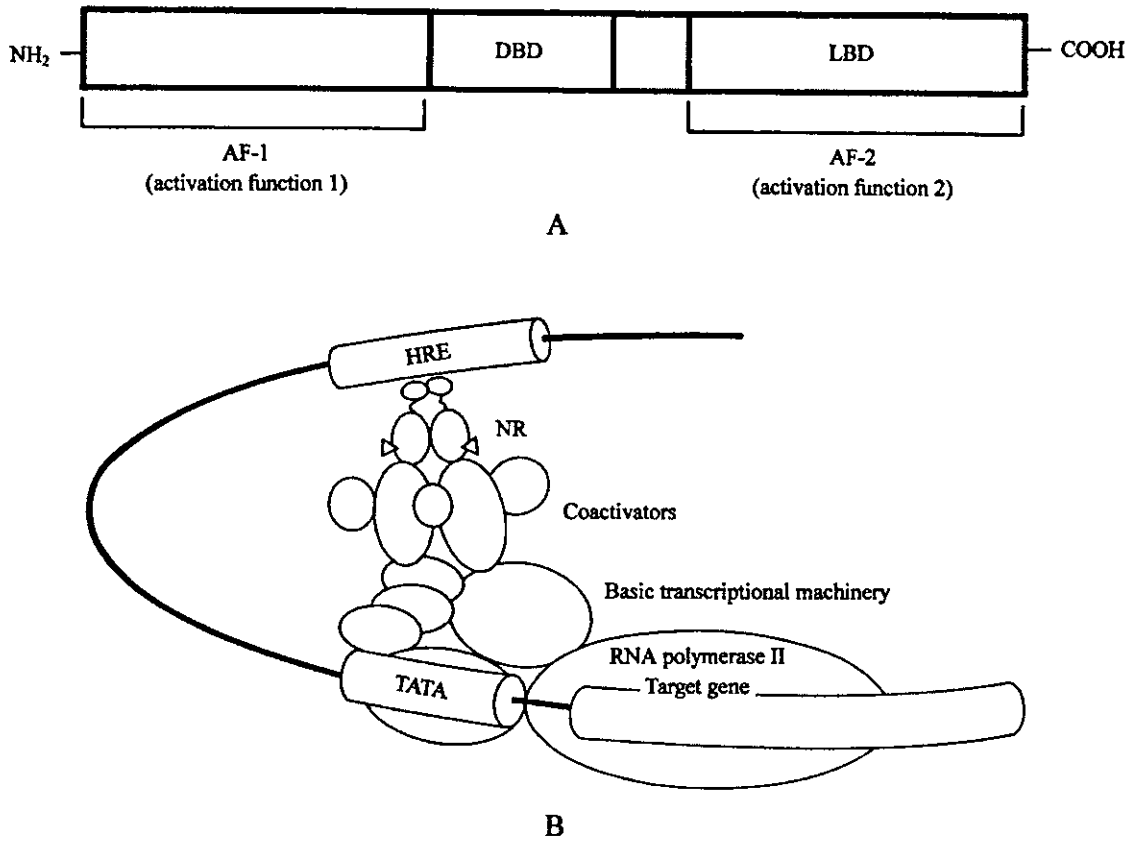

Figure 1. (A) Structure of nuclear receptors (NRs). DBD, DNA-binding domain; LBD, ligand-binding domain. (B) Interactions among the nuclear receptor (NR), coactivators and basic transcriptional machinery.

for exerting the full nuclear receptor transactivation capacity (9). Recently, it was found that rather than operating individually these coregulators operate together by forming an enormous protein complex. For example, a complex composed mainly of CBP/p300 and the p160 system, has histone acetyltransferase (HAT) activity that acetylates a basic amino acid of the histone protein and alters the chromatin structure. This makes it easier for transcription factors to be recruited on the DNA and thus promotes transcription. However, complexes such as the DRIP/TRAP complex do not have HAT activity. On the contrary, the corepressor SMRT/NCoR complex represses transcription activity by coupling with the nuclear receptor that is unbound to the ligand and recruits the histone deacetylation enzyme (HDAC), which has an opposing effect to HAT on the promoter. It is thus speculated that once ligand binds to the nuclear receptor, the corepressor complex dissociates from the receptor and the coactivators are recruited onto the promoter (2, 3).

AIS and Coactivator Disease

Of several steroid hormone resistance syndromes such as AIS, primary glucocorticoid resistance due to GR abnormalities, and type II vitamin D resistance due to vitamin D receptor (VDR) abnormalities, AIS is thought to be the most common. AIS occurs in genetically 46XY males who have testis but suffer from various degrees of virilization failure because of androgen insensitivity (10). This disease can be classified into four types depending on the degree of virilization failure as follows: complete AIS, partial AIS, Reifeinstein syndrome and male infertility. Patients with complete AIS show male pseudohermaphroditism, that is, they have female external genitalia and female type breast development. Patients with Reifenstein syndrome have hypospadias, a micropenis and gynecomastia. Most cases of AIS are the result of *AR* gene mutations located on chromosome Xq11-12. More than 300 AIS mutations have been identified. The degree of AR dysfunction due to *AR* gene abnormality generally correlates with the clinical severity (feminization) (10, 11).

We previously examined a complete AIS patient without an *AR* gene mutation (5). The patient also expressed a normal androgen binding capacity in the pubic skin fibroblast. Therefore, it was postulated that the pathogenesis of this patient was due to an unknown factor that might have modulated AR function. The degree of transcriptional activation by the normal AR in the genital fibroblast of the patient was less than 9% of that in the fibroblasts of normal subjects. However, the fibroblasts of another complete AIS patient with an *AR* gene mutation (Arg 840→His) were similar to those of the normal subjects. The degree of transcriptional activation by the AR-GR chimera that consisted of the N-terminal and DNA-binding domains of the AR and the C-terminal domain of the GR, in the genital fibroblast of the patient was 12 to 17% of that in the fibroblasts of the normal and complete AIS patient. In contrast, the degree of transcriptional activation by the GR and GR-AR chimera that consisted of the N-terminal and DNA-binding domains of the GR and the C-terminal domain of the AR was similar among the three types of fibroblasts. Furthermore, the degree of ligand-independent transcriptional activation by the N-terminal domain fragment including the AF-1 region of the AR in the genital fibroblasts of this patient was approximately 20% of that in the fibroblasts of the normal and complete AIS patients. However, the degree of transcriptional activation by the N-terminal domain fragment of the GR was similar among the three types of fibroblasts. The degree of ligand-dependent transcriptional activation by the C-terminal domain fragments of the AR and GR was similar among the three types of fibroblasts.

Together, these findings suggest that transmission of the transactivating signal from the AF-1 region of the AR to the basal transcriptional machinery might be impaired in this patient. Finally, as a result of the AR AF-1 binding protein investigation of the pubic skin fibroblasts using glutathione-S-transferase (GST) pull down analysis, it was revealed that the absence of 90 kDa protein, which is present in normal fibroblasts, is most likely the cause of AIS in this case. From these findings, we proposed a new clinical concept of steroid hormone resistance, a coactivator disease, in which a physiologically indispensable AF-1-specific coactivator crucial to the transactivation activity of AR is responsible for complete AIS (Fig. 2) (5).

Other Kinds of Coregulator Disease

Rubinstein-Taybi (RTS) syndrome

Rubinstein-Taybi (RTS) syndrome is another coregulator disease. In 1963, RTS syndrome was reported as a disease that presents autosomal dominant inheritance and clinically characterized by, for example, short stature, craniofacial malformation, heart malformation and mental retardation (12). In 1995, CREB (cAMP response element binding protein)-binding protein *CBP* gene defects was reported as the cause of this disorder (6). CBP is a protein originally identified as a coactivator that stimulates the phosphorylation-dependent transcriptional activation of CREB. CBP also works as a nuclear integrator when various classes of transcription factors crosstalk with each other (13). In a CBP heterozygous (CBP +/–) mouse created by gene targeting, several bone malformations and cardiac anomalies similar to those of RTS were observed, supporting a correlation between RTS and CBP (14). However, how CBP abnormality causes RTS has not been well established. Unlike CBP +/– mice, CBP –/– embryos did not exhibit cardiac anomalies (15), suggesting that at least the cardiac anomaly in CBP +/– mice might be caused by the dominant-negative action of the truncated CBP mutant (16). The impaired HAT activity observed in the CBP mutant might also be a cause of RTS in humans (17). However, since it has been reported that only 20% of 194 patients with RST were found to have *CBP* gene mutations there is the possibility that other gene mutations are also involved in the diverse RST symptoms (18).

Multiple hormone resistance

A case involving sisters with resistance to multiple steroid hormones such as glucocorticoid, androgen and mineralocorticoid, revealed the possibility that some abnormalities in the coactivators that interact with GR, MR and AR might be present (4, 19, 20). Investigations showed that SRC-1 null mice exhibited partial resistance to sex hormones, estrogen, progesterone and androgen with impaired development of testis, and uterine and mammary glands (21). However, since no abnormalities of the known coregulators such as SRC-1, TIF-2, AIB1 and SRA were found, the etiology of these cases remains unknown (19, 20).

Refetoff syndrome

Resistance to thyroid hormone (RTH) is an inherited disorder called Refetoff syndrome that involves reduced tissue sensitivity to the thyroid hormone. Most cases associated with RTH are caused by *TRβ* gene mutations. The resulting mutant receptors suppress the functioning of normal TR as a result of a dominant negative effect. It has been reported that dissociation of corepressors such as NcoR and SMART or recruitment of the coactivator SRC-1 are impaired in mutant TRβs (22, 23). However, some patients with RTH do not have *TRβ* gene mutations. Since SRC-1 null mice exhibited a phenotype similar to RTH, it can, however, be speculated that some coregulator abnormalities are present in these patients (24). Nevertheless, no known coregulator defects such as SRC-1 mutations have been found in such cases.

TRAP complex-related phenotypes

Another coactivator complex, the human TR-associated protein (TRAP) complex was originally identified through its intracellular ligand-dependent association with TRα. TRAP-related complexes have also been isolated and identified as being completely or partly SRB/MED-containing cofactor complex (SMCC) or DRIP/TRAP complexes and so on (25). The TRAP complex lacks HAT activity but does interact with RNA polymerase II and is thought to be a component of an

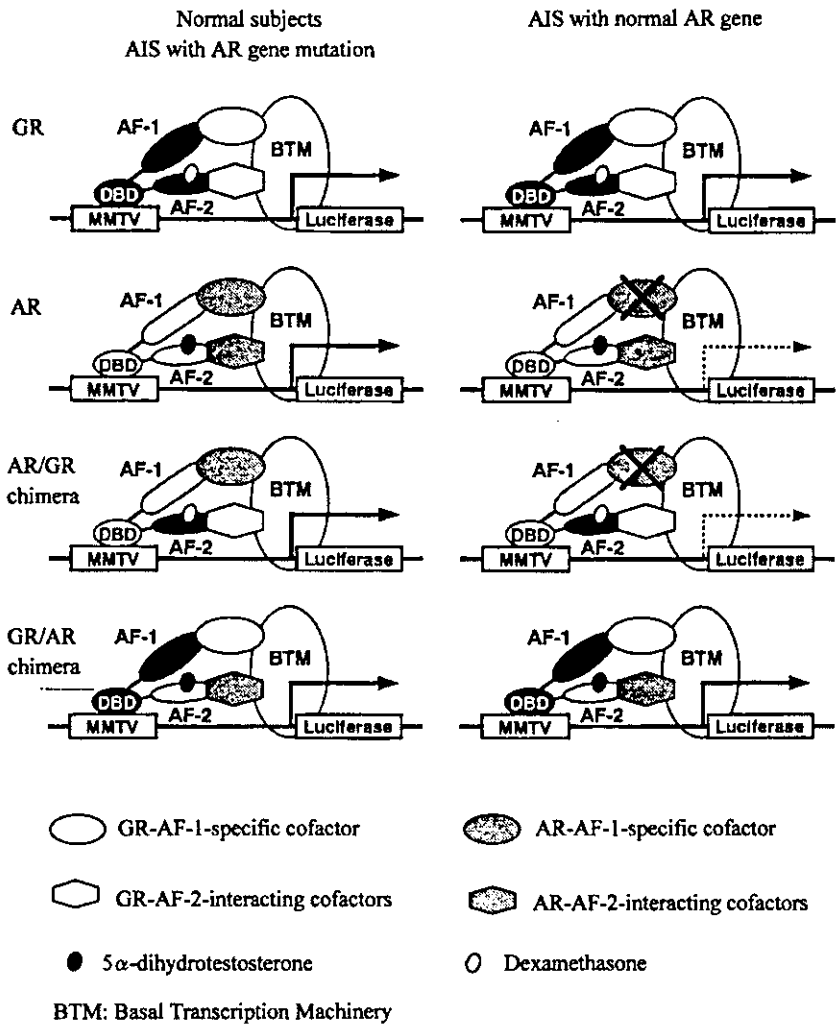

Figure 2. Schematic representation of the coactivator defects in AIS patients (ref. 5). In the fibroblasts of AIS patients with normal genes, transactivation by the transfected AR or AR-GR chimera was low because of defects in an AR AF-1 specific coregulator essential to transmission of the transactivating signal. In the fibroblasts of AIS patients with an AR gene mutation ($Arg^{840} \rightarrow His$), the endogenous AR is inactive, but the transfected AR and AR-GR chimera are active, because the AR-AF-1-specific cofactor is intact. The transfected GR and AR-AR chimera are active among the three types of fibroblasts, since the GR AF-1 specific coregulator is intact. LBD: ligand-binding domain, BTM: basal transcriptional machinery.

RNA polymerase II holoenzyme. The TR complex interacts with a variety of activators that include not only nuclear receptors such as TR and VDR but also others such as p53, VP16, Sp1 and so on. As suggested by yeast mediator functions, this mammalian complex appears to function through direct interactions with both DNA-bound activators and RNA polymerase II, thus mediating the activator-enhanced recruitment of RNA polymerase II and other general transcription factors to the promoter. TRAP 220 is a component of the TRAP complex, and a mammalian homologue of the yeast mediator that shows diverse coactivation functions, interacting directly with nuclear receptors and RNA polymerase II. Complete disruption of the murine *Trap 220* gene produced null mutants that died during early gestation as a result of heart failure while exhibiting impaired neuronal development. Primary embryonic fibroblasts derived from these null mutants showed a prominent decrease in thyroid hormone receptor function that was restorable by ectopic TRAP 220. In

Table 1. Coregulator-related Diseases

A. Coregulator disease	
Rubinstein-Taybi syndrome	CBP gene deletions or mutations
Androgen insensitivity syndrome	AR AF-1 specific coactivator abnormality
Multiple hormone resistance	Absence of any defects in the coregulators examined
Refetoff syndrome	Absence of any defects in the coregulators examined
X-linked dementia and hypothyroidism	TRAP230
B. Diseases with coregulator abnormalities or altered interactions with the coregulators	
a. Altered expression or phosphorylation of the coregulators	
Breast cancer	increased expression of AIB1
	phosphorylation of AIB1
	increased expression of cyclin D1
	increased expression of SRA isoform
Prostate cancer	increased expression of TIF2 or SRC-1
	synergistic action of BRCA1 with the AR-TIF2 complex
	activation of cyclin E for AR transcription
Adrenal tumor	correlation between COUP-TF-1 and N-CoR mRNA expression
Huntington's disease	increased expression of CA150
Meningioma	increased expression of TIF2
AIDS	activation of Vpr for GR transactivation
b. Altered binding affinity with the coactivators	
Prostate cancer	increased binding affinity between the mutant ARs and TIF2
Kennedy's disease	decreased binding affinity between the AR
	polyglutamine repeats and TIF2 or SRC-1
Refetoff syndrome	increased binding affinity between the mutant TRβs and SRC-1
c. Altered binding affinity with the corepessors	
Refetoff syndrome	impaired dissociation of corepressors from the mutant TRβs

addition, haploinsufficient animals showed growth retardation and pituitary hypothyroidism (7, 25). Furthermore, it was reported that a mutation within the CAG repeat of the TRAP 230 subunit gene of the TRAP complex is correlated with X-linked dementia and hypothyroidism syndrome (7, 26). These results suggest a close functional association between the TRAP complex and pituitary-thyroid axis.

Various Clinical Situations That are Thought to be Related to Coregulators

It is well recognized that proliferations of mammary and uterine cancers are estrogen-dependent whereas those of prostate cancer are androgen-dependent. In such hormone-dependent cancers, it has been suggested that the degree of expression or phosphorylation of steroid hormone receptors and coregulators in cancerous tissues is closely associated with the extent of cancer cell proliferation or the prognosis. Although some changes in the expression of steroid hormone receptors such as ER, progesterone receptor (PR) or AR occur in accordance with tumorigenesis, it is reported that these changes are regulated by the expression of the coactivators or corepressors. In addition, it has been shown that signal crosstalk between the steroid hormone receptor and growth factor provokes phosphorylation of both the steroid hormone receptor and coregulators, consequently leading to more powerful activation of the steroid hormone receptors. One such example is AIB1, which is a coactivator and is especially overexpressed in mammary cancer (27). The mechanism by which growth factors such as insulin-like growth factor (IGF), which is an epidermal growth factor (EGF), promote mammary cancer proliferation involves MAPK activation by these growth factors leading to phosphorylation of ER (28) and AIB1 (27). The phosphorylated AIB1 couples tightly with p300/CBP leading to the enhancement of ER-mediated transactivation as a result of increased HAT activity (29). While ER also stimulates the expression of a cell cycle regulator, cyclin D1, AIB1 enhances the ER-mediated transcriptional activity of Cyclin D1 gene promoter. It is reported that Cyclin D1 is overexpressed from the early stages of breast cancer, suggesting that AIB1 also plays an important role in the regulation of Cyclin D1-associated proliferation mechanisms during the development of breast cancer (30, 31). Similar effects have also been reported with other coregulators, namely SRC-1 and TIF-2 on the estrogen-dependent transcription of Cyclin D1.

The mechanisms associated with the onset of leukemia, involve chromosomal translocation resulting in the formation of a chimera-type fusion protein composed of hematopoietic transcription factor, which leads to the suppression of hematopoietic differentiation as a result of a dominant negative effect. A co-repressor complex is involved in this mechanism. For example, the hematopoietic transcription activation factor, AML1, is associated with the onset of leukemia because it forms fusion proteins as a result of chromosomal translocations such as t (8, 21), t (12, 21), t (3,

21). Furthermore, the fusion protein, AML-ETO, that results from t (10, 23) translocation recruits corepressors such as NCoR and SMRT and the corepressor complex, thus suppressing differentiation of the hematopoietic cell and resulting in the production of leukemia (AML-M2) (32).

Since it is difficult to refer to each disease that is thought to be associated with coregulators, a summary of possible coregulator-related diseases is presented in Table 1.

Conclusion

The discovery of AR coregulator disease in association with AIS has verified the importance of the AF-1 region during AR transcription activities. Identification of the exact molecule that underlies this disease will be an important issue in future studies. Coregulators are essential in determining the tissue-specific actions and the specific biological functions of nuclear receptors. Therefore, extensive research of coregulators is expected to clarify the mechanisms associated with nuclear receptors as well as the pathogenesis of steroid resistance syndrome and hormone-dependent cancers.

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Activation of Peroxisome Proliferator-Activated Receptor-γ and Retinoid X Receptor Inhibits Aromatase Transcription via Nuclear Factor-κB

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Our previous studies demonstrated that a peroxisome proliferator-activated receptor (PPAR)-γ ligand, troglitazone (TGZ), and/or a retinoid X receptor (RXR) ligand, LG100268 (LG), decreased the aromatase activity in both cultured human ovarian granulosa cells and human granulosa-like tumor KGN cells. In the present study, we further found that a combined treatment of TGZ+LG decreased aromatase promoter II (ArPII) activity in both ovarian KGN cells and fibroblast NIH-3T3 cells in a PPARγ-dependent manner. Furthermore, the inhibition of both aromatase activity and the transcription of ArPII by TGZ+LG was completely eliminated when nuclear factor-κB (NF-κB) signaling was blocked by specific inhibitors, suggesting NF-κB, which is endogenously expressed in both fibroblast and granulosa cells, might be a mediator of this inhibition. Interestingly, activation of NF-κB by either forced expression of the p65 subunit or NF-κB-inducing kinase up-regulated ArPII activity. Positive regulation of aromatase by endogenous NF-κB was also suggested by the fact that NF-κB-specific inhibitors suppress basal activity of the aromatase gene. A concomitant formation of high-order complex between NF-κB p65 and ArPII was also observed by chromatin immunoprecipitation assay. Although activation of PPARγ and RXR affected endogenous expression levels of neither inhibitory κBα nor p65, it impaired the interaction between NF-κB and ArPII and the p65 based transcription as well. Altogether, these results indicate that activation of a nuclear receptor system, constituted by PPARγ and RXR, down-regulates aromatase expression through the suppression of NF-κB-dependent aromatase activation and thus provide a new insight in the mechanism of regulation of the aromatase gene. (*Endocrinology* 146: 85–92, 2005)

THE BIOSYNTHESIS OF estrogens is catalyzed by the enzyme complex referred to as aromatase cytochrome P-450, which aromatizes the A ring of C19 androgens to the phenolic A ring of C18 estrogens, resulting in loss of the C19 angular methyl group as formic acid (1). In humans, aromatase is present in many tissues, including ovary (2, 3), testis (4, 5), placenta (2), and brain (6, 7). The gene encoding the aromatase (*CYP19*) is extraordinarily long (more than 120 kb), with a coding region of approximately 30 kb, containing nine translated exons (II-X). One reason for this long gene is that the transcription of aromatase in different tissue is regulated by different promoters (8) (ovary: promoter II; placenta: promoter I.1; and adipose tissue: promoter I.4). The aromatase promoter II (ArPII) functions in the ovary under the control of FSH. In cooperation with Ad4BP/SF-1, FSH, via the cAMP-protein kinase A (PKA) pathway, stimulates aromatase gene expression in the ovary through promoter II.

It has been determined that estrogens contribute to the growth and development of some estrogen-dependent neoplasm, including breast, endometrial cancers, and some ovarian cancers (9, 10). Estrogens, especially those produced locally in the adipose stoma cells, exert a definite role in stimulating proliferation of breast tumor cells (11). In normal breast adipose tissue, the estrogen-producing aromatase gene is driven by a distal promoter I.4 (8), whereas in breast adipose tissue containing a tumor, there is a switch in the promoter, whereby the aromatase expression is regulated through the proximal promoter II. This shift results in elevated aromatase expression in the tumor or surrounding breast adipose tissue and subsequently elevated production of estrogen in local breast adipose tissue, thus leading to the development of breast cancer (12–16). These findings highlight the importance of promoter II, especially in breast cancer.

Peroxisome proliferator-activated receptor (PPAR)-γ is a nuclear receptor that has an essential role in adipogenesis and glucose homeostasis in response to its ligands, which are either naturally existing ligands like 15-deoxy-$\Delta^{12,14}$ prostaglandin J2 or synthetic thiazolidinediones. Besides relatively

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Abbreviations: APDC, Ammonium pyrrolidinedithiocarbamate; ArPII, aromatase promoter II; CAPE, caffeic acid phenethyl ester; ChIP, chromatin immunoprecipitation; CMV, cytomegalovirus; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; IκBα, inhibitory κBα; LG, LG100268; NF-κB, nuclear factor-κB; NIK, NF-κB-inducing kinase; NS, normal saline; PKA, protein kinase A; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; SDS, sodium dodecyl sulfate; TGZ, troglitazone.

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well-known PPARγ-expressing tissues like adipose tissue, adrenal gland, and spleen (17–19), ovary (20) and granulosa cells (21, 22) also express an abundant amount of PPARγ, whose physiological role in these tissues is largely unknown. We previously reported that the PPARγ ligand, troglitazone (TGZ), especially together with the retinoid X receptor (RXR) ligand, LG100268 (LG), dose-dependently inhibits aromatase activity in granulosa cells (21, 23, 24).

In the present study, we extended our study to clarify the underlying mechanism whereby activation of a nuclear receptor system constituted by PPARγ and RXR down-regulates the aromatase gene. Herein we report an involvement of the transcriptional factor nuclear factor-κB (NF-κB) in the above mechanism as well as its importance in the regulation of aromatase expression through promoter II.

Materials and Methods

Materials

TGZ and LG were obtained from Sankyo Pharmaceuticals (Tokyo, Japan), and Ligand Pharmaceuticals Inc. (San Diego, CA), respectively. Caffeic acid phenethyl ester (CAPE), forskolin, and TNFα were all purchased from Sigma-Aldrich (St. Louis, MO). Ammonium pyrrolidinedithiocarbamate (APDC) was purchased from Wako (Osaka, Japan). All the above compounds (except CAPE and TNFα, which were dissolved in 50% ethanol and normal saline, respectively) were dissolved in dimethyl sulfoxide (DMSO), and the final concentration of solvents (DMSO, 50% ethanol or normal saline) in the cell growth medium was 0.1% (vol/vol). An equal volume of solvents was added to control cultures during cell treatment with chemicals.

Cell culture

We established a human ovarian granulosa-like tumor cell line, KGN, from a 63-yr-old female patient with invasive granulosa cell carcinoma (25). The cells grew as an adherent monolayer with stable proliferation. The cells possess properties similar to those of normal granulosa cells, including the expression of functional FSH receptor and a relatively high aromatase activity, which is PKA dependent (25). The cells were maintained in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) in an atmosphere of 5% CO_2 at 37 C. NIH-3T3 cells were purchased from the Japanese Cell Research Bank (Tokyo) and maintained in DMEM (high glucose) supplemented with 10% FBS at 37 C.

Aromatase assay

The aromatase activity was determined by measuring the $[^3H]H_2O$ released on conversion of [1β-^{3}H]androstenedione to estrone, as described previously (21). The cells were precultured in 6-well plates in DMEM/F12 with 5% dextran-coated, charcoal-treated FBS for 48 h before treatment with chemicals. After the cells were treated with TGZ+LG, [1β-^{3}H]androstenedione was added, and the cells were then further incubated for 6 h. In the case of combined treatment with the NF-κB inhibitors, CAPE or APDC was added to cultures 2 h before an 8-h treatment with TGZ+LG. A second-round treatment consisting of 2 h of CAPE (or ADPC) followed by 8 h of TGZ+LG was carried out before addition of [1β-^{3}H]androstenedione. Extraction of medium (2.0 ml) and measurement of radioactivity in $[^3H]H_2O$ for aromatase activity were done as described previously (21). The amount of radioactivity was then standardized by protein concentration, which was determined using a micro-BCA kit (Pierce Chemical Co., Rockford, IL) and expressed as picomoles per milligram protein per 6 h.

Plasmid constructions

The 4.0-kb ArPII was amplified by PCR from genomic DNA. After confirmation of the entire sequence by direct sequencing, the fragment was subcloned into PGL3-Basic vector (Promega, Madison, WI) to make the luciferase reporter plasmid PGL3-ArPII, in which the luc+ gene is driven by the 4.0-kb fragment of human ArPII. To construct the NF-κB luciferase reporter plasmid, pGL3-tk was first constructed by cloning the −109 to +37 region of the herpes virus thymidine kinase promoter into the *Bgl*II and *Hin*dIII sites of the pGL3-basic vector (Promega). A pair of oligonucleotides, 5′-TGGAAATTCCTGGAAATTCCTGGAAATTCC-3′ and 5′-TCGAGGAATTTCCAGGAATTTCCAGGAATTTCCA-3′, were annealed together, thus resulting in double-stranded oligonucleotides with both a blunt end and a *Xho*I compatible overhang, which were then ligated into the *Sma*I and *Xho*I sites of tk-Luc, thus giving rise to pGL3-NF-κB containing three copies of the NF-κB sites. The *Renilla* luciferase reporter plasmid phRL-cytomegalovirus (CMV), serving as an internal control in the dual-luciferase reporter assay, was purchased from Promega. Human p65 expression vector, pcDNA-p65, was provided by Dr. C. Scheidereit (Max Delbruck Center for Molecular Medicine, Berlin, Germany). pcDNA-NF-κB-inducing kinase (NIK) was provided by Dr. D. Wallach (Department of Biological Chemistry, The Weizmann Institute of Science, Rehovot, Israel). All plasmids were prepared from an overnight bacterial culture using the QIAfilter plasmid maxikit (Qiagen, Valencia, CA).

Relative luciferase reporter assay

For the relative luciferase reporter assay, 1.5×10^5 cells/well in 1 ml growth medium were seeded into 12-well plates, and 0.8 μg of PGL3-ArPII (or PGL3-NF-κB) and 2.0 ng of phRL-CMV were transiently cotransfected in each well using the Superfect transfection reagent (Qiagen) following the manufacturer's protocol. In the case of cotransfection, 0.15 μg of expression vector for p65 (pcDNA-p65) or NIK (pcDNA-NIK) was also added; the total amount of plasmid DNA added to each well was equalized using the empty vector: pcDNA-3.1. Twenty-four hours after transfection, the cells were treated with TGZ+LG for 24 h at the concentrations indicated in each figure. The cells were then lysed in 100 μl/well passive lysis buffer, and the luciferase assay was performed in accordance with the protocol of the dual-luciferase reporter assay system, using a Lumat LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). The firefly luciferase activity, produced by PGL3-ArPII in identically treated triplicate samples, was normalized for the *Renilla* luciferase activity produced by phRL-CMV. The data shown are representative of at least three independent experiments. In the case of cotreatment with the NF-κB inhibitors, cells were preincubated for 2 h with CAPE (working concentration 20 μg/ml) or APDC (working concentration 100 ng/ml) and then incubated for 10 h with TGZ+LG. Another round of 2 h of CAPE plus 10 h of TGZ+LG was carried out before the cells were lysed for luciferase assay.

Western blotting

NIH-3T3 and KGN cells treated with either TGZ+LG or DMSO were grown to subconfluent phase, washed with PBS, and actively lysed in 500 μl lysis buffer. Samples were subjected to electrophoresis on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were incubated with either a rabbit polyclonal antibody against the p65 subunit of NF-κB [NF-κB p65 (c-20): sc-372, Santa Cruz Biotechnology, Santa Cruz, CA] or a rabbit polyclonal antibody against inhibitory κBα (IκBα) (c-21: sc-371, Santa Cruz Biotechnology) and subsequently with a horseradish peroxidase-linked goat antirabbit IgG secondary antibody (Cell Signaling Technology, Beverly, MA). Detection was carried out using the ECL+Plus Western blotting detection system (Amersham Biosciences, Buckinghamshire, UK). Membranes were then visualized using a STORM 860 scanner (Molecular Dynamics, Sunnyvale, CA). Images were finally analyzed using ImageQuant software (Molecular Dynamics).

ChIP assays

These were performed by the chromatin immunoprecipitation (ChIP) assay kit (Upstate Biotechnology, Lake Placid, NY), according to the protocol provided by manufacturer with some modifications. Briefly, KGN cells were seeded in 10-cm^2 dishes and treated overnight with 10 μM TGZ + 1 μM LG or the solvent DMSO. After an additional treatment of 10 ng/ml TNFα or its solvent normal saline (NS) for 1 h, cells were cross-linked with 1% formaldehyde for 60 min, washed with chilled PBS, resuspended in 200 μl SDS lysis buffer, and sonicated six times for 10

sec each at 60% maximum setting of the sonicator (Handy Sonic-UR-20P, TOMY SEIKO Co., Ltd., Tokyo, Japan). Sonicated cell supernatant was diluted 10-fold, and 1% (20 μl) of the total diluted lysate was used for total genomic DNA as input DNA control. The rest (1980 μl) was then subjected to immunoclearing by 75 μl salmon sperm DNA/protein A agarose-50% slurry for 30 min at 4 C. Immunoprecipitation was performed for overnight at 4 C with 3 μg p65 antibody (Santa Cruz Biotechnology). For negative control, normal rabbit IgG (Santa Cruz Biotechnology) was used instead of p65 antibody. Precipitates were washed sequentially for 5 min each in low salt, high salt, LiCH immune complex wash buffers, and finally washed twice with Tris/EDTA buffer. Histone complexes were then eluted from the antibody by freshly prepared elution buffer (1% SDS, 0.1 M $NaHCO_3$). Histone-DNA cross-links (including the input samples) were reversed by 5 M NaCl at 65 C for 4 h. DNA fragments were extracted with a PCR purification kit (Qiagen). One microliter from a 30-μl DNA extraction was used for PCR and primed by sequences as follows: forward, 5′-GGG AAG AAG ATT GCC TAA AC-3′; reverse, 5′-TGT GGA AAT CAA AGG GAC AG-3′; the PCR size was 401 bp.

Real-time PCR

Immunoprecipitated DNA samples were then set to real-time PCR analysis to quantify the relative amount to their corresponding input controls with a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instruction. Briefly, 1 μl immunoprecipitated DNA sample (or H_2O as negative control), was placed into a 20-μl reaction volume containing 1 μl of each primer (10 μM) and 2 μl LightCycler-FastStart DNA Master SYBR Green I (Roche), which includes nucleotides, Tag DNA polymerase, and buffer. PCR products were visualized on a 2% agarose gel and finally validated by direct sequencing. Input samples were amplified simultaneously as the internal controls. Real-time PCR data for each immunoprecipitated sample were calculated as a ratio to its corresponding input sample. Briefly, threshold values (crossing line) obtained where fluorescent intensity was in the geometric phase, cycle number at the crossing point of an immunoprecipitated sample (Cip), and the corresponding input sample (Cco) were determined via LightCycler software version 3.5. The relative amount to input sample of the immunoprecipitated sample (Aip) was calculated by the formula of: $Aip = 2^{(Cco-Cip)}$.

Statistics

One-way ANOVA followed by Scheffé's test was used for multigroup comparisons.

Results

TGZ+LG inhibited ArPII dose-dependently

We previously reported that TGZ+LG inhibit aromatase activity, and consistently the estrone production, in a dose-dependent manner (23). We also found that TGZ+LG down-regulated aromatase mRNA by both decreased transcription and increased degradation. Here a 4.0-kb fragment of human ArPII was inserted into PGL3-Basic to make the luciferase reporter PGL3-ArPII and then used to further address whether TGZ, LG, or TGZ+LG interfered with the transcription of aromatase from promoter II. KGN cells were transfected by Superfect with PGL3-ArPII as well as the internal control phRL-CMV. As shown in Fig. 1, on the addition of increasing concentration of TGZ+LG, the relative luciferase activity was decreased in a concentration-dependent manner. Although they were weaker, TGZ or LG alone also manifested inhibitory effects on ArPII. The results indicate that the inhibitory effect of TGZ+LG on aromatase gene is directly mediated by the inhibition of promoter II activity.

PPARγ is critical for the TGZ+LG inhibition

To further clarify the involvement of PPARγ in the regulation of ArPII, the same experiment as described above was carried out in NIH-3T3 cells, which lack endogenous expression of PPARγ (26). As shown in Fig. 2A, neither TGZ (or LG) alone nor combined treatment of TGZ+LG could decrease the expression of the PGL3-ArPII reporter, even when the concentration was raised to 10 μM for TGZ and 1.0 μM for LG, when the cells had been cotransfected with PGL3-ArPII+phRL-CMV and pcDNA3.1, the empty vector. However, on the exogenous cotransfection of the PPARγ expression vector, either TGZ or LG alone significantly decreased ArPII activity in a concentration-dependent manner, and combined treatment of TGZ+LG caused a sharper decrease in expression from the promoter. This phenomenon nicely mimicked what was observed in KGN cells, which possess endogenous PPARγ. These data clearly demonstrate the involvement of PPARγ in the inhibition of ArPII.

NF-κB inhibitors abolished the inhibition of TGZ+LG on aromatase gene

Due to the absence of a PPARγ-RXR-responsive element in the aromatase promoter II (23), we previously suggested that PPARγ might inhibit the promoter by an indirect mechanism (23). This hypothesis is supported by recent work (27), which showed that there was no binding of PPARγ and RXRα heterodimers to the promoter. A series of studies pointed out the inhibitory effect of PPARγ activation on NF-κB-dependent transcription system (28–30). We thus tested the possibility that PPARγ activation inhibits aromatase gene through the NF-κB system by using specific inhibitors for NF-κB: CAPE (31) and APDC (32). CAPE specifically inhibits NF-κB binding to DNA and also prevents the translocation of the p65 subunit of NF-κB to the nucleus and delays IκBα

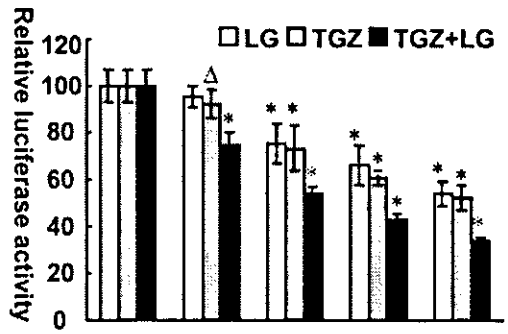

FIG. 1. TGZ+LG inhibits aromatase promoter II. The luciferase reporter PGL3-ArPII, wherein the luc+ gene is driven by a 4.0-kb segment of the human ArPII, was transfected into KGN cells, which were seeded in 12-well plates 1 d earlier. The cells were treated with an increasing concentration (as indicated) of TGZ, LG, or TGZ+LG 1 d after transfection for 24 h. Cells were then lysed and a dual-luciferase reporter assay was performed. The ArPII-mediated firefly luciferase signal was normalized using *Renilla* luciferase, which was constitutively expressed by the internal control phRL-CMV vector. Data expressed in mean ± SD was from identically treated triplicate samples of three independent experiments. Δ, $P < 0.05$; *, $P < 0.01$, compared with basal level of the same treatment group.

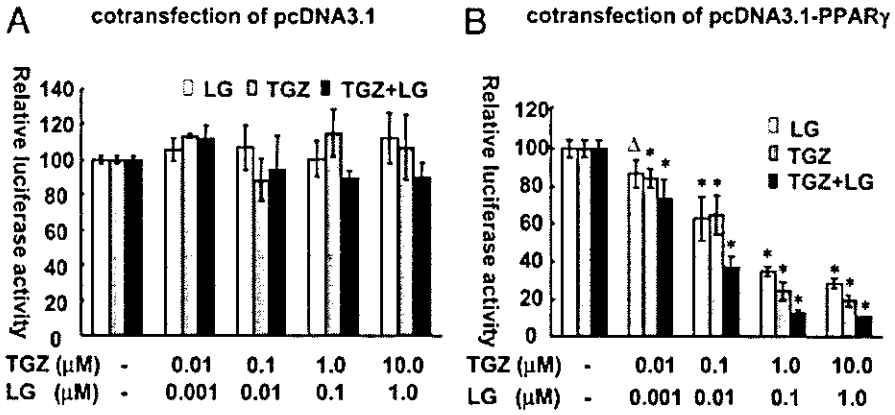

FIG. 2. TGZ+LG inhibits ArPII in a PPARγ-dependent manner. PPARγ-deficient NIH-3T3 cells were transfected with PGL3-ArPII; either a human PPARγ2 expression vector pcDNA3.1-PPARγ2 or the empty control pcDNA3.1 vector was cotransfected. Cells were then treated with TGZ, LG, or both for 24 h. Neither TGZ (or LG) alone nor TGZ+LG inhibited the promoter in the absence of PPARγ, whereas exogenous coexpression of the nuclear factor restored the inhibition. TGZ+LG synergistically inhibited the promoter in the presence of PPARγ. Δ, $P < 0.05$; *, $P < 0.01$, compared with basal level of the same treatment group.

resynthesis (31). As described above, cotransfection of PGL3-ArPII and pcDNA-PPARγ in NIH-3T3 cells allowed direct assessment of PPARγ mediation of the inhibitory effect on ArPII activity. CAPE was applied to this model to test the possible involvement of the NF-κB system in the inhibition. As shown in Fig. 3A, NIH-3T3 cells were treated with 20 μg/ml CAPE or 50% ethanol for 2 h before combined treatment with TGZ+LG, which lasted for 8 h. To gain a clearer inhibition, a second round of 2 h of CAPE plus 8 h of TGZ+LG was carried out before the cells were subjected to luciferase assay. Pretreatment of the cells with CAPE completely abolished the inhibitory effect of PPARγ activation, whereas the inhibition was still present with pretreatment with only the solvent for CAPE, 50% ethanol. A similar result was observed when CAPE was replaced by another NF-κB inhibitor, APDC (data not shown).

We next assessed whether the inhibition of aromatase activity caused by TGZ+LG also disappears on treatment with CAPE.

KGN cells were treated with CAPE in the same manner as above before aromatase activities were assayed. As shown in Fig. 3B, on pretreatment with 50% ethanol, the solvent for CAPE, TGZ+LG significantly inhibited aromatase activity, whereas once the cells were pretreated with CAPE, no decrease of aromatase activity was seen. These results indicate the mediation of NF-κB in the down-regulation of aromatase activity by TGZ+LG at the transcriptional level of promoter II.

NF-κB up-regulates ArPII

In the experiments described in Fig. 3, we noticed that on treatment of CAPE, even basal levels of both ArPII and aromatase activity were decreased, suggesting that NF-κB might be a positive regulator of aromatase gene. We tested this possibility by further experiments. As shown in Fig. 4A, cotransfection of the p65 subunit of NF-κB directly stimulated ArPII by 4-fold in NIH-3T3 cells. A similar phenomenon was observed in KGN cells (data not shown). NIK, which causes degradation of IκBα by phosphorylation of the latter at serine 176 and thus activates p65 (33), was used to specifically induce the endogenous activation of NF-κB. Figure 4B shows that PGL3-NF-κB, (positive control, contains three repeats of the NF-κB consensus elements) was augmented by NIK 2.80-fold. PGL-ArPII was also up-regulated 2.25 times by NIK. In the case of PGL3-Basic (negative control), NIK exhibited no effect.

TGZ+LG interfered with the interaction between NF-κB and ArPII

ChIP assay is a powerful technique to determine *in vivo* binding of transcription factors to target genes' promoters on

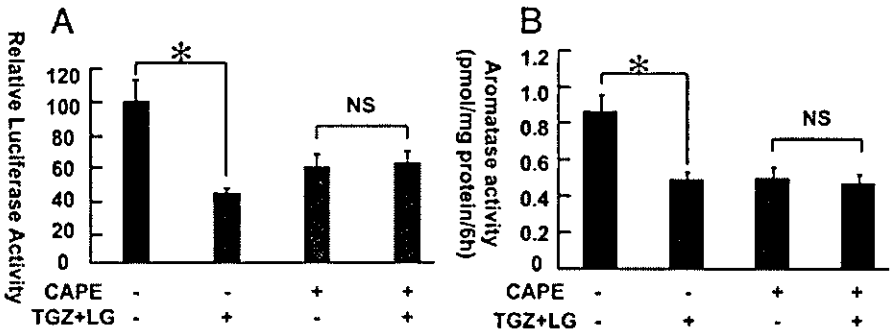

FIG. 3. TGZ+LG inhibition disappeared on NF-κB blockage. A, PGL3-ArPII was cotransfected with pcDNA3.1-PPARγ2 into NIH-3T3 cells. Cells were first treated with or without 20 μg/ml CAPE for 2 h and then with TGZ+LG or the solvent DMSO for 8 h. A second round of 2 h of CAPE (or the solvent 50% ethanol) and 8 h of TGZ+LG (or DMSO) treatment was carried out before cells were lysed for the luciferase assay. CAPE abolished the inhibitory effect of PPARγ activation and decreased the basal activity of the promoter as well. B, Aromatase activity assayed in KGN cells. Cells were treated with chemicals in the same manner as described in A. TGZ+LG inhibition of aromatase activity also disappeared on NF-κB blockage. *, $P < 0.01$; NS, not significantly different.

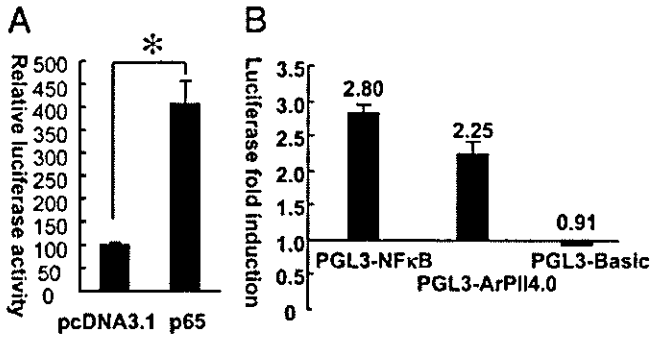

FIG. 4. NF-κB up-regulated ArPII. A, NIH-3T3 cells were transfected with PGL3-ArPII. pcDNA-p65 or pcDNA3.1 was cotransfected. ArPII activity was stimulated 4-fold by p65. B, pcDNA-NIK was transfected to NIH-3T3 cells to trigger the endogenous activation of NF-κB, whose effect on ArPII was evaluated by cotransfection of PGL3-ArPII. PGL3-NF-κB, a reporter containing three repeats of NF-κB elements, and PGL3-basic was also included as positive and negative controls, respectively. The *bars* represent the relative effect of NIK on each reporter; namely, the NIK-mediated reporter activity was divided by the control pcDNA3.1 empty vector-mediated reporter activity. *, $P < 0.01$.

chromatin. We used this assay to evaluate the interaction of NF-κB with ArPII, especially the recruitment of p65 to the promoter region. KGN cells pretreated either with an overnight 10 μM TGZ+1 μM LG or their solvent, DMSO, were challenged with 10 ng/ml TNFα or its solvent NS for 1 h before being subjected to ChIP assay with an antibody against p65. Enrichment of ArPII DNA sequences in the chromatin immunoprecipitates, which indicates association of p65 to the promoter within intact chromatin, was visualized by PCR amplification. Based on our primitive promoter deletion analysis data, which show that a 600-bp ArPII reporter already responds positively to p65 and NIK, we designed the PCR to amplify a 401-bp region of ArPII (−403 to −2, upstream of ovary exon 2, GenBank accession no. D21241). As shown in Fig. 5A, although a weak band was amplified (30 cycles) in the absence of antibody (lane 1, which may represent the nonspecific binding of the ArPII chromatin region to normal rabbit IgG), an increase in the relative intensity of ArPII PCR band amplified from samples treated with p65 antibody indicated binding between the transcription factor and the promoter (lanes 2–5). Among cells not pretreated with TGZ+LG, 1 h TNFα challenge seemed slightly increased band intensity (lane 3 *vs.* 2). Pretreatment of TGZ+LG clearly weakened the PCR band intensity (lane 4), suggesting a decreased occupancy by p65 on ArPII. However, the decrease was not observed in cells challenged with TNFα (lane 5). Control amplification was with total input DNA (Fig 5A, *lower panel*). There was no change in the amplification of input DNA in all cases.

To further objectively tell the difference between different groups of cells, we performed real-time PCR to quantify the relative amount of immunoprecipitated ArPII copies to input control for each sample. Figure 5B shows that presence of p65 antibody significantly increased the relative copy number of immunoprecipitated ArPII DNA segments, indicating that p65 associates with ArPII. And TGZ+LG pretreatment significantly reduced the relative copy number, suggesting the association was impaired. However, TNFα restored the TGZ+LG reduced relative ArPII copy number, although the cytokine did not change the copy number from cells not pretreated with TGZ+LG. Consistent with data presented in Fig. 4, these results indicated that NF-κB may interact with ArPII *in vivo*, and activation of PPARγ/RXR may interfere with the interaction.

The interference of PPARγ activation on the endogenous expression of NF-κB in KGN cells

The endogenous expression of the NF-κB system in KGN cells was tested by Western blotting, using antibodies against the p65 subunit and IκBα, and was positively controlled using NIH3T3 cells, whose endogenous NF-κB has already been proven (34). The KGN cells were treated with or without 24 h of 10 μM TGZ + 1.0 μM LG, actively lysed, and subjected to Western blotting. Figure 6 (*upper panel*) shows endogenous expression of IκBα in KGN cells and the *lower panel* the endogenous expression of the p65 subunit of NF-κB.

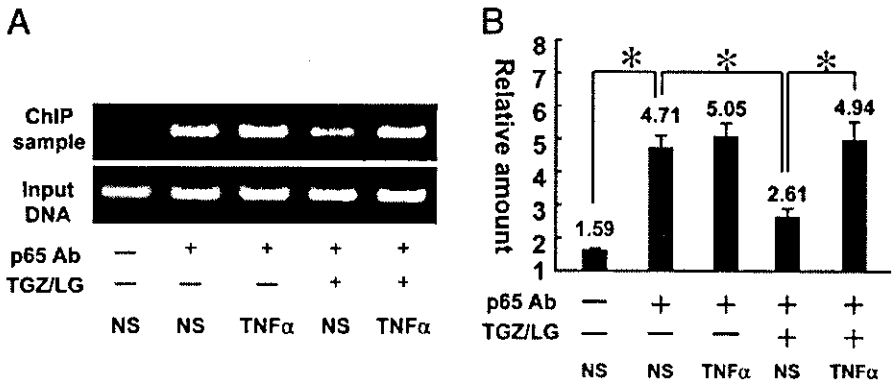

FIG. 5. ChIP assay of p65 binding to ArPII. KGN cells pretreated with or without an overnight 10.0 μM TGZ+1.0 μM LG were challenged with 10 ng/ml TNFα or its solvent, NS, for 1 h. ChIP assay was then performed with anti-p65 antibody or normal rabbit IgG as negative control. Enrichment of ArPII-specific DNA sequence in immunoprecipitated DNA pool indicating association of p65 with ArPII within intact chromatin was visualized by PCR. A, PCR was performed on immunoprecipitated DNA pool with normal rabbit IgG [p65 Ab (−)], p65 Ab, and purified input DNA (input). *Upper panel*, PCR amplified ArPII-specific bands from cells under various treatments as indicated. *Lower panel*, ArPII PCR bands from input controls. B, Real-time PCR was performed to quantify the amount of immunoprecipitated ArPII DNA copy number from cells under various treatments relative to their corresponding input controls. *, $P < 0.01$; NS, not significantly different.

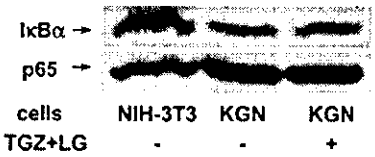

FIG. 6. TGZ+LG did not alter the endogenous expression of IκBα and p65. KGN cells were treated with 10.0 μM TGZ+1.0 μM LG for 24 h and were then subjected to Western blotting analysis for the IκBα and p65 subunits of NF-κB. NIH-3T3 cells were used as a positive control. KGN cells highly expressed both IκBα and p65, and 24 h of TGZ+LG did not apparently alter the protein expression levels.

Neither of these two proteins' expression was altered by a 24-h treatment of TGZ+LG, suggesting that PPARγ activation does not interfere with NF-κB function via down-regulation of p65 subunit expression or up-regulation of the IκB protein.

PPARγ activation suppresses NF-κB transactivation

As shown above, PPARγ activation does not apparently change the protein level of NF-κB but impairs the interaction between the transcription factor and ArPII. We subsequently studied the possible interference of PPARγ activation on NF-κB transactivation in KGN cells. As shown in Fig. 7, in KGN cells, cotransfection of 0.15 μg/well of pcDNA-p65 stimulated PGL3-NF-κB production (0.8 μg/well) approximately 4-fold, whereas the p65-augmented PGL3-NF-κB signal was decreased in a concentration-dependent manner on cotreatment with an increasing concentration of TGZ+LG. Thus, activation of PPARγ-RXR heterodimers by TGZ+LG resulted in inhibitory effects on NF-κB-mediated transcription. Namely, the final net outcome effect of PPARγ activation is a down-regulation of NF-κB transactivation activity.

Discussion

The physiological significance of the mysteriously high expression of PPARγ in ovarian granulosa cells is largely unknown. We previously reported that the synthetic PPARγ ligand, TGZ, in a concentration corresponding to human

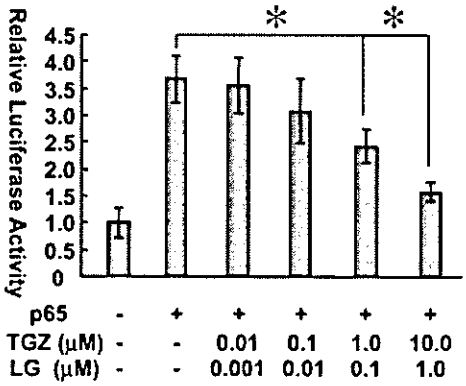

FIG. 7. TGZ+LG inhibited transactivation of NF-κB. KGN cells were transfected with PGL3-NF-κB, which contains three repeats of NF-κB elements. A p65 expression vector (pcDNA-p65) or the pcDNA3.1 empty vector was also transfected. Cells cotransfected with p65 were further treated with increasing concentrations of TGZ+LG for 24 h. TGZ+LG was shown to decrease p65-stimulated PGL3-NF-κB signal in a concentration-dependent pattern. *, $P < 0.01$.

plasma TGZ concentration after oral administration of a therapeutic dosage, caused a significant decrease in aromatase activity as well as mRNA level in human ovarian granulosa cells (21). The effect was enhanced synergistically by the specific ligand (LG) for RXR, the PPARγ partner. Consistently, TGZ+LG inhibited estrogen production in KGN cells (23), and TGZ reduced estrogen levels in patients with polycystic ovary syndrome, which suggested the *in vivo* relevance of the inhibition (35). In the present study, we further demonstrated that the aromatase promoter II, which is specially used in ovary, is also inhibited by TGZ+LG, indicating that inhibition occurs at the transcriptional level. It was recently reported that 15-deoxy-$\Delta^{12,14}$ prostaglandin J2, which is believed to be the endogenous ligand for PPARγ, inhibits aromatase activity through a PPARγ-independent, but redox-sensitive, mechanism (36). However, TGZ and LG, the synthetic ligands for PPARγ and RXR, respectively, seem to exert an inhibitory function in a PPARγ-dependent way because the inhibition of ArPII could not be observed in PPARγ-deficient NIH-3T3 cells unless the nuclear receptor is exogenously expressed. It is noteworthy that even for LG-induced inhibition, PPARγ was required, suggesting that inhibition requires PPARγ-RXR heterodimers. The important involvement of PPARγ in the regulation of the aromatase gene was also strengthened by a recent report that demonstrated that an environmental toxin, a commonly used plasticizer, di-C2-ethylhexyl phthalate, decreased aromatase expression through both PPARγ and PPARα in granulosa cells (37).

The aromatase gene is unique in that expression of the gene in different tissues is driven by different promoters in a tissue-specific pattern (8). Promoter II is typically used to drive the gene in ovarian granulosa cells, especially before menopause. Local estrogen production in breast adipose tissue has a definite mitogenic role in breast tumors (15, 16), and local estrogen levels in breast tumors were found 10 times higher than that in the circulation of postmenopausal women (38). Although in normal adipose tissue aromatase is mainly produced via the promoter I.4 (15, 39), the local accumulation of estrogen in breast adipose tissue containing a tumor is largely due to a critical shift in promoter usage from I.4 to II (12–14, 40). In this study, we found that TGZ+LG, in a PPARγ-dependent manner, dose-dependently inhibited ArPII activity in ovarian KGN cells as well as in fibroblast NIH-3T3 cells, suggesting that the PPARγ inhibitory effect on ArPII might be universal. These data highlighted the importance of ArPII and the therapeutic potency of TGZ+LG.

Due to the lack of an apparent consensus about PPARγ-responsive element on ArPII, we hypothesized that the inhibition mechanism might be indirect (23). This idea is supported by a recent study, which shows that PPARγ is unable to bind ArPII (27).

It has been proven that the ArPII gains its maximal activity when both PKA and protein kinase C are activated by cotreatment with forskolin and tetradecanoyl phorbol acetate (41, 42). NF-κB is one of the transcriptional factors that can be activated by the activation of the protein kinase C pathway (34). Whereas on the other hand, activation of PPARγ can regulate inflammatory responses by suppressing the activation of the transcriptional factor of NF-κB (28–30). In the

present study, we tested the hypothesis that PPARγ activation may exert its inhibitory effect on ArPII by inhibiting NF-κB, which is endogenously expressed in ovarian granulosa cells and breast tissues as well (43). In this study the inhibitory effect of PPARγ-RXR activation on both ArPII and aromatase activity was found to sharply disappear on treatment with NF-κB blockers (either CAPE or APDC), suggesting that NF-κB might be the mediator of this inhibition. If this is the case, NF-κB should logically be a positive regulator of ArPII. In line with this, the basal ArPII activity as well as the aromatase activity was decreased on CAPE treatment, and activation of the NF-κB system by either forced expression of p65 or cotransfection of NIK to activate endogenous NF-κB stimulated ArPII activity. Consistently, ChIP assay also showed the interaction between NF-κB and ArPII. However, no classical consensus NF-κB-responsive element was detected on the promoter. Nevertheless, because there is an instance that NF-κB may bind a DNA motif, which is not related to the classical NF-κB consensus sequence (44), we suppose that there might exist putative ArPII-specific binding sites for p65, which are to be further delineated.

Although we observed no effect of TGZ+LG on endogenous expressions of either IκBα or p65, which is considered one possible mechanism by which NF-κB system is regulated (45). Treatment of TGZ+LG apparently weakened the interaction between p65 and ArPII, suggesting activation of PPARγ may interfere with the formation of high-order complex between NF-κB and aromatase gene at chromatin level. This is probably further explained by the finding that activated PPARγ can physically interact with p65 and results in inhibition of NF-κB (46, 47). And probably as an outcome of the impaired transcription factor-promoter association, we found that PPARγ activation by TGZ+LG suppressed the transactivation ability of NF-κB. The suppression by the PPARγ-RXR nuclear receptor system may also possibly be related to the fact that the nuclear receptors compete for limited amounts of the general coactivators, cAMP response element-binding protein and steroid receptor coactivator-1, as we previously reported (48).

Considering our previous finding that activation of a PPARγ-RXR nuclear receptor system by TGZ+LG inhibits aromatase by accelerating mRNA degradation, we report in the present study that TGZ+LG inhibited transcriptional activity of the ArPII in a PPARγ-dependent manner. These data reinforce the potential use of synthetic PPARγ and RXR agonists for therapeutic applications in diseases in which estrogens, locally or systematically, play prominent pathogenic roles, especially in diseases like breast cancer. In addition, we found that the inhibition disappeared on blockage of NF-κB, which was found in turn to positively regulate aromatase. Notably, activation of NF-κB has been found involved in the proliferation and metastasis of breast cancer cells (43), for which, although several mechanisms have been suggested, we suppose that stimulation of aromatase might be an additional one. Classically, regulation of ArPII involves PKA-cAMP response element-binding protein (49) and the orphan nuclear receptor steroidogenic factor 1 (Ad4BP/SF-1) (50), but the actual regulation may be much more complicated, at least in that nuclear receptors like PPARγ, RXR, and their cross-talk with the transcriptional factor NF-κB might also play some important roles.

Acknowledgments

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The Orphan Nuclear Receptors NURR1 and NGFI-B Modulate Aromatase Gene Expression in Ovarian Granulosa Cells: A Possible Mechanism for Repression of Aromatase Expression upon Luteinizing Hormone Surge

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Ovarian granulosa cells play pivotal roles in many aspects of ovary functions including folliculogenesis and steroidogenesis. In response to FSH and LH, the elevation of intracellular cAMP level in granulosa cells leads to activation of multiple ovarian genes. Here, we report findings from a genome-wide study of the cAMP-responsive gene expression profiles in a human granulosa-like tumor cell line, KGN. The study identified 140 genes that are either activated or repressed by 2-fold or greater after stimulation by the adenylyl cyclase activator forskolin. The induction patterns of some cAMP-responsive genes were further analyzed by quantitative real-time PCR. Consistent with previous observations, the LH-responsive genes, such as the nuclear receptor 4A subfamily (NURR1, NGFI-B, and NOR-1), were rapidly but transiently induced, whereas the FSH-responsive gene CYP19 encoding aromatase was induced in a delayed fashion. Interestingly, ectopic expression of NURR1 or NGFI-B severely attenuated the cAMP-responsive activation of the ovary-specific aromatase promoter. Reduction of the endogenous NURR1 or NGFI-B by small interfering RNA significantly elevated aromatase gene expression. The *cis*-elements responsible for NURR1/NGFI-B-mediated repression were mapped to the minimal aromatase promoter sequence that confers camp responsiveness. Furthermore, the DNA-binding domain of NURR1 was required for the repression. Taken together, these results strongly suggest a causal relationship between the rapid decline of aromatase mRNA and induction of nuclear receptor subfamily 4A expression, which concomitantly occur upon LH surge at the later stages of ovarian follicular development. (*Endocrinology* 146: 237–246, 2005)

OVARIAN FOLLICULAR DEVELOPMENT and function are controlled by multiple components along the hypothalamic-pituitary-ovarian axis, including the gonadotropins produced by the pituitary gland and a plethora of ovarian genes activated in response to the peptide hormones (1, 2). In particular, FSH and LH play pivotal roles in both promoting follicular maturation and inducing gene expression involved in hormonal action (3, 4). Upon binding to their cognate receptors, the pituitary hormones activate the receptor-coupled adenylyl cyclase that leads to an increased intracellular cAMP level. Numerous studies have firmly established that cAMP activates the protein kinase A-dependent pathway and other signal transduction cascades, which ultimately control differentiation of ovarian granulosa cells and expression of ovarian genes (5, 6). Thus, cAMP can be viewed as a molecular switch that mediates diverse FSH/LH-stimulated functions of ovarian follicles. A comprehensive understanding of its impact on ovarian gene expression will shed light on the molecular and biochemical nature of the signaling cascades involved.

Many downstream target genes of FSH and LH in ovarian granulosa cells have been identified over the years (1, 3). Extensive studies of the gonadotropin-mediated ovarian gene expression have also revealed a high degree of complexity in the regulatory pattern (1). First, the action of gonadotropins at a defined follicular development stage appears to be mediated by multiple intracellular signal transduction pathways and many *trans*-acting transcription factors. Furthermore, FSH-responsive gene activation occurs in a sequential order, with rapid activation of the immediate-early genes, such as c-*fos* and serum glucocorticoid kinase, but delayed expression of some other genes including aromatase (CYP19) and inhibin α (7–9). Finally, the effects of gonadotropins on ovarian gene expression are modulated by additional factors that are associated with specific stages of follicular development, ovulation, and luteinization. For instance, FSH induces a robust expression of aromatase in granulosa cells of preovulatory follicles but not preantral follicles (1). On the other hand, many FSH-stimulated genes in granulosa cells are down-regulated in response to the LH surge during the early stages of luteinization (10, 11). The molecular basis for modulating the FSH effect during follicle maturation remains to be better understood.

Nuclear receptor subfamily 4A (NR4A) is a group of orphan nuclear receptors that includes NGFI-B, NURR1, and

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Abbreviations: AF, Activation function; DBD, DNA-binding domain; LBD, ligand-binding domain; NR4A, nuclear receptor subfamily 4A; PII, ovary-specific promoter II; SF-1, steroidogenic factor 1; siRNA, small interfering RNA.

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NOR-1 (12, 13). The genes that encode the NR4A subfamily are considered immediate-early genes because they are rapidly induced by a variety of stimuli including cAMP, phorbol ester, and growth factors (12, 13). All three members of the NR4A subfamily share significant sequence similarity, recognize common DNA sequences, and play important and partially redundant roles in the nervous, endocrine, and immune systems. Of particular relevance to ovarian follicular maturation, NGFI-B is expressed in cultured ovarian granulosa cells and corpora lutea but not in small antral or preovulatory follicles (1). In addition, NGFI-B has been shown to activate steroidogenic gene expression during luteinization (14), thus indicating a functional relevance of the stage-specific expression of NGFI-B in follicular maturation. The preovulatory surge of LH, which triggers the transition from preovulatory follicles to ovulatory and luteinizing follicles, induces the rapid and transient expression of ovarian NGFI-B as well as the other two members of the NR4A subfamily (15). As well documented, the LH surge also leads to a rapid decline in the expression of multiple FSH-stimulated genes (*e.g.* aromatase) (10, 11). However, it remains unclear as to whether the concomitant induction of the NR4A family and repression of aromatase expression upon the LH surge is causally related.

In this report, we conducted a series of transcription studies using a recently established human granulosa tumor-derived cell line, KGN (16). Previous extensive characterization of this cell line demonstrates that it maintains most physiological functions of granulosa cells *in vivo*, such as steroidogenesis, cell growth, and apoptosis (16–19). We first performed a genome-wide study of the cAMP-stimulated gene expression profile in the granulosa cells. Our study uncovers 140 genes that are either up-regulated or down-regulated upon forskolin treatment. We then followed up the microarray study by exploring the functional significance of the rapid and transient induction of the NR4A gene family in granulosa cells. Our work suggests a regulatory function of NGFI-B and NURR1 in ovarian gene expression. Specifically, they strongly inhibit transcription driven by the ovary-specific aromatase promoter. Based on these results, we propose that the LH-induced NR4A genes may play a critical role in down-regulating FSH-responsive gene expression, thus promoting the transition from preovulatory follicles to corpora lutea.

Materials and Methods

Cell line and plasmids

The culture condition for the human ovarian granulosa-like tumor cell line KGN has been previously described (16). To construct the expression vectors for NURR1 and NGFI-B, cDNA of both genes was amplified by RT-PCR from total RNA of forskolin-treated KGN cells and subsequently cloned into the *Hin*dIII and *Xho*I (or *Eco*RI) sites of pcDNA3-FLAG plasmid. A similar strategy was used to construct the deletion mutants of NURR1. The 5′ primers used for amplifying the deletion fragments contain an additional sequence that encodes the nuclear localization sequence of the simian virus 40 large T antigen. The resulting expression vectors contain the nuclear localization sequence-encoding sequence situated between the FLAG tag and NURR1 fragments. To construct the various aromatase luciferase reporter plasmids, the corresponding genomic fragments were amplified from the human genomic DNA (Clontech, Palo Alto, CA) and subcloned into the restriction sites *Xho*I and *Hin*dIII located upstream of the luciferase gene in pTRE-luc plasmid (BD Bioscience Clontech) to replace the TRE-CMV sequence. The identity of all inserts was verified by sequencing. The hCYP11B2 luciferase reporter and the synthetic NR4A-responsive luciferase reporter $p(Bla)_8luc$ were described previously (20, 21).

Microarray hybridization experiments and data analysis

KGN cells were treated with either vehicle or forskolin (Sigma, St. Louis, MO) at a final concentration of 25 μM for 3 h. The cultures were harvested and processed for further analyses. RNA was isolated, and microarray hybridization was performed in duplicates as described previously (22), except that Affymetrix (Santa Clara, CA) microarray chips (HG-U133A) were used. Microarray data were analyzed using the Dchip software package previously described (23).

Real-time PCR

KGN cells were treated with forskolin for the time period as indicated in the figures. Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA was reverse transcribed using the random primers of the ImPrompII kit from Promega (Madison, WI). The real-time PCR assay was conducted following the manufacturer's procedures (Applied Biosystems, Foster City, CA). Essentially, the fluorescent dye SYBR Green was used in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Real-time PCR for the aromatase gene shown in Fig. 5B was conducted using a FAM-labeled probe/primer set. The sequences of the specific PCR primers were as follows (5′ to 3′): Arom (SYBR), forward: TTCCCCA-AACCCAATGAATTT; reverse: ACTTTCCTGCACAGCCACG; Arom (TaqMan), forward: CCTCAATACCAGGTCCTGGC; reverse: CAGG-AATCTGCCGTGGGA; probe: *6FAM*-ACTGCATGGGAATTGGACCC-CTCAT-*TAMRA*; reverse: GACGGTGCCATGGAATTTG; StAR, forward: TGCCTGAGCAGAAGGGTGTC; reverse: AGCACCATGCAAGT-GGGAC; NGFI-B, forward: AACCCCGCCTCTTCCTCC; reverse: TA-TTGGGCTTGGATACAGGGC; NURR1, forward: GCTGGGTGTCAT-CTCCACTCA; reverse: GCCCATGTCGACTCCAACC; NOR-1, forward: CCTTCTCCTCCAATCTGCATG; reverse: CAGCCTGGTCAGTGG-GACA; Fra1, forward: CTGCAGGCGGAGACTGACA; reverse: TTCC-AGCACCAGCTCTAGGC; GADD45α, forward: CGGACCTGCACTG-CGTG; reverse: TCCATGTAGCGACTTTCCCG; GADD45β, forward: GGTGTACGAGTCGGCCAAGT; reverse: GATTTGCAGGGCGATG-TCA; KLF4, forward: ATCTCAAGGCACACCTGCG; reverse: CCTG-GTCAGTTCATCTGAGCG; and β-actin, forward: CCAGATCATGTT-TGAGACCTTCAAC; reverse: CCAGAGGCGTACAGGGATAGC.

Dual luciferase reporter assay

KGN cells were transfected using FuGene 6 (Roche, Indianapolis, IN) according to the manufacturer's instructions. On the following day, cells were treated overnight with either vehicle or forskolin at a final concentration of 25 μM. Typically, 250 ng of firefly luciferase reporter plasmid, 10–250 ng of the NR4A expression vectors, and 1 ng of phRL-CMV Renilla luciferase plasmid (Promega) were used. The dual luciferase activity was measured using the reagents from Promega. The firefly luciferase values were normalized against those of the Renilla luciferase. The presented data were averages of quadruplets.

Small interfering RNA (siRNA) knockdown

Transient transfection of the luciferase or NGFI-B- or NURR1-specific siRNA oligonucleotides (200 nmol per well in a six-well dish) was conducted using Oligofectamine (Invitrogen). The luciferase (catalog no. D-001100-01-80) and NURR1-specific oligonucleotides (catalog no. D-003427-04) were purchased from Dharmacon (Lafayette, CO). Two consecutive transfections were carried out, followed by forskolin treatment for 24 h.

Immunoblotting

KGN cells were treated with forskolin as indicated. Total cell lysates were prepared by lysing the cells in the sodium dodecyl sulfate sample buffer. Equal amount of total proteins was resolved by SDS-PAGE, and immunoblotting was performed following the manufacturer's instruc-

tions using anti-NURR1 (catalog no. SC5568; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and anti-NGFI-B (catalog no. 554088; BD PharMingen, San Diego, CA) or anti-FLAG antibodies (F-3165; Sigma).

Results

Whole-genome analysis of cAMP-regulated genes

The KGN granulosa-like tumor cell line was recently established (16). It exhibits properties similar to those of ovarian granulosa cells *in vivo* including cAMP-inducible aromatase expression (16–19), thus validating its utility in studying granulosa cell differentiation and steroidogenesis. To conduct a genome-wide search for cAMP-responsive genes in KGN cell line, asynchronously growing cells were treated with either vehicle or 25 μM forskolin for 3 h. Total RNA was isolated from the mock- or forskolin-treated cells. Fluorescence-labeled cDNA was used to cohybridize Affymetrix microarray chips (HG-U133A) that contain 14,000 well-characterized human open reading frames. Two independent cohybridizations were performed, and data were analyzed. A total of 140 genes displayed over 2.0-fold difference in expression between the mock- and forskolin-treated samples at the 95% confidence level ($P \leq 0.05$). Among these genes, 56 were stimulated upon forskolin treatment (Table 1), whereas the remaining 84 genes were downregulated after the treatment (Supplemental Table 1, published on The Endocrine Society's Journals Online web site, http://endo.endojournals.org). The list of cAMP-stimulated genes includes those with known functions in the cAMP-mediated signal transduction pathway (*e.g.* CREM) and ovarian follicles (*e.g.* aromatase and inhibin βA). The cAMP-responsive gene set entails genes involved in a diverse range of cellular and biological processes. Interestingly, many of the forskolin-activated genes are transcription regulators, some of which have been implicated in regulation of gonadal gene expression, such as NURR1, NOR-1 (15), and GATA-4 (24). On the other hand, some of the forskolin-repressed genes are known for their functions in stress response and inhibition of cell proliferation (*e.g.* GADD45α and GADD45β) (25).

Confirmation of the microarray data by real-time PCR

Using real-time RT-PCR analysis, we confirmed the effect of forskolin on the expression of five cAMP-activated genes (NURR-1, NOR-1, aromatase, StAR, and KLF4) and three cAMP-repressed genes (GADD45α, GADD45β, and FRA1). Because the two most activated genes in the microarray belong to the NR4A orphan receptor family (18.9-fold for NR4A2/NURR1 and 15.7-fold for NR4A3/NOR-1), we also included NR4A1/NGFI-B, the third member of the NR4A family, in the real-time PCR study. Total RNA was isolated from the KGN cells treated with either vehicle or 25 μM forskolin. As shown in Fig. 1, A–F, mRNA levels of NGFI-B, NURR1, NOR-1, aromatase, StAR, and KLF4 were increased to various degrees after forskolin treatment, which is consistent with their being cAMP-activated genes. In contrast, transcripts of GADD45α, GADD45β, and FRA1 were reduced in response to forskolin (Fig. 1, G–I), thus corroborating the repressive effect of forskolin on these genes as observed in the microarray study.

Several distinct patterns of induction were observed among the cAMP-activated genes. The three immediate-early genes that encode NR4A subfamily (NGFI-B, NURR1, and NOR-1) all showed rapid but transient expression pattern, reaching the peaks of expression by 1–3 h after forskolin treatment and declining significantly by 6 h (Fig. 1, A–C). Furthermore, the protein expression patterns of NGFI-B and NURR1 closely followed the patterns of the mRNA (Fig. 2). The transient expression pattern of NR4A in the forskolin-treated KGN cells mirrors the expression of these genes previously observed in LH-treated rat preovulatory follicles (15). In contrast to NR4A, induction of two important steroidogenic genes (StAR and aromatase) occurred in a delayed fashion (Fig. 1, D and E). Again, this is in agreement with the previous reports of delayed expression patterns of these genes in response to gonadotropins (7). Distinct from the expression patterns of the first two subgroups of cAMP-activated genes, KLF4 was induced as rapidly as the NR4A genes, but its mRNA level decreased at a slower pace than the levels of the NR4A genes (compare Fig. 1F with Fig. 1, A–C). The differential induction patterns likely result from gene-specific regulation at both transcriptional and posttranscriptional levels.

Ectopic NURR1 and NGFB-I represses transcription from the aromatase promoter

The real-time PCR study clearly indicates that the decline of the NR4A mRNA levels coincides with a surge in the aromatase expression at the later time points of forskolin treatment. This is reminiscent of the changes in the mRNA abundance of these genes upon the ovarian LH surge in preovulatory follicles, where the rapid decrease in aromatase mRNA level is accompanied by enhanced expression of the NR4A proteins (10, 11, 15). However, the function of the NR4A family members in folliculogenesis and steroidogenesis is poorly understood. In light of the regulatory role of the NR4A in transcription, we tested a possible causal relationship between NR4A and aromatase expression by cotransfecting into the KGN cells the NURR1 expression vector and a luciferase reporter construct driven by the ovary-specific promoter II (PII) of the aromatase gene. As shown in Fig. 3A, NURR1 severely mitigated the cAMP-induced transcriptional activation of the aromatase promoter in a dose-dependent manner. In contrast, the same amounts of NURR1 resulted in strong stimulation of the CYP11B2 promoter (Fig. 3B), a known NURR1-activated promoter (20). This suggests a promoter-specific repression of NURR1 on the aromatase promoter.

We also extended the luciferase-based reporter assay to include NGFI-B, another member of the NR4A subfamily (Fig. 4). The NGFI-B expression vector impaired transcription from the aromatase PII promoter to a similar extent as NURR1 (Fig. 4A). In contrast, the CYP11B2 promoter and a synthetic NR4A-responsive promoter p(Bla)$_8$luc harboring tandem repeats of the NR4A binding site (21) were robustly stimulated by NGFI-B and NURR1 (Fig. 4, B and C). Intriguingly, ectopic expression of NOR-1 did not result in an appreciable level of repression (data not shown), and the molecular basis of this awaits further exploration. Thus, at least two members of the NR4A subfamily may play an important

TABLE 1. List of forskolin-induced genes (2-fold or more)

No.	Gene	Accession no.	Folds	Functions
1	NURR1	NM_006186	18.9	Regulation of transcription, signal transduction
2	NOR-1	NM_173198	15.7	Regulation of transcription, cell growth, and/or maintenance
3	PP5395	NM_021732	8.3	Promotes MAPK activation and ENaC down-regulation
4	CREM	NM_001881	7.5	Binds the cAMP-response element
5	KLF4	NM_004235	5.9	Transcriptional activator; binds the CACCC sequence, involved in the differentiation of epithelial cells
6	RPS11	NM_001015	5.4	Protein biosynthesis
7	ZNF331	NM_018555	5.4	May play a role in renal development
8	IL11	NM_000641	5.2	Cytokine, cell-cell signaling
9	β4GalT	NM_001497	5.0	Catalyzes the production of lactose
10	EIF5A	NM_001970	4.9	Promoting the formation of the first peptide bond
11	TM4SF1	NM_014220	4.9	Unknown function
12	SLC7A8	NM_012244	4.1	Amino acid-polyamine transporter activity
13	DKFZP434 B044	NM_031476	4.0	Unknown function
14	CYP19A1	NM_031226	3.9	Aromatization of androgens
15	TCP8	NM_030751	3.9	Inhibits interleukin-2 gene expression
16	ID2	NM_002166	3.9	Inhibitor of DNA binding
17	XP32	AK005081	3.8	Skin-specific protein
18	HOXA5	NM_019102	3.2	Development regulation of transcription
19	GNAL	NM_002071	3.0	G proteins, modulators in various transmembrane signaling systems
20	STC1	NM_003155	3.0	Calcium ion homeostasis, response to nutrients
21	H3F3B	NM_005324	2.9	Chromosome organization and biogenesis
22	BCL3	NM_005178	2.9	Cytoplasmic sequestering of NF-κaB
23	RPS20	NM_001023	2.8	A component of the 40S subunit of ribosome
24	NKX3-1	NM_006167	2.8	A putative prostate tumor suppressor
25	INHBA	NM_002192	2.8	Inhibiting the secretion of follitropin by the pituitary gland
27	ING1	NM_198219	2.8	Induce cell growth arrest and apoptosis
28	CALR	NM_004343	2.7	Acts as a major Ca(2+)-binding (storage) protein in the lumen of the endoplasmic reticulum
29	DIPA	NM_006848	2.6	Affect HDV replication *in vitro*
30	NPYIR	NM_000909	2.6	Neuropeptide Y receptor activity
31	EMS1	NM_005231	2.6	Regulating the interactions between components of adherens-type junctions
32	RAB20	NM_017817	2.4	Plays a role in apical endocytosis/recycling
33	RPS10	NM_001014	2.3	RNA binding, structural constituent of ribosome
34	AQP3	NM_004925	2.3	Forms a water-specific channel
35	RPL38	NM_000999	2.3	A component of the 60S subunit in ribosome
36	NPC1	NM_000271	2.3	Mutation results in Niemann-Pick C disease
37	RPL37A	NM_000998	2.3	A component of the 60S subunit in ribosome
38	DAF	NM_000574	2.2	Prevents the formation of c4b2a and c3bbb, the amplification convertases of the complement cascade
39	FOSB	NM_006732	2.2	Dimerize with proteins of the JUN family
40	GATA4	NM_002052	2.1	Transcriptional activator, plays a crucial role in cardiogenesis
41	CASP9	NM_001229	2.1	Involved in the activation cascade of caspases responsible for apoptosis execution
42	KCNJ13	NM_002242	2.1	Inward rectifier potassium channel activity
43	TUBA1	NM_006000	2.1	A highly conserved homolog of a rat testis-specific α-tubulin
44	ELL2	NM_012081	2.1	Elongation factor that can increase the catalytic rate of RNA polymerase II transcription
45	PTP4A1	NM_003463	2.1	Belongs to a small class of prenylated protein tyrosine phosphatases
46	DDIT4	NM_019058	2.0	Regulated in development and DNA damage responses
47	GAB2	NM_012296	2.0	The principal activator of phosphatidylinositol-3 kinase in response to activation of the high-affinity IgE receptor
49	CALM1	NM_006888	2.0	Calcium-modulated proteins
50	CTBP1	NM_001328	2.0	A transcriptional repressor
51	SPEC1	NM_020239	2.0	A small protein effector of Cdc42
52	GPRC5C	NM_022036	2.0	A transmembrane receptor
53	MOX2	NM_005944	2.0	Costimulates T-cell proliferation
54	IGFBP1	NM_000596	2.0	Prolongs the half-life of the IGFs
55	TLE3	NM_005078	2.0	Transcriptional corepressor
56	GNAL	NM_020412	2.0	G-proteins-coupled receptor

role in down-regulating transcription of key steroidogenic genes in granulosa cells.

Knockdown of endogenous NURR1 leads to increased expression of the endogenous aromatase genes

To investigate the role of endogenous NURR1 in modulating forskolin-induced activation of the native aromatase gene, we transiently transfected KGN cells with an siRNA oligonucleotide that is known to specifically knock down NURR1 expression (Dharmacon) and examined its effect on the endogenous mRNA level of aromatase. As expected, the real-time PCR analysis showed a substantial reduction of the NURR1 mRNA level by the NURR1-specific siRNA (compare lane 2 with lane 3 and lane 5 with lane 6 in Fig. 5A). Importantly, the NURR1 knockdown resulted in a significant increase in both the basal and forskolin-stimulated expres-

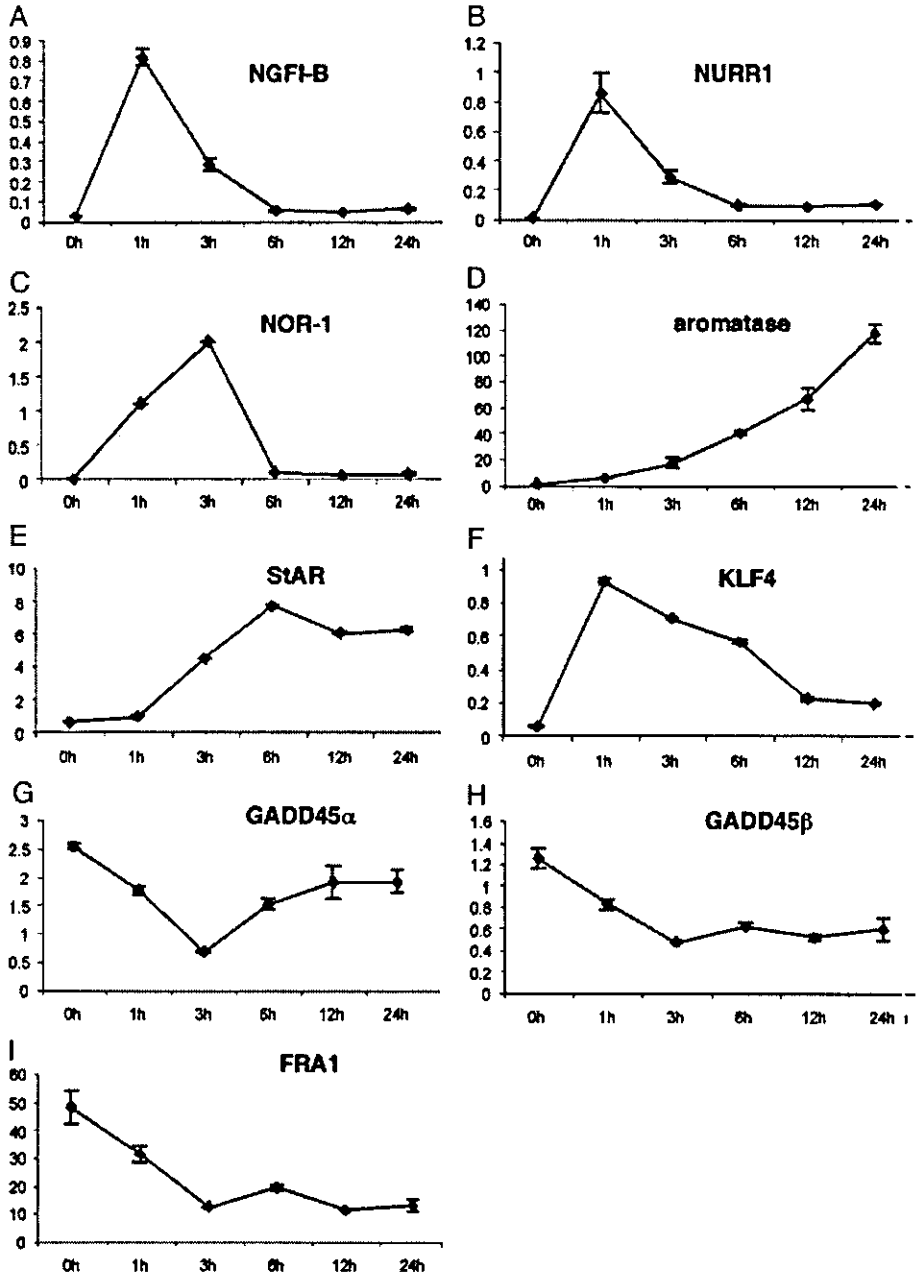

FIG. 1. Confirmation of the microarray data on several forskolin-responsive genes by real-time RT-PCR. KGN cells were treated with vehicle or forskolin (25 μM) for various periods of time. Real-time RT-PCR was conducted on the total RNA to assess the transcription level of several forskolin-responsive genes. The results are averages of duplicates that are normalized against β-actin mRNA levels.

sion of the aromatase gene (compare lane 2 with lane 3 and lane 5 with lane 6 in Fig. 5B). Reduction of NGFI-B by siRNA also led to a similar elevation of the endogenous aromatase mRNA (data not shown). Thus, the functional study clearly indicates that the NR4A proteins can act as transcription repressors to modulate aromatase gene activation in granulosa cells.

Further characterization of the NGFI-B- and NURR1-mediated transcriptional repression

To map the *cis*-element in the aromatase PII promoter that mediates the NR4A repression, a series of 5′ deletions of the promoter constructs were generated (Fig. 6A) and tested in the luciferase assay for their responsiveness to NURR1 and NGFI-B. As shown in Fig. 6B, deletion of most of the distal sequences of the PII promoter (*e.g.* between −1016 bp and −213 bp) reduced the magnitude of the forskolin-mediated transcription activation (compare lane 1 with lane 10). However, the same deletions did not abrogate the repressive effect of NGFI-B and NURR1 (compare lanes 1–3 with lanes 10–12 in Fig. 6B). The −213 bp construct of the aromatase promoter contains the binding sites for steroidogenic factor 1 and a cAMP-responsive element-like sequence, two critical *cis*-elements for conferring cAMP-responsive transcription of the aromatase PII promoter (26–29). Thus, our data indicate that the same promoter-proximal region in the aromatase PII

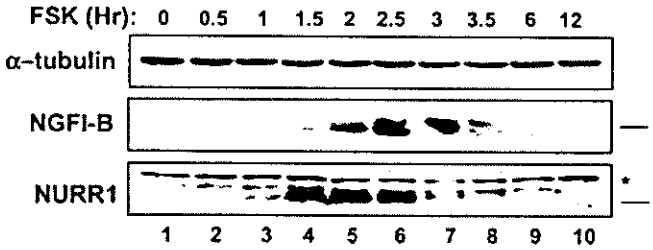

FIG. 2. Forskolin (FSK)-induced expression of NURR1 and NGFI-B proteins. An equal amount of whole-cell lysates was analyzed by immunoblotting for the presence of NGFI-B and NURR1 proteins, the positions of which are indicated by *bars*. The *band indicated by an asterisk* in the anti-NURR1 blot was not related to NURR1. In addition, α-tubulin was shown as a loading control.

promoter also mediates the transcription repression by NGFI-B and NURR1.

Like other nuclear receptors, members of the NR4A subfamily can be divided structurally into an N-terminal activation function (AF) 1 region, a central DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD; also known as AF2) (12). Multiple lines of evidence suggest that the N-terminal domain of NR4A proteins is mainly responsible for transactivation, whereas the C-terminal domain may modulate nuclear localization and transcriptional activation (30–34). In the current study, deletion of the C-terminal LBD moderately enhanced the activation of CYP11B2 by NURR1 (compare lane 2 with lane 5 in Fig. 7C). The same construct also efficiently repressed transcription of the aromatase promoter (compare lane 2 with lane 5 in Fig. 7B). However, further deletion that removed the DBD impaired both NURR1-mediated activation and repression (lane 4 in Fig. 7, B and C). Interestingly, an N-terminal deletion construct that lacks the entire AF1 domain was still capable of at least partial transcriptional activation and repression (lane 3 of Fig. 7, B and C). As indicated in Fig. 7D, all deletion constructs were expressed at least to the same level as the wild-type protein. Thus, the DBD appears essential for NURR1-mediated transcriptional regulation, whereas either AF1 or LBD might also be required for NURR1 functions in both gene activation and repression.

Discussion

The gonadotropin-responsive, cAMP-mediated signal transduction pathways play a central role in ovarian follicular and luteal development. In the past decade, gene-specific approaches have uncovered a number of important ovarian genes that are intimately controlled by the intracellular cAMP level. We carried out a genome-wide study of the cAMP-responsive gene expression profiles in ovarian granulosa cells. Our microarray analysis revealed 140 genes that were either activated or repressed by the cAMP-dependent signaling cascades. These include genes that are known to be cAMP- and gonadotropin-regulated genes, as well as numerous genes that have not been reported previously to be cAMP-responsive genes. Gene-specific real-time PCR analysis not only verified the microarray finding but also indicated distinct patterns of cAMP-mediated gene activation. In particular, members of the NR4A subfamily of the orphan nuclear receptors were robustly and transiently up-regulated, whereas expression of aromatase responded to the cAMP surge in a delayed fashion. Importantly, our work shows that NR4A proteins are capable of curtailing aromatase expression by inhibiting transcription from the ovary-specific aromatase PII promoter. Thus, the combination of genome-wide and gene-specific approaches shed light on the functional relationship among multiple cAMP-responsive genes in ovarian granulosa cells.

A recent miroarray study of gene expression in rat Sertoli cells has revealed a large number of FSH-responsive genes (35). By comparing the current forskolin-regulated gene sets in human granulosa cells with the published gene profile in rat Sertoli cells, we found several cAMP-repsonsive genes that are common to both microarray data sets. These include NURR1, NOR-1, and CREM. Of particular interest is the finding that NGFI-B was one of the most up-regulated genes from the published microarray study in rat Sertoli cells. Furthermore, the expression of NGFI-B in both human KGN and rat Sertoli cells followed a similar pattern in response to the cAMP surge. Given the analogous functions of ovarian granulosa cells and testicular Sertoli cells in folliculogenesis and spermatogenesis, respectively, these results suggest that the

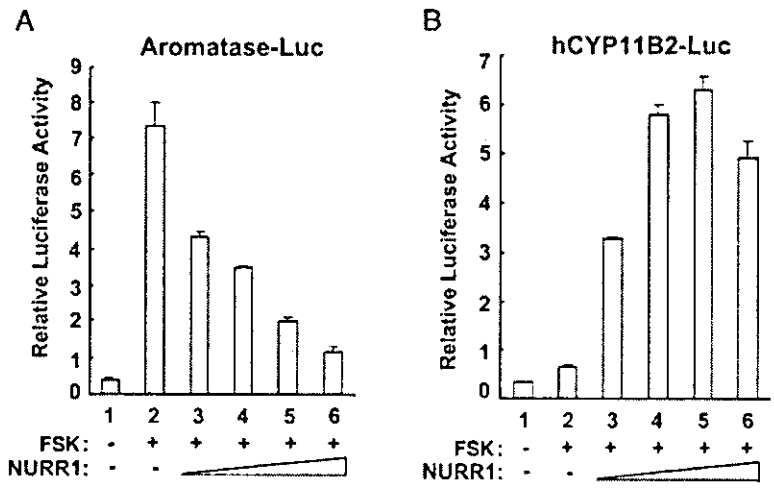

FIG. 3. Ectopic expression of NURR1 in KGN cells inhibits transcription from the ovary-specific PII promoter of the aromatase gene. Luciferase reporter plasmids (aromatase promoter in A and hCYP11B2 promoter in B) were cotransfected into the KGN cells with an increasing amount of the NURR1 expression vector (10, 30, 100, and 250 ng for lanes 3–6). The total amount of expression vector was kept constant in each experiment using the pcDNA3 empty vector. The results are averages of four different sets of data. FSK, Forskolin.

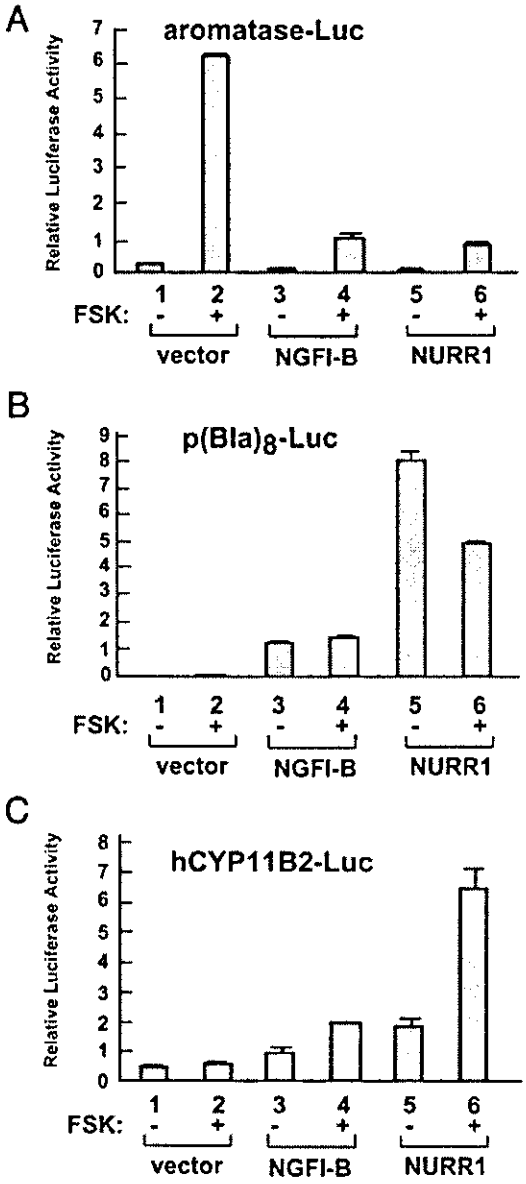

FIG. 4. Both NURR1 and NGFI-B repress transcription in a promoter-specific manner. A luciferase reporter plasmid harboring the aromatase promoter (A), a synthetic NR4A-responsive promoter (B), or the hCYP11B2 promoter (C) were cotransfected with various expression vectors. The transfected cells were subsequently treated with forskolin (FSK), and luciferase assay was conducted.

NR4A proteins may play a physiological function in both male and female reproductive development. It is also worth noting that a significant proportion of the activated genes from the two microarray studies does not overlap. Moreover, although most FSH-responsive genes in rat Sertoli cells are induced genes, more than 50% of the forskolin-affected genes in KGN cells are repressed. This could be due to several underlying reasons, including the differences in species, gender, culture system, or the number of genes represented in the arrays.

In addition to providing a comprehensive picture of gene

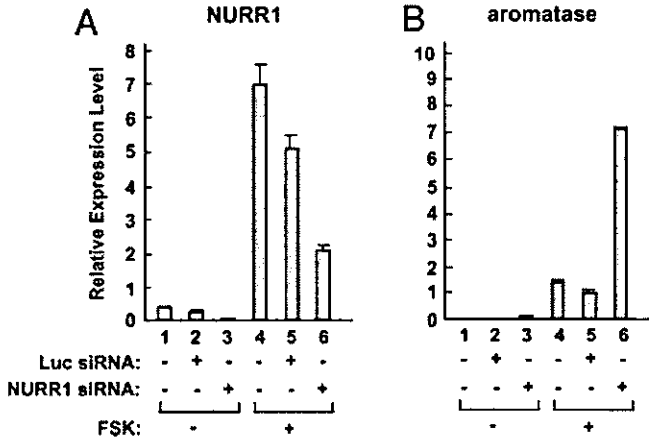

FIG. 5. Reduction of endogenous NURR1 in KGN cells enhances the basal and forskolin-induced expression of the aromatase gene. A, Quantitative real-time PCR analysis of the NURR1 mRNA level in the mock (buffer), control (luciferase), and NURR1-specific siRNA-transfected cells. B, Real-time PCR determining the aromatase transcript levels in the mock, control, and NURR1 knockdown cells. Results are averages of triplicates that are normalized against the transcript levels of β-actin.

expression profiles, information obtained from the gene array study may also aid in the understanding of the intricate interplay among specific ovarian genes. As an initial step toward elucidating the physiological significance of the cAMP-mediated induction of the NR4A orphan nuclear receptors, we showed that at least two members of this family, NGFI-B and NURR1, strongly inhibited transcription from the ovary-specific promoter (PII) of the aromatase gene. The functional relationship between NR4A proteins and aromatase as revealed in the tissue culture system may bear significant relevance to the maturation of ovarian follicles. A large body of studies in animal models and tissue culture systems has firmly established that hormone-dependent gene activation in ovarian follicles occurs in a sequential and stage-specific manner (1). In particular, granulosa cells of preovulatory follicles respond to the ovarian LH surge by undergoing two major physiological events, ovulation and luteinization. One of the biochemical hallmarks for luteinization is the rapid and dramatic reduction of the mRNA levels of many FSH-stimulated genes including aromatase (1, 10, 11). Intriguingly, NGFI-B and the other members of the NR4A subfamily are strongly stimulated by LH, which appears to activate both the cAMP/protein kinase A pathway as well as the calcium/protein kinase C pathway (15) (1). Consistently, it has been shown that NGFI-B mRNA is present in cultured ovarian granulosa cells and corpora lutea but not in small antral or preovulatory follicles (1). The strong inhibitory effect of NR4A on aromatase expression rationalizes the inverse correlation of the expression of these two genes upon LH surge, thus providing the first evidence for a causal relationship between these two gene regulatory events.

It has been well documented that different FSH-responsive ovarian genes *in vivo* follow distinct induction patterns in response to tonic secretion of FSH (1, 2, 6). In contrast to the immediate-early genes, such as Sgk and cyclin D2, the aro-