Orphan Nuclear Receptor Nur77 Induces Zinc Finger Protein GIO1-1 Gene Expression, and GIOT-1 Acts as a Novel Corepressor of Orphan Nuclear Receptor SF-1 via Recruitment of HDAC2*

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Kruppell-associated box (KRAB) domain-containing proteins consist of potential transcriptional regulation modules. Previously, gonadotropin-inducible ovarian transcription factor-1 (GIOT-1) was identified as a novel KRAB-containing zinc finger protein and shown to have transcriptional repression activity. Here, we demonstrate that orphan nuclear receptor Nur77 regulates GIOT-1 gene expression through Nur77 binding directly to GIOT-1 promoter and overexpression of GIOT-1 represses the SF-1 transactivation, and specific interaction assays showed that Nur77 directly binds to the GIOT-1 promoter. Electrophoretic mobility shift and chromatin immunoprecipitation assays showed that Nur77 directly binds to the GIOT-1 promoter. Recruitment of HDAC2 in the presence of Nur77 recruits the corepressor nuclear receptor corepressor to repress SF-1 transactivation, and specific interaction knockdown assays showed that Nur77 directly binds to the GIOT-1 promoter. Recruitment of HDAC2 in the presence of Nur77 recruits the corepressor nuclear receptor corepressor to repress SF-1 transactivation, and specific interaction knockdown assays showed that Nur77 directly binds to the GIOT-1 promoter. Recruitment of HDAC2 in the presence of Nur77 recruits the corepressor nuclear receptor corepressor to repress SF-1 transactivation, and specific interaction knockdown assays showed that Nur77 directly binds to the GIOT-1 promoter. Recruitment of HDAC2 in the presence of Nur77 recruits the corepressor nuclear receptor corepressor to repress SF-1 transactivation, and specific interaction knockdown assays showed that Nur77 directly binds to the GIOT-1 promoter. Recruitment of HDAC2 in the presence of Nur77 recruits the corepressor nuclear receptor corepressor to repress SF-1 transactivation, and specific interaction knockdown assays showed that Nur77 directly binds to the GIOT-1 promoter. Recruitment of HDAC2 in the presence of Nur77 recruits the corepressor nuclear receptor corepressor to repress SF-1 transactivation, and specific interaction knockdown assays showed that Nur77 directly binds to the GIOT-1 promoter. Recruitment of HDAC2 in the presence of Nur77 recruits the corepressor nuclear receptor corepressor to repress SF-1 transactivation, and specific interaction knockdown assays showed that Nur77 directly binds to the GIOT-1 promoter.
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Mechanisms that modulate the activity of SF-1 are not well characterized.

Kruppel-type zinc finger proteins belong to the largest known family of transcription factors (33, 34). These proteins are characterized by Cys2-His2 zinc finger motifs, often repeated in tandem, that fold around a zinc ion (35-37). About one-third of mammalian Cys2-His2 zinc finger proteins contain a conserved domain of ~75 amino acids called KRAB (Kruppel-associated box) (38). The KRAB domain is divided into an A and B box in which the KRAB-A domain itself harbors the transcriptional repression activity and the KRAB-B box seems to be dispensable (39, 40). This domain is located at the N-terminal of a Cys2-His2 zinc finger protein and confers strong distance-independent transcriptional repression activity (39-43). Moreover, the KRAB domain is a protein-protein interaction motif and possesses transcriptional repression function (39, 40). This domain is predicted to form an amphipathic helix that may interact with components of the basal transcriptional complex or other cellular proteins (38). Many KRAB domain zinc finger proteins are known to physically associate with a RING domain-containing corepressor protein known as KAP1 (KRAB-associated protein-1)/KRI-1 (KRAB-A interacting protein)/TIF-1β (transcription intermediary factor β) (42, 44, 45). KAP1 can enhance KRAB-A mediated repression and silence the transcriptional activity when the KAP1-KRAB-domain protein complex directly tethers to DNA (41-43). This silencing activity may result from recruitment of histone deacetylase (HDAC) complexes, such as nuclear receptor corepressor (46).

Recently, a novel KRAB domain-containing protein (RBaK) has been shown to function as a transcriptional repressor by interacting with retinoblastoma to repress E2F-dependent genes and prevent DNA synthesis (47). This protein has been identified as a new Cys2-His2 zinc finger protein family member. The expression of RBaK is regulated by SF-1 in ovarian granulosa cells and the function in other tissues are still not determined (11).

In this study, we have demonstrated that the SF-1 binding zinc finger protein, GIOT-1, is a novel target of LH-mediated Nur77 repression (38, 48). We have determined the interacting region of SF-1 and GIOT-1 and have shown that GIOT-1 can recruit HDAC2 and HDAC5 to SF-1 transactivation. Novel cross-talk between GIOT-1 and SF-1 nuclear receptors will provide new insights into the roles of KRAB-containing zinc finger proteins in nuclear receptor transactivation.

EXPERIMENTAL PROCEDURES

Hormone and Reagents—Ovine luteinizing hormone (LH-s-26; 2,300 IU/mg) was obtained from the National Hormone and Pituitary Distribution Program, NIDDK, National Institutes of Health (Baltimore, MD). Trichostatin A (TSA) was purchased from Sigma.

Cell Culture—HEK 293 and a mouse Leydig cell line, K28, were maintained in Dulbecco’s modified Eagle’s medium in the presence of 10% fetal bovine serum (Invitrogen), respectively. The rat Leydig cell line, R2C, was maintained in F10 medium supplemented with 15% horse serum and 15% fetal bovine serum (Invitrogen), respectively. The rat Leydig cell line was maintained in Dulbecco’s modified Eagle’s medium in the presence of 10% fetal bovine serum (Invitrogen), respectively. The rat Leydig cell line was maintained in Dulbecco’s modified Eagle’s medium in the presence of 10% fetal bovine serum (Invitrogen), respectively. The rat Leydig cell line was maintained in Dulbecco’s modified Eagle’s medium in the presence of 10% fetal bovine serum (Invitrogen), respectively. The rat Leydig cell line was maintained in Dulbecco’s modified Eagle’s medium in the presence of 10% fetal bovine serum (Invitrogen), respectively.
CGCCACAAGAGTCTG-3', which amplify a 250-bp region (−206 to +44) spanning the Nur77/SF-1 binding site of the rat GIOT-1 gene promoter. As a negative control, PCRs were performed using β-actin primer pairs (sense, 5'-GAGACCTTCAACACGCCGC-3', and antisense, 5'-CCGTACGGGCTTCTCGTCTC-3'), which amplify a 362-bp region spanning exon 4 of the β-actin gene.

**GST Pull-down Assay**—[35S]Methionine-labeled proteins were prepared using pcDNA3-HA vectors containing cDNAs encoding for full-length and deletion constructs of SF-1, DAX-1, SHP, and Nur77, and the TnT-coupled transcriptional translation system with conditions as described by the manufacturer (Promega). GST-fused GIOT-1 and GST alone was expressed in *Escherichia coli* BL21(DE3) strain and purified using glutathione-Sepharose 4B beads (Amersham Biosciences). In *vitro* protein–protein interaction assays were carried out as described previously (11, 50). For interaction between GIOT-1 and HDACs, GST-fused GIOT-1 and GST alone were expressed in *E. coli* BL21(DE3) strain and purified using glutathione-Sepharose 4B beads (Amersham Biosciences). Recombinant GST or GST fusion proteins were incubated with pre-washed glutathione-Sepharose 4B beads (Amersham Biosciences) while shaking for 30 min at room temperature and washed three times for 5 min at 4 °C with phosphate-buffered saline. To test for GIOT-1 and HDAC interaction, a GST pull-down assay was carried out with 1 × 10^7 K28 Leydig cells that were harvested, washed, and lysed in NP lysis buffer (50 mM Tris, pH 8.0/150 mM NaCl/5 mM EDTA/1% Nonidet P-40/1 mM PMSF/protease inhibitors). K28 extract was incubated with GST or GST-GIOT-1 fusion proteins bound to glutathione beads at 4 °C overnight. The beads were washed three times for 5 min with 1 ml of Nonidet P-40 lysing buffer while shaking for 30 min at room temperature and washed three times for 5 min with 1 ml of phosphate-buffered saline. Lysed proteins were separated by 4–12% SDS-PAGE, transferred to polyvinylidene difluoride (Invitrogen), and incubated with primary polyclonal rabbit HDAC antibodies (HDAC1, -2, -3, -5, -6, and -8, Zymed laboratories Inc.). After extensive washing, blots were visualized by chemiluminescence.

**siRNA Experiments**—siHDAC2-I to -IV and siGIOT-1-I to -IV were manufactured by Samchully Pham. Target sequences of siHDAC2-I, 5'-CAATCTAACTGTCAAAGGTCATGC-3', and antisense, 5'-UCCCGGAUGACUCAUAACUUTT-3', respectively. Target sequences of siGIOT-1-I, 5'-AGCACCTTTTCTGTTCAA-3', and 5'-AGCAUAAGGAGUUUGAAAGT-3', respectively. siRNA duplexes targeting HDAC2 and GIOT-1 mRNAs were transfected using Oligofectamine (Invitrogen) according to the manufacturer's instructions. Total RNA was prepared and used for RT-PCR. To detect the expression level of HDAC2, GIOT-1, CYP17, and β-actin mRNA, the following primers were used. For HDAC2, 5'-CAATCTAACTGTCAAAGGTCATGC-3' (forward) and 5'-TGAAGTCTGGTCTCAAATAACTCA-3' (reverse) were used for PCR amplification (28 cycles). For GIOT-1, 5'-CTCTGTGCCCCATTTCTCTTT-3' (forward) and 5'-CTCTTGTTCTCATGTT-3' (reverse) were used for PCR amplification (30 cycles). For CYP17, 5'-AGACCTTTTCTGGTCAA-3' (forward) and 5'-GGCTTTTGGGATTCTC-3' (reverse) were used for PCR amplification (28 cycles). For β-actin mRNA, 5'-CGTGAAAAAGATGACCAGATCATGTT-3' (forward) and 5'-GCTTGGGCTTCTGATG-3' were used for PCR amplification (23 cycles).

**RESULTS**

*GH and Nur77 Regulate GIOI-1 Promoter Activity*—A recent study showed that GIOI-1 gene expression is rapidly and strongly induced by
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pregnant mare serum gonadotropin or human chorionic gonadotropin in a testicular Leydig cell line (48), which led us to investigate the mechanism mediating the stimulatory effect of LH on GIOT-1 gene expression in Leydig cells. First, we examined whether GIOT-1 promoter activity is regulated by LH. To map the promoter region of GIOT-1, which is required for LH-mediated activation, a series of 5′ deletions of GIOT-1 promoter fused to a reporter gene were used in a transient transfection assay with or without LH treatment. As shown in Fig. 1, LH-mediated induction of GIOT-1 promoter activity was retained with deletion of the promoter up to −161 but lost with the −122 promoter. These results indicate that the sequences required for the LH response lie in the region between −161 and −122, which contains a potential Nur77 binding site (NBRE, AAAGGTC). To further investigate a role for the Nur77 protein in the LH-mediated stimulation of the GIOT-1 promoter, we cotransfected a Nur77 expression vector with Nur77 deletion constructs. We observed a significant stimulation of GIOT-1 promoter activity with deletion up to −161 following coexpression of Nur77 (Fig. 1C). However, a marked decrease in Nur77-mediated stimulation of the GIOT-1 promoter was observed upon deletion to −122. The results from Fig. 1 (B and C) suggest that Nur77-mediated transactivation of the GIOT-1 promoter may be directly mediated through the NBRE site.

To address whether the potential NBRE site in the GIOT-1 promoter is responsible for Nur77-mediated activation of the GIOT-1 promoter, an NBRE mutant (AAAGGTC to AAATTTCA) within the −282 GIOT-1 promoter construct (MT-282) was used in transient transfection assays. The participation of Nur77 in the induction of expression by LH was also confirmed by using a dominant-negative mutant of Nur77 (DN-Nur77), which completely abrogated transactivation activity of not only Nur77, but also other members of the Nur77 family, including Nurr-1 and NOR1 (Fig. 2B), with either a DN-Nur77 expression vector and were treated with or without Nur77-expressing adenovirus (AD-Nur77), RT-PCR was performed to detect GIOT-1 mRNA in rat Leydig R2C cells, and K28 cells were cotransfected with 200 ng of WT-282 or MT-282 reporter along with increasing amounts of expression vectors of Nur77 family members, pCMX-Nurr1 (100 ng), pCMX-NOR1 (100 and 200 ng, NOR1). Data are the mean ± S.E. of triplicate assays.

It has been reported that the Nur77 subfamily members, Nurr-1 and NOR1, also transactivate the NBRE-containing promoter, and they are induced by LH treatment in the testis (2). To explore the possibility that Nurr-1 or NOR1 is also involved in GIOT-1 gene expression, we cotransfected other Nur77 subfamily members with the GIOT-1 promoter reporter. Although the degree of activation was variable among Nur77 family members, GIOT-1 gene expression was increased by both Nurr-1 and NOR1 (Fig. 2B). These results suggest that the Nur77 family plays a major role in GIOT-1 gene transcription in the testis.

**LH and Nur77 Induce GIOT-1 Gene Expression in Testicular Leydig Cell Lines**—Our previous study demonstrated that LH rapidly increases the expression of Nur77 mRNA in testicular Leydig cells (2). Moreover, GIOT-1 expression has been well documented in ovarian granulosa cells (48). However, the hormonal regulation of GIOT-1 expression in Leydig cells has not been addressed. Therefore, we analyzed the regulation of GIOT-1 expression by LH in a testicular Leydig cell line, K28. Northern blot analysis revealed that GIOT-1 mRNA was barely detectable basally. However, the GIOT-1 mRNA level was significantly increased within 30 min, peaked at around 1 h, and then returned to basal levels at 3 h after LH treatment.

Based on the observation that overexpression of Nur77 stimulates the activity of the GIOT-1 promoter in transient transfection assays (Fig. 1B), we examined whether adenovirus-mediated Nur77 overexpression could increase the transcription of the GIOT-1 gene. Following infection with or without Nur77-expressing adenovirus (AD-Nur77), RT-PCR was performed to detect GIOT-1 mRNA in rat Leydig R2C cells, which are constitutively steroidogenic and express Nur77 (54). As...
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FIGURE 3. LH and Nur77 Induce GIOI-1 gene expression in K28 testicular Leydig cells. A. expression of GIOI-1 in the testicular Leydig cells. K28 cells were cultured in serum-free conditions for 24 h. These quiescent cells were then treated with LH (200 ng/ml) for up to 24 h. Total RNA (20 μg) was analyzed by Northern blotting using as cDNA probe for GIOI-1. The migration distances of 28 S and 18 S ribosomal RNA (left) and the GIOI-1 transcript (right) are indicated. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. B. adenovirus-mediated Nur77 overexpression stimulates GIOI-1 mRNA expression. R2C cells were infected with adenoviral vector expressing Nur77 (50 multiplicity of infection). Total RNA was isolated from cells analyzed by RT-PCR.

FIGURE 4. Nur77 binds to the GIOI-1 promoter in vitro and in vivo. A. EMSA of NBRE in GIOI-1. Labeled oligonucleotide was incubated with 10 μg of nuclear extracts from LH-treated (LH) or LH-ununtreated (CTL) K28 cells. Cells treated with LH for 2 h (LH) were incubated with the GIOI-1 probe with a 25- or 50-fold excess of unlabeled GIOI-1 probe competitor (S.C.) and a 25- or 50-fold excess of unlabeled mutated GIOI-1 probe (N.S.C.), and 2 μg of Nur77-specific antibody (Nur77-Ab) or SF-1-specific antibody (SF-1-Ab) was added to the reaction mixture before the addition of labeled probe. Free indicates running of labeled probe only, and the arrow indicates the Nur77-DNA complex. B, Nur77 was recruited to the endogenous GIOI-1 promoter in the Leydig cells. Chromatin immunoprecipitation assays were performed with R2C cells. Anti-Nur77 and anti-SF-1 or anti-HA antibodies were used for immunoprecipitations, and the immunoprecipitates were analyzed by PCR using a pair of specific primers spanning a region containing the Nur77/SF-1 binding site of the GIOI-1 gene promoters. A control PCR for nonspecific gene promoters was used as an internal control. The migration distances of 28 S and 18 S ribosomal RNA (left) and the GIOT-1 transcript (right) are indicated. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as an internal control. The migration distances of 28 S and 18 S ribosomal RNA (left) and the GIOT-1 transcript (right) are indicated. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The migration distances of 28 S and 18 S ribosomal RNA (left) and the GIOT-1 transcript (right) are indicated. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Nur77 Directly Binds and Activates the GIOT-1 Promoter—To investigate whether LH-mediated induction of GIOT-1 gene expression involves DNA binding of Nur77, we performed EMSAs. Nuclear extracts were prepared from mouse testicular Leydig K28 cells with or without LH treatment for 1 h. As shown in Fig. 4A, an intense shifted band was observed in the LH-treated lane. This protein-DNA complex was completely abolished with 50-fold excess of unlabeled specific NBRE probe but not with 50-fold excess of oligomer containing the mutated NBRE motif. To determine whether Nur77 is present in this complex, we preincubated whole cell extracts with an antibody against Nur77. The addition of anti-Nur77 prevented the formation of the LH-induced Nur77-DNA complex, whereas anti-SF-1 had no effect. To determine whether Nur77 or SF-1 actually binds to the GIOT-1 promoter in Leydig cells in vivo, we performed chromatin immunoprecipitation assays with rat Leydig R2C cells. As shown in Fig. 4B, PCR amplification of a region, from −206 to +44 of the GIOT-1 promoter, containing a NBRE motif, indicated Nur77 recruitment to the promoter region in vivo. Similarly, SF-1 was also bound to the NBRE-containing region of the GIOT-1 gene promoter but relatively weakly compared with Nur77. No signal was detected from the control PCR for nonspecific immunoprecipitation using anti-HA antibody. In addition, primers to the β-actin coding region did not produce any amplified bands, demonstrating the specificity of the immunoprecipitations. These results suggest that Nur77 directly binds to the GIOT-1 promoter, activating its gene expression.

GIOT-1 Functions as a Corepressor of Orphan Nuclear Receptor SF-1—Previous studies have demonstrated that GIOT-1 has a potent corepressor activity (48). To determine whether GIOT-1 plays a regulatory role in the transactivation of orphan nuclear receptors, we performed transient transfection assays with Nur77 or SF-1 along with GIOT-1 expression vectors in K28 cells. Coexpression of increasing amounts of GIOT-1 with a constant amount of SF-1 caused progressive repression of SF-1-mediated transactivation in a dose-dependent manner (Fig. 5A). However, GIOT-1 coexpression showed no significant effect on the Nur77 transactivation (Fig. 5B).

To determine whether the inhibitory effect of GIOT-1 on SF-1-mediated transactivation is due to direct repression of SF-1 activity, we used a SF-1 protein fused to Gal4-DBD (Gal4-SF-1). In HEK293 cells, Gal4-SF-1 increased by ~15-fold the activity of the reporter gene driven by Gal4 binding sites. Interestingly, coexpression of GIOT-1 significantly decreased Gal4-SF-1 transactivation in a dose-dependent manner (Fig. 5C). In contrast, GIOT-1 was unable to repress the transactivation activity of Gal4-Nur77 fusion protein. Taken together, these results demonstrated that GIOT-1 directly inhibits the transactivation activity of SF-1 but not that of Nur77.

WITHDRAWN
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It has been reported that the N-terminal repeat region of GIOT-1, which contains a KRAB-A domain, has transcriptional repression (48). Therefore, we tested two parts of GIOT-1, the N-terminal KRAB-A domain (GIOT-1-KRAB-A) and C-terminal zinc finger domain (GIOT-1-zinc finger), for interaction with SF-1 (Fig. 6D). GST pull-down assays revealed that GIOT-1-KRAB-A retained the ability to interact with SF-1 to the same extent as full-length GIOT-1 (Fig. 6E), suggesting that the N-terminal KRAB-A domain of GIOT-1 is responsible for the SF-1 interaction.

Because the GIOT-1-KRAB-A domain is required for the interaction with SF-1, we investigated whether GIOT-1-KRAB-A alone could repress SF-1-mediated transactivation. Transient transfection assays were performed with SF-1 in the presence of GIOT-1-KRAB-A or GIOT-1-zinc finger (Fig. 6F). As expected from interaction assays, coexpression of the GIOT-1-zinc finger failed to reduce SF-1-mediated transactivation, whereas GIOT-1-KRAB-A caused progressive suppression of SF-1-mediated transactivation. These results suggest that GIOT-1-KRAB-A is involved in both the interaction with SF-1 and the repression of its transactivation.

To verify a physical interaction between GIOT-1 and SF-1 in vitro, we performed mammalian two-hybrid analyses using GIOT-1 fused to the Gal4 DNA-binding domain (Gal4-GIOT-1), SF-1 fused to the VP16 activation domain (VP16-SF-1), and Gal4-tk-Luc as a luciferase reporter (Fig. 6G). SF-1 produced luciferase activity but not with Gal4 alone. However, coexpression of VP16-SF-1 with Gal4-GIOT-1 and SF-1-H26 resulted in a decrease in luciferase activity compared with Gal4 alone. This suggests that GIOT-1 repression activity on SF-1 transactivation by repression of TIF1 expression. These results suggest that the repressive activity of GIOT-1 involves the recruitment of HDAC.

To gain a further insight into the mechanism of GIOT-1-mediated repression, we analyzed the association of specific HDACs with GIOT-1. Bacterially expressed GST-GIOT-1 was incubated with K28 Lysd cell extracts, and HDAC proteins bound to GST-GIOT-1 were detected by Western blot analysis. As shown in Fig. 7C, the endogenous HDAC2 associated with GST-GIOT-1, whereas HDAC1, -3, -5, -6, and -8 did not (Fig. 7C). To address the effect of HDAC2 on GIOT-1 action, we coexpressed either HDAC1 or HDAC2 with GIOT-1 and analyzed their effect on the GIOT-1-mediated repression of SF-1 transactivation (Fig. 7D). Transient transfection assays showed a strong repression of SF-1 transactivation with coexpression of GIOT-1 and HDAC2 compared with expression of GIOT-1 alone, indicating that the two proteins have an additive effect on the repression of SF-1 transactivation.

**Figure 5. Repression of SF-1 transactivation by GIOT-1**—To determine whether GIOT-1 directly interacts with SF-1, we performed GST pull-down assays. [35S]Methionine-labeled SF-1, DAX-1, SHP, and Nur77 produced by in vitro translation were incubated with the GST fusion protein of GIOT-1 or GST alone. As shown in Fig. 6A, GIOT-1 specifically interacted with SF-1 and not with other nuclear receptors. GST alone did not interact with SF-1. These results are consistent with the data from functional analyses by transient transfection assays. The region of SF-1 responsible for the interaction with GIOT-1 was also assessed by GST pull-down experiments. [35S]Methionine-labeled SF-1 and its deletion mutants (Fig. 6B) produced by in vitro translation were allowed to bind the GST-fused GIOT-1 (GST-GIOT-1) or GST. GST-GIOT-1 interacted with SF-1 LBD as well as full-length SF-1 but not with SF-1DBD or SF-1ΔAF2 (Fig. 6C). These results suggest that GIOT-1 directly interacts with SF-1 through the AF-2 domain of SF-1.
FIGURE 6. Specific interaction between SF-1 and GIOT-1. A, GST pull-down assay. Purified GST-GIOT-1 (lanes 5–8) or GST alone (lanes 9–12) bound to glutathione-Sepharose beads were incubated with 35S-labeled SF-1 (lanes 5 and 9), DAX-1 (lanes 6 and 10), SHP (lanes 7 and 11), and Nur77 (lanes 8 and 12). After extensive washing, the reactions were analyzed by SDS-PAGE, and bound protein was visualized by autoradiography. The input represents 10% of the labeled SF-1 (lane 1), DAX-1 (lane 2), SHP (lane 3), and Nur77 (lane 4), used for the pull-down assay. B, schematic representation of the functional domains of SF-1. DBD, DNA-binding domain; LBD, ligand binding domain; AF2, activation function 2. C, analysis of GIOT-1 interaction domain of SF-1. Purified GST-GIOT-1 (lanes 5–8) or GST alone (lanes 9–12) bound to glutathione-Sepharose beads was incubated with 35S-labeled GIOT-1 (lanes 5 and 8), GIOT-1-KRAB-A (lanes 6 and 9), and GIOT-1-Zinc Finger (lanes 7 and 9). After extensive washing, the reactions were analyzed by SDS-PAGE, and bound protein was visualized by autoradiography. The input represents 10% of the labeled GIOT-1 (lane 1), GIOT-1-KRAB-A (lane 2), and GIOT-1-Zinc Finger (lane 3), used for the pull-down assay. D, schematic representation of the functional domains of GIOT-1. KRAB-A, Kruppel-associated box-A. E, analysis of SF-1 interaction domain of GIOT-1. Purified GST-SF-1 (lanes 4–6) or GST alone (lanes 7–9) bound to glutathione-Sepharose beads were incubated with 35S-labeled GIOT-1 (lanes 4 and 7), GIOT-1-KRAB-A (lanes 5 and 8), and GIOT-1-Zinc Finger (lanes 6 and 9). After extensive washing, the reactions were analyzed by SDS-PAGE, and bound protein was visualized by autoradiography. The input represents 10% of the labeled GIOT-1 (lane 1), GIOT-1-KRAB-A (lane 2), and GIOT-1-Zinc Finger (lane 3), used for the pull-down assay. F, KRAB-A domain is required for SF-1 repression. HEK293 cells cultured on 24-well plates were transfected with 250 ng of Gal4-tk-Luc reporter, 100 ng of pCMV-H9252-gal, and increasing amounts (100 and 300 ng) of pcDNA-GIOT-1 (GIOT-1) or pcDNA-HA-GIOT-1-KRAB-A (GIOT-1-KRAB-A) or pcDNA-HA-GIOT-1-Zinc Finger (GIOT-1-Zinc Finger). G, in vivo interaction. K28 cells were transfected with 250 ng of Gal4-tk-Luc reporter, 100 ng of pCMV-GAL4 DBD alone (Gal4), or pSG-GIOT-1 (Gal4-GIOT-I) with or without VP16-SF-1 (100 ng). Transfection efficiency was normalized using β-galactosidase activity. Data are the mean ± S.E. values of at least three assays.
To investigate the role of endogenous HDAC2 in regulating the transcriptional repression activity of GIOT-1, we conducted a mammalian one-hybrid assay comprising Gal4-GIOT-1 with or without transfection of a HDAC2 siRNA duplex. The expression of endogenous HDAC2 was markedly decreased by transfection of the HDAC2 siRNA duplex, siHDAC2-I, but not significantly by siHDAC2-II, siHDAC2-III, or siHDAC2-IV (Fig. 7E). The repression effect of siHDAC2-I was dose-dependent. In the cells transfected with siHDAC2-I and knocked down for endogenous HDAC2, the GIOT-1-mediated transactivation was increased similar to the Gal4 control (Fig. 7E). Transfection with siHDAC2-II, siHDAC2-III, and siHDAC2-IV did not affect the GIOT-1 transactivation. These observations suggest that the expression level of the intracellular HDAC2 gene may determine the transcriptional repression activity of GIOT-1.
To further investigate the role of GIOT-1 in SF-1-mediated gene activation, we examined the mRNA expression of CYP17, a well-known SF-1 target gene, in testicular Leydig cells after knockdown of endogenous GIOT-1 with GIOT-1 siRNAs (Fig. 7F). Of four tested siRNAs, each targeted to a different region of GIOT-1, siGIOT-1-II, and siGIOT-1-III were found to knock down the expression of GIOT-1 in R2C cells, as monitored by quantitative real-time RT-PCR. The repression effects of siGIOT-1-II and siGIOT-1-III occurred in a dose-dependent manner. Interestingly, reduction of GIOT-1 levels with siGIOT-1-II and siGIOT-1-III enhanced the expression of CYP17 in R2C cells. These results provide strong evidence that endogenous GIOT-1 plays a role in the modulation of SF-1 transcriptional activity in testicular Leydig cells. Taken together, these results suggest that GIOT-1 represses SF-1 transactivation *in vivo* by recruiting HDAC2.

**DISCUSSION**

The stimulatory effect of FSH on the gene expression of GIOT-1 in the ovarian granulosa cells has been previously characterized (49). However, the regulation of GIOT-1 expression in testicular Leydig cells has not been studied yet. In addition, the cellular function of GIOT-1 remains uninvestigated. In this report, we have identified the GIOT-1 gene as a novel target of the orphan nuclear receptor Nur77. We have demonstrated that LH stimulated GIOT-1 expression in testicular Leydig cells via Nur77, which binds to the specific response element in the GIOT-1 promoter. Furthermore, we demonstrate that GIOT-1 functions as a corepressor of SF-1 by recruiting HDAC2.

In contrast to other transcription factors that are constitutively expressed and activated by post-translational modifications, the expression of Nur77 is tightly regulated by extracellular signals and its post-translational modification is also important for its function (56). Because Nur77 has been reported to have high selectivity for its response element compared with other members of the Krüppel-associated box family, our results strongly suggest that Nur77 plays an important role in GIOT-1 transcription in testicular Leydig cells and binds to an element similar to, but distinct from, Nur77's recognition sequence. However, Nur77 is present at low levels in fetal tissues and becomes rapidly induced in response to testicular stimulation. This indicates that LH-mediated GIOT-1 gene induction most likely involves Nur77 rather than SF-1. Moreover, our observations of Nur77 recruitment to the GIOT-1 promoter and the enhancement of GIOT-1 gene expression upon adenovirus-mediated overexpression of Nur77 support the hypothesis that Nur77 plays an important role in GIOT-1 gene transcription.

It has been reported that SF-1 binds to the GIOT-1 promoter and that phosphorylation of SF-1 by protein kinase A increases its ability to enhance GIOT-1 gene expression (49). Thus, it is likely that activated protein kinase A phosphorylates SF-1 leading to enhanced transcription of GIOT-1. We previously showed that, in testicular Leydig cells, LH treatment rapidly and dramatically increased the levels of Nur77 mRNA and protein, and protein kinase A increased Nur77 expression (2). In addition, both Nur77 and SF-1 have been implicated in the regulation of 21-hydroxylase (CYP21) transcription (58). Furthermore, Nur77 protein level is significantly elevated in SF-1 ± adrenal (59). Together, these findings suggest that a balanced function of two proteins may be important for GIOT-1 gene expression. Nevertheless, Nur77 and SF-1 are not functionally equivalent, because subtle changes of the consensus DNA-binding sequence affect the transactivation of Nur77 and SF-1 differentially. Therefore, we propose that the effects of LH on GIOT-1 transcription occur through two possible pathways: increasing Nur77 gene expression and activating SF-1 through phosphorylation, although SF-1 alone did not significantly stimulate GIOT-1 promoter activity (Fig. 2A).

In this report, we demonstrate that GIOT-1 is a novel corepressor of SF-1. The KRAB-A domain of GIOT-1 exhibits transcriptional repression activity, which is consistent with the function of the KRAB-A domain described previously (39, 40). We also demonstrate that GIOT-1 interacts with SF-1 via the AF-2 domain, repressing its transactivation. These results indicate that the direct interaction between SF-1 and GIOT-1 is independent of additional factors. Although the possibility that other domains of SF-1 also participate in the binding of GIOT-1 cannot be excluded, our results clearly showed that the SF-1 AF-2 domain is an absolute requirement for the interaction with GIOT-1.

A functional connection between GIOT-1-mediated repression and HDAC activity was implied when the HDAC inhibitor, TSA, blocked GIOT-1 repression. In this study, we demonstrate that GIOT-1 can recruit HDAC2 and that the transcriptional repression of GIOT-1 is dependent on HDAC2 activity; this is comparable with the report that the transcriptional repression mediated by KRAB-A-KAP1 may result from association with the HP1 family (60–63), a family of non-histone proteins and proteins with an established gene-silencing activity. Our results elucidate a molecular mechanism for GIOT-1-mediated transcriptional repression activity of SF-1 (Fig. 7). However, the possible participation of components of other corepressor complexes in the GIOT-1-mediated SF-1 repression remains to be determined. In addition, further study will be required to assess the generality of the inhibitory mechanism of GIOT-1 and determine whether other mechanisms may contribute to the inhibitory effect of GIOT-1.

In conclusion, here we provide direct experimental evidence that orphan nuclear receptor Nur77 is involved in LH-mediated GIOT-1 gene expression in testicular Leydig cells and that GIOT-1 acts as a novel corepressor of the orphan nuclear receptor SF-1. To our knowledge, GIOT-1 is the first member of the KRAB-containing protein reported to interact with SF-1. The identification of HDAC2 involvement also provides new insight into the molecular mechanism by which the corepressor GIOT-1 mediates its repression. GIOT-1-mediated repression of SF-1 transactivation may play an important role in the regulation of SF-1 target genes during development and function of reproductive and steroid-producing tissues. Studies are currently underway to assess these possibilities.

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