DAX-1 Functions as an LXXLL-containing Corepressor for Activated Estrogen Receptors*

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We have discovered that the orphan receptor DAX-1 (NROB1) interacts with the estrogen receptors ERα and ERβ. Interaction occurs with ligand-activated ERs in solution and on DNA and is mediated by the unique DAX-1 N-terminal repeat domain. Each of the three repeats contains a leucine-rich receptor-binding motif, known as the LXXLL motif, which is usually found in nuclear receptor coactivators. We have demonstrated that DAX-1 functions as an inhibitor of ER activation in mammalian cells and suggest a mechanism involving two sequential events, occupation of the ligand-induced coactivator-binding surface and subsequent recruitment of corepressors. Accordingly, we propose that DAX-1 itself acts as a corepressor for ERs. Because DAX-1 is coexpressed with ERs in reproductive tissues, these interactions could play significant roles by influencing estrogen signaling pathways. Our results point at functional similarities between DAX-1 and the orphan receptor SHP (NROB2) in that they have acquired features of transcriptional coregulators that are unique for members of the nuclear receptor family.

To transduce hormone and metabolic signaling to target genes, nuclear receptors require transcriptional cofactors that are collectively referred to as coregulators (1). These proteins exist in multiple complexes, possess multiple enzymatic activities, and bridge receptors to chromatin components or to the basal transcription machinery or to both. Multiple candidate proteins exist that are believed to be critical for the proper function of nuclear receptor signaling (1–3). The majority of coregulators bind to the receptor ligand-binding domain (LBD),1 which is able to adopt different conformations depending on the ligand status and thereby discriminate between coactivators and corepressors. Functional and structural studies in particular elucidated the precise mechanisms of coactivator interaction with the ligand-inducible activation domain (AF-2) via short leucine motifs known as LXXLL or NR-Box (4–8).

We became interested in coregulators in particular that influence the transcriptional activity of estrogen receptors (ER). Two related subtypes, ERα and ERβ, play crucial roles in sex development and reproduction in multiple physiological processes as well as in cancer (9–11). Previous research has provided detailed insights into structural and functional aspects of their interplay with coregulators (8, 12–14). Although agonist binding usually is associated with ER activation caused by coactivator recruitment, regulatory mechanisms have been proposed that could play a role in modulation and feedback control of estrogen signaling (15, 16). Recent work has revealed an unexpected role of the orphan receptor SHP (NROB2) in inhibiting transactivation of ERs (17, 18). Particularly, we have provided evidence that SHP, which consists only of an LBD and thus cannot bind target genes directly, has instead acquired a novel coregulator function by antagonizing the interactions of ERs with associated coactivators (18, 19).

The closest relative to SHP within the nuclear receptor family is the orphan receptor DAX-1 (NROB1) (20), which has a homologous LBD but contains a unique three repeat domain in the N terminus representing a novel type of single strand DNA/RNA-binding domain (21–23). Mutations in the human gene encoding DAX-1 cause adrenal hypoplasia, a rare inherited male disorder that frequently is associated with hypogonadotropic hypogonadism (22). Intriguingly, many mutations abolish the potent silencing function within the LBD and have lost the ability to recruit corepressors such as N-CoR and Alien (24–27). Multiple evidence suggests key roles for DAX-1 in mammalian sex development, reproduction, and steroidogenesis (28–33). DAX-1 is predominantly expressed in adrenal, ovary, testes, hypothalamus, and pituitary and functionally antagonizes SF-1, an essential orphan receptor for the development of the hypothalamic-pituitary-gonadal axis and regulator of male-specific gene expression (27, 28, 30, 32–35). Together with demonstration of direct interaction, these findings suggest DAX-1 specifically acts as a cofactor for SF-1 (25, 32).

In this study we have provided evidence that DAX-1 may play roles in regulation of ER transactivation. We have demonstrated that DAX-1 directly binds to ERα and ERβ via the N-terminal repeat domain that contains LXXLL motifs. Functionally, DAX-1 inhibits the transcriptional activity of liganded ERs by a sequential mechanism, possibly involving the recruitment of corepressors. Accordingly, we propose that DAX-1 itself acts as an ER corepressor.

**EXPERIMENTAL PROCEDURES**

Phasmid—GST-DAX-1 DBD (aa 1–253) was made by recloning an EcoRI/SalI fragment from pGAL4-DAX-1 DBD (see below) into pGEX4T-1 (Amersham Pharmacia Biotech). GST-DAX-1 R3 (aa 115–199) was cloned by inserting PCR-generated fragments into EcoRI/SalI cut pGEX4T-1. GST-DAX-1 mut carrying the AXXAL mutation (see Fig. 1B) was generated by PCR mutagenesis. NR-Box peptide expression
Functional and Direct Interactions of DAX-1 with Estradiol Receptors

DAX-1 contains putative NR boxes in its N-terminal repeat region. A, schematic structure of DAX-1 and SHP showing the homology in their LBDs and the location of putative NR boxes. Black boxes indicate consensus LXXLL motifs and gray boxes indicate variants with leucine +4 substitutions. B, illustration of different DAX-1 constructs that are used in this study. C, alignment of NR boxes found in human DAX-1 and SHP.

RESULTS

Interaction of DAX-1 with Liganded ERs via the N-terminal Repeat Domain—Inspection of the human DAX-1 sequence revealed that each of the three N-terminal repeats contains a leucine-rich motif resembling the NR-box (Fig. 1A). Whereas the third motif (Box 3) matches the consensus LXXLL core with leucines in critical +1, +4, and +5 positions, the other two motifs contain methionine at the +4 position (Fig. 1C). Intriguingly, only Box 3 is conserved between species (e.g. mouse and human) DAX-1 has extensive amino acid homology (i.e. 70–80% identity with the leucine core) to the SHP-Box1 (Fig. 1, A and C), which we have previously demonstrated to be functional as an ER-binding motif (19). Therefore, we reasoned that the DAX-1 repeat region, which functions as a DNA/RNA-binding domain (21, 23), may additionally mediate binding to ERs.

We made several constructs expressing NR-Box-containing DAX-1 fragments fusing to GST (Fig. 1B) and assessed their binding to radiolabeled ERs in pull-down assays (Fig. 2). First, GST-DAX-1 DBD was found to interact with estradiol-bound ERα and ERβ (Fig. 2A) as well as with various other receptors (data not shown), indicating that the N-terminal repeat region of DAX-1 indeed may serve as receptor-binding domain. Second, because only repeat 3 contains a perfect LXXLL motif, we assessed binding of ERs to the repeat 3 region alone (Fig. 2B). Both ERs bound equally well, and estradiol enhanced the interactions. Third, to see whether these interactions were mediated by the LXXLL motif, we assessed binding to 14-mer NR-Box peptides fused to GST (Fig. 2C). No binding was observed using GST (G) alone. The input (I) represents 10% of the amount of labeled protein used in each pull-down assay.
analyzed in the context of the three repeat domain or the entire DAX-1 protein (data not shown).

To investigate whether DAX-1 can interact with DNA bound and liganded ER dimers in vitro, we performed gel shift experiments using purified proteins (Fig. 3A). Whereas estradiol in the presence of GST control protein induced a characteristic DNA complex caused by conformational changes upon ligand binding (lanes 1 and 2), addition of GST-DAX-1 R3 (see Fig. 1B) protein led to a significant upshift (compare lane 4 with lanes 2 and 3) indicating ternary complex formation. For comparison, the LXXLL domain of TIF2 fused to GST (6) promoted a ligand-dependent supershift as expected (lane 6). Ternary complex formation was similarly observed with ERs homodimers, with RXR heterodimers and with monomeric SF-1 (data not shown). These data indicate that DAX-1 interaction with other nuclear receptors is not interfering with dimerization and DNA binding but instead resembles coactivator-type interactions with the AF-2 domain via LXXLL motifs. Further evidence for the DAX-1 interaction with ERs was observed in two additional experimental settings. First, in a communoprecipitation assay, whole-cell extracts expressing FLAG-tagged ERs and wild-type DAX-1 were incubated with an αFLAG-affinity matrix and after washing, analyzed for the presence of DAX-1 protein (Fig. 3B). Overexpressed DAX-1 (lanes 2 and 4) as well as a protein possibly representing endogenous DAX-1 (lanes 1 and 3) were specifically coprecipitated only when FLAG-ERs were present but not in their absence (lanes 5 and 6). Second, in a yeast two-hybrid assay (Fig. 3C), GAL4-DAX-1 strongly interacted with both activation domain (GAD)-tagged ERs but not with GAD alone. Apparent differences between ERα and ERβ with regard to their ligand-independent interaction in two-hybrid assays are not a peculiarity of the DAX-1 interaction but have been observed with SHP as well (19).

**Functional Consequences of the DAX-1 Interaction with ERs**—To investigate functional consequences of the interactions of DAX-1 with ERs on the activity of estrogen-responsive promoters, we performed transient transfections (Fig. 4). We compared three different DAX-1 expression constructs: (i) wild-type DAX-1, which is known to be a nuclear protein and a potent transcriptional repressor (21, 23, 24, 27, 32), (ii) the naturally occurring DAX-1 R267P mutation, which is nonpressing possibly caused by its inability to bind the corepressors N-CoR and Alien (24, 26), and (iii) a GAL4-DAX-1 fusion protein lacking the N-terminal receptor interaction domain but containing the repressor function within the LBD. We observed that DAX-1 WT and surprisingly also DAX-1 R267P inhibited ERα (Fig. 4A) or ERβ activity (Fig. 4B), respectively, in a dose-dependent manner. However, the repression-defective DAX-1 variant appeared to be less effective than the wild-type DAX-1, particularly when using higher amounts of ER expression plasmid (data not shown), indicating that active repression may contribute to the inhibitory effects. Inhibition did not occur with GAL4-DAX-1 LBD lacking the N-terminal repeat domain, indicating that the inhibitory effect requires a direct interaction of DAX-1 with ERs and furthermore that the DAX-1 LBD cannot serve as an ER interaction domain.

To obtain further evidence for a possible recruitment of corepressors to ERs via DAX-1 as bridging protein, we performed a mammalian two-hybrid experiment in analogy to the experiments described for SF-1 (25). As seen in Fig. 4C, GAL4-ERα and VP16 activation domain-tagged N-CoR encompassing the DAX-1 interaction domain were cotransfected in the absence or presence of DAX-1. Consistent with the inability of N-CoR to bind to ERs in the presence of agonists (37, 38), we found that VP16-N-CoR in the absence of cotransfected DAX-1 had no effect on estradiol-induced ERα activity. However, VP16-N-CoR could partially restore reporter gene activity in the presence of inhibitory amounts of DAX-1. Because control Western blot analysis shows equal DAX-1 protein levels in both the absence or presence of VP16-N-CoR (Fig. 4C, lower), this result suggests that DAX-1 may serve as a bridging protein between liganded ERs and the co-repressor N-CoR (see Fig. 5).

**DISCUSSION**

The results presented in this study provide insights into previously uncovered aspects of DAX-1 structure and function and substantially expand the regulatory potential of DAX1. The interactions with ERs may be physiologically relevant because DAX-1 is expressed in multiple estrogen target tissues (9, 39). Summarizing the results from independent immuno-
histochemical analyses (40, 41), it is quite striking that ERα and ERβ proteins are apparently differentially expressed in distinct cell types of male and female reproductive tissues. For example, in testis ERα seems predominantly expressed in Leydig cells, whereas high ERβ expression seems restricted to Sertoli and germ cells. In ovary, ERβ is mainly expressed in granulosa cells, whereas ERα expression is much lower and restricted to interstitial theca cells. Although a comparative analysis of DAX protein expression needs to be accomplished, the known mRNA expression pattern suggests DAX-1 coexpression in all of these cell types (22, 29, 31, 32, 35). Notably, until now little is known about female-specific roles of DAX-1. Gene inactivation in mice surprisingly did not affect ovarian development and fertility but instead caused male infertility (28), a phenotype which intriguingly has been observed in mice lacking ERα (42). Moreover, developmental studies suggest coexpression of ERs and DAX-1 in testis and ovary during certain stages of embryogenesis (35, 43). Possibly, DAX-1 serves as a tissue- or stage-specific ER coregulator involved in modulation of estrogen signaling. Comparatively, much less is known about expression of ERs in adrenal gland, a major site of DAX-1 function in steroidogenesis. However, estrogens are known to affect adrenal development, and ERs have been detected in the cortex of fetal primate glands (44) as well as in all cell types of the adult rat gland (45). Because DAX-1 expression could be hormonally regulated (29), feedback mechanisms in steroidogenesis involving DAX-1 may principally resemble the recently discovered feedback loop in bile acid biosynthesis involving SHP (46, 47). Furthermore, our results indicate nuclear receptor binding as a novel feature of the DAX-1 repeat domain and thereby it reveals additional functional similarities between DAX-1 and SHP, the only two members of the nuclear receptor family that have acquired characteristics of transcriptional coregulators (18, 19, 48). Interestingly, the chicken DAX-1 homolog apparently lacks the entire mammalian repeat domain but contains in its short N terminus a single ILYSIL motif (49). This possibly suggests that binding to nuclear receptors is evolutionarily conserved between species whereas binding to DNA is not. Intriguingly, the mechanism we propose here for ERs may provide an alternative explanation for the inhibitory effects of DAX-1 on retinoic acid receptor transcription first reported in the original study (22), supporting the idea that DAX-1 may serve broader functions in nuclear receptor signaling (25, 39).

In Fig. 5 we provide a discussion model by integrating our

\[ \text{Fig. 4. DAX-1 inhibits transcriptional activation of ERs in mammalian cells possibly by recruitment of corepressors.} \]

\[ \text{800 ng of ERE-TATA-Luc reporter and 10 ng of ERα (A) or ERβ (B) expression plasmids, respectively, were cotransfected with increasing amounts (10 ng, 50 ng, 100 ng) of pSG5-DAX-1, DAX-1 R267P, or GAL4-DAX-1 LBD expression plasmids into COS-7 cells as described under "Experimental Procedures." C, coexpression of a VP16-tagged N-CoR fragment (DAX-1 interaction domain, aa 1689–2453) partially restores DAX-1 inhibition. COS-7 cells were cotransfected with 500 ng of UAS-tk luc reporter, 100 ng of GAL4-ERα, and 50 ng of VP16-N-CoR expression plasmids in the absence or presence of 1 μg of pSG5-DAX-1. Western blot analysis (lower panel) shows equal DAX-1 protein levels irrespective of VP16-N-CoR expression. All values represent the mean ± S.D. from triplicate transfections and were reproduced in at least three independent experiments.} \]

\[ \text{Fig. 5. Model describing the interplay of DAX-1 with upstream and downstream targets.} \]

\[ \text{DAX-1 is known to recruit corepressors. It is currently unknown whether ligand binding could convert DAX-1 into an activator because of its recruitment of coactivators. The unique DAX-1 repeat domain may bind single-stranded DNA regions in DAX-1 target genes directly. Alternatively, this domain may bind nuclear receptors such as ERs and SF-1 and thereby regulate target genes for these receptors indirectly. Additionally, DAX-1 may exert nongenomic functions such as RNA binding. For further discussion, see text.} \]

\[ \text{\textsuperscript{2} J. Å. Gustafsson and M. Warner, unpublished data.} \]
findings with current theories of DAX-1 mechanisms of action. In this model, DAX-1 is envisaged to mediate functional interactions through LXLL motifs and possesses an intrinsic silencing function, it is likely that DAX-1 may inhibit receptor activation by a sequential mechanism involving coactivator displacement and subsequent corepressor recruitment. Therefore, DAX-1 itself may be defined as an LXLL-containing corepressor and shares this feature with SHP. Although DAX-1 is a true orphan receptor, endogenous ligands could possibly activate DAX-1 from a repressor to an activator and thereby possibly activate, for example, estrogen target genes. Indeed, indirect evidence for a role of ligand-activated DAX-1 comes from the recent discovery of a patient with X-linked congenital adrenal hypoplasia carrying a missense mutation in the activation domain helix 12 (50), which is dispensable for corepressor binding but indispensable for coactivator binding to liganded receptors (1). Also, the DAX-1 helix 12 contains a glutamate residue that is conserved in all ligand-activatable receptors and was suggested to be critical for coactivator LXLL binding (7). Future structural information may be required to reveal the presence of a ligand-binding pocket. Until then, it is an exciting possibility that the coregulatory potential of DAX-1 could be hormonally, metabolically, or pharmacologically regulated, a novel aspect of both receptor and coregulator function.

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