Orphan nuclear receptors share sequence homology with members of the nuclear receptor superfamily, but ligands are unknown or unnecessary. A novel orphan receptor, estrogen receptor-related protein 3 (ERR3), was identified by yeast two-hybrid screening, using the transcriptional coactivator glucocorticoid receptor interacting protein 1 (GRIP1) as bait. The putative full-length mouse ERR3 contains 458 amino acids and is closely related to two known orphan receptors ERR1 and ERR2. All the ERR family members share an almost identical DNA-binding domain, which has 68% amino acid identity with that of estrogen receptor. ERR3 bound specifically to an estrogen response element and activated reporter genes controlled by estrogen response elements, both in yeast and mammalian cells, in the absence of any added ligand. A conserved AF-2 activation domain located in the hormone-binding domain of ERR3 was primarily responsible for transcriptional activation. The ERR3 AF-2 domain bound GRIP1 in a ligand-independent manner both in vitro and in vivo, through the LXXLL motifs of GRIP1, and GRIP1 functioned as a transcriptional coactivator for ERR3 in both yeast and mammalian cells. Expression of ERR3 in adult mouse was restricted; highest expression was observed in heart, kidney, and brain. In the mouse embryo, no expression was observed at day 7, and highest expression occurred around the 11–15 day stages. Although ERR3 is much more closely related to ERR2 than to ERR1, the expression pattern for ERR3 was similar to that of ERR1 and distinct from that for ERR2, suggesting a unique role for ERR3 in development.

The nuclear receptor (NR) superfamily is composed of both constitutive and ligand-inducible transcription factors that regulate pivotal gene networks important for eukaryotic cell growth, development, and homeostasis (1–4). The most well-studied members of the NR superfamily are receptors for steroid hormones, thyroid hormone, vitamin D, and retinoid acid, but the family also includes a group called orphan receptors whose cognate ligands are unknown or unnecessary. At present, orphan receptors are by far the largest subclass of the family, although some orphan receptors later may be removed from this subclass after the identification of their ligands (5, 6). Most orphan receptors are composed of three structural domains which are characteristic of NRs (7–9). The N-terminal domain, which often contains a transcriptional activation function, is usually not conserved among the family members, and is absent in some NRs. The C-terminal domains of NRs share 10–60% homology among the family members; this domain is responsible for binding ligands in the hormonally regulated NRs, and it also contributes to transcriptional activation. The centrally located DNA-binding domain (DBD) is typically composed of two four-cystine zinc fingers and is responsible for specific recognition of enhancer or hormone response elements in the promoter region of target genes. The DBD is the region which shares the highest homology among the NR family members (40–90%); its sequence is often used to classify NRs into subfamilies.

Although the biological functions of most orphan NRs are largely unknown, they are likely to play some important roles, since their sequences are usually highly conserved among mammals and even other taxa, and their expression in either embryonic or adult tissues often occurs in very restricted spatial and temporal patterns (5). For example, steroidogenic factor 1 regulates many of the enzymes that synthesize steroids and plays a key role in the development and differentiation of the adrenal gland and gonads (10–12). Hepatic nuclear factor 4 is preferentially found in the liver where it controls the expression of liver-specific genes (13). Orphan receptors may act by different mechanisms (5). Some act as enhancer element-bound transcriptional activators for specific genes, in a manner similar to the well-documented mechanism of hormone-binding NRs. Other orphan receptors may regulate the function of other NRs, by competing for the same enhancer and some NRs lack an N-terminal activation function. The AF-2 activation domain within the C-terminal hormone-binding domain (HBD) of NRs is also important for transcriptional activation and is highly conserved among essentially all NRs that activate transcription (15–17). Recently, a new class of proteins called transcriptional coactivators have been found to play key roles in mediating transcriptional activation by NRs (18, 19). While transcriptional coactivators for the AF-1 do-
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mains are mostly unknown, quite a few putative coactivators for the AF-2 domain have been reported, and some were shown to enhance or be required for transcriptional activation by NRs, including some orphan receptors. The best characterized coactivators for NRs are three genetically distinct but structurally and functionally related proteins of approximately 160 kDa known as NR coactivators or p160 coactivators: SRC-1 (20, 21), GRIP1 (22, 23) (also known as transcriptional intermediary factor 2 (24)), and p300/CPB interacting protein (25) (also known as ACTR (26), receptor-associated coactivator 3 (27), amplified in breast cancer 1 (28), and thyroid hormone receptor activator molecule-1 (29)). The physical interactions between NRs and these coactivators are quite conserved: the conserved AF-2 domains of apparently all NRs that function as transcriptional activators form a conserved hydrophobic groove that accommodates an LXXLL motif (where L is leucine and X can be any amino acid) found in several copies in the p160 coactivators (30–35). Classical NRs require binding of ligand before they can interact with coactivators and activate transcription (21, 23, 24). However, at least some orphan receptors have been found to bind coactivators and activate transcription in the absence of any added ligand. For example, orphan receptor CARβ apparently functions as a transcriptional activator in the absence of ligand, but the binding of its ligand results in the dissociation of bound coactivators, thus providing a negative regulatory mechanism for the ligand (36).

Since the first two orphan NRs, estrogen receptor related proteins 1 and 2 (ERR1 and ERR2), were identified 10 years ago based on their sequence homology with the DBD of estrogen receptor (ER) (37), a large number of new orphan NRs have been identified (5, 6). Orphan NRs have recently been one of the most rapidly growing families of proteins, and characterization of their functions has contributed to our understanding of important biological processes. This paper reports the identification of mouse ERR3, a novel member of the ERR subfamily of orphan NRs, by a new approach which is based on the interaction of the receptor with its transcriptional coactivator GRIP1. We tested the ability of ERR3 to act as a transcriptional activator, both in mammalian cells and in yeast, identified a functional domain responsible for transcriptional activation, and explored the role of coactivators in ERR3 function. A comparison of the sequence homology and expression patterns for the three ERR proteins suggests distinct developmental and/or regulatory roles for all three.

MATERIALS AND METHODS

Plasmids—Mammalian expression vectors: pSG5.HA was constructed by inserting AATTACCCGCGATGCGCTACCATACGATTGTTCCTGACTATCGCATGGGATTCTCGA, containing a translation start signal and codons for hemagglutinin (HA) epitope tag YPYDV- DYA (38) (both underlined), into the EcoRI/BamHI sites of pSG5 (Stratagene); the original EcoRI site was thus inactivated, and a new EcoRI site and an XhoI site were introduced after the HA coding sequence. The multiple cloning site following the HA tag contains EcoRI, XhoI, BamHI, and BglII sites. The following ERR3 expression vectors were constructed by inserting the indicated PCR amplified fragment into pSG5.HA: pSG5.HA-ERR3AF-1, an XhoI/BglII fragment encoding ERR3-28–458, pSG5.HA-ERR3a, a XhoI/BamHI fragment encoding ERR3-28–458 and pSG5.HA-ERR3AF-2, an XhoI/ BamHI fragments encoding ERR3-28–458 and ERR3-292–458 respectively. Expression vectors for ERR3-292–458 containing L449A/F450A or M453A/L454A amino acid substitutions were made by including relevant point mutations in PCR primers, and then inserting the PCR amplified genomes into the XhoI/BglII sites of pSG5.HA-ERR3, encoding full-length ERR3, was constructed by inserting the PCR amplified C-terminal region of ERR3 into the BamHI/BglII sites of pSG5.HA-ERR3-28–458 by Reporter genes EREII-LUC GL450 (39), MMTV-LUC, MMTV(ERE)-LUC, and MTV(ERE)-LUC (40), and expression vectors pSG5.GRIP1 for full-length GRIP1 (30), pCMX.ACTR for ACTR (26), pHE0 for ER (41), pSV40.AR for androgen receptor (AR) (42), and pCMX.TR6/1 for thyroid hormone receptor β1 (TRβ1) (43) were described previously. Expression vector pSG5.HA-SRC-1a for full-length SRC-1a was made by inserting Smal/SalI fragment at the EcoRI site (which was blunted by filling with Klenow polymerase) and the XhoI site of pSG5.HA.

Bacterial expression vectors: pBG79.GRIP1a encoding a Gal4DBD/ GRIP1 fusion protein was made by inserting a GRIP1 EcoRI/XhoI fragment into EcoRI/SalI sites of pGBT9 (CLONTECH), pGAD10. ERR3 encoding the fusion protein of the Gal4 activation domain (Gal4AD) and mouse ERR3 amino acids 28–458 were isolated by yeast two-hybrid screening (see below). pGBT9. ERR3 encoding the Gal4DBD/ERR3 fusion protein was made by subcloning an EcoRI fragment encoding the ERR3-28–458, from pGAD10. ERR3 (derived by partial digestion with EcoRI) into pGBT9. Vectors for fusion proteins of Gal4 DBD and ERR3 AF-2 mutations were made by a strategy similar to that described above for the corresponding mammalian vectors containing ERR3 AF-2 mutations; the resultant PCR amplified fragments coding for ERR3 HBD (amino acids 214–458) were inserted into the BamHI site of pGBT9. Vectors for full-length GRIP1 (pBRIP1b1) and for fusion proteins of Gal4AD with wild type GRIP1 and its NR Box mutants were described previously (23, 30). Yeast expression vector pGBT9.p300 for the fusion protein of Gal4DBD and p300 (amino acids 1856–2414) was described previously (44). Yeast expression vectors pRS314-GR NT795 encoding full-length rat glucocorticoid receptor (GR) and pRS314-HER_encoding full-length human ER were constructed by first inserting a ClaI/BamHI fragment containing the yeast gpd promoter into pRS314 (trp1-1 cen-5). A BamHI fragment encoding rat GR or a BamHI/SalI fragment encoding human ER was then inserted.7 YEp46-HTR1 encoding full-length human TRβ1 was described previously (46), as were yeast reporter genes ERE1-CYC-LacZ at 47, GREG-CYC-LacZ at 48, and F2-CYC-LacZ (containing a thyroid hormone response element (TRE)) at

8 S. P. Bohem, personal communication.

RESULTS

Identification of Mouse ERR3—To identify proteins that interact with transcriptional coactivator GRIP1, a fragment of

8 S. P. Bohem, personal communication.
GRIP1 (amino acids 5–765) was used as bait to screen a mouse 17-day embryo cDNA library by the yeast two-hybrid system. The screening was performed in the absence of any hormone in order to avoid isolating classical hormone-regulated NRs, which interact with GRIP1 in a strictly hormone-dependent manner (21, 23, 24, 30). As a result of the screening, a 1.5-kilobase cDNA clone was identified. A BLAST search (50) of GenBank using the predicted amino acid sequence indicated that the known proteins with the highest sequence homology were two orphan NRs, ERR1 and ERR2 (37) (Fig. 1, A and B). Therefore, the new protein was named estrogen receptor-related protein 3 (ERR3). Like other orphan NRs, ERR3 exhibited typical structural features of the NR family (Fig. 1B): a putative DBD, which is almost identical to that of ERR1 and ERR2; a putative HBD, sharing 57% amino acid identity with ERR1 and 73% with ERR2; and an N-terminal domain which shares very little homology with ERR1 but 58% amino acid identity with ERR2. The regions of sequence similarity and divergence between ERR3 and the other ERR proteins were finely dispersed throughout the entire lengths of the proteins (Fig. 1A), indicating that ERR3 is not a splicing variant of either ERR1 or ERR2, and thus is a novel member of the ERR subfamily of orphan NRs.

Since the original ERR3 cDNA sequence isolated from the
yeast two-hybrid screening was presumably only a partial sequence, it was used to perform a BLAST search of GenBank EST data base. A human cDNA sequence (GenBank Accession Number W26274) overlapped with the 5’-end of our partial mouse ERR3 cDNA clone, and the overlapping portions shared 93% identity; PCR primers designed from this presumed human ERR3 sequence were then used to amplify a DNA fragment from the mouse 17-day embryo cDNA library by PCR. The amplified DNA fragment was identical to the original mouse ERR3 sequence in their overlapping region and had a 5’-extension relative to the original mouse cDNA clone. The combination of both sequences resulted in a cDNA sequence of 1567 nucleotides (GenBank Accession Number AF117254), which contains an open reading frame of 458 amino acids and is proposed to encode the full-length mouse ERR3. The putative start codon was designated because of an upstream stop codon in the same reading frame and because the sequence around the putative start codon resembled the consensus for a translation start signal (51). The same PCR primers were also used to amplify a DNA fragment from a human brain cDNA library. The resulting cDNA sequence (not shown, GenBank accession number AF117255) corrected several likely sequencing mistakes from the original human EST cDNA, and encoded the N-terminal 129 amino acids of a human ERR3 protein which is identical to that part of mouse ERR3. During our characterization of mouse ERR3, Eudy et al. (52) reported the isolation of a human cDNA sequence ESRRG, which encoded a protein sequence sharing 98% amino acid identity with mouse ERR3 codons 24–458; no functional analysis of ESRRG was reported. Our mouse and human ERR3 cDNA sequences are totally unrelated to that of ESRRG before our ERR3 amino acid 24 (amino acid 1 in ESRRG), indicating that ESRRG and ERR3 are probably splicing variants produced from the same gene.

RNA blot analysis of adult mouse tissues, using the N-terminal region of ERR3 as a probe, indicated the presence of a 5.7-kilobase transcript of ERR3 in selected tissues, with the highest expression in the heart, brain, and kidney, much lower expression in the liver, and no detectable expression in other tested tissues (Fig. 2A). In mouse embryo, ERR3 was not expressed at the 7-day stage; highest expression was detected during the 11–15-day period, and decreased expression at 17 days (Fig. 2B). Eudy et al. (52) reported a different distribution of ERR3 transcripts in adult mouse and human tissues. However, we have observed the same expression pattern (Fig. 2A) in two independent adult mouse tissue RNA blots.

**Activation of ERE-controlled Reporter Genes by ERR3 Without Any Added Ligand**—Since ERR3 contains a putative DBD which shares 68% amino acid identity with the DBD of ER, we tested whether ERR3 could bind a functional estrogen response element (ERE). In yeast, while Gal4AD by itself did not produce detectable β-galactosidase activity from an ERE-controlled reporter gene, a Gal4AD/ERR3 fusion protein activated the same reporter gene (Table I). Since Gal4AD cannot bind the ERE, the activation of the reporter gene must result from the binding of ERR3 to the ERE and the subsequent activation by the tethered Gal4AD. Co-expression of the Gal4AD/ERR3 fusion protein with reporter genes controlled by glucocorticoid response elements (GRE) or a TRE caused only a very weak activation of the reporter gene, indicating that the binding of ERR3 with ERE is DNA sequence-specific (Table I). All those reporter genes were functional in yeast in the presence of the appropriate NRs and hormones.

When ERR3 was transiently expressed in mammalian cells, it activated a reporter gene controlled by a basal herpesvirus thymidine kinase promoter and two EREs, and the activity increased with the amount of ERR3 expression vector transfected (Fig. 3A). ERR3 also activated a MTV(ERE)-LUC reporter gene controlled by a modified mouse mammary tumor virus (MMTV) promoter with an ERE substituted for the original GREs (Fig. 3B). ERR3 increased the activity of this reporter gene 100-fold but only caused a very weak activation of reporter genes with a native MMTV promoter (containing GREs) or a modified MMTV promoter with a TRE substituted for the GREs. Thus ERR3 selectively activated reporter genes regulated by EREs, and the ERE-dependent activation effect was observed with different types of basal promoters. Note that activation of the reporter genes by ERR3 occurred in the absence of any exogenously added hormone. For these experiments the serum used in the growth medium was pretreated by charcoal/dextran adsorption, which removes many potential small ligands including steroids. Adding some known hormones, estradiol or dihydrotestosterone, to the growth medium at a concentration of 100 nM did not change the activity of

| ERE | GRE | TRE |
|-----|-----|-----|
| Gal4 AD | −a | − | − |
| Gal4 AD/ERR3 | ++b | ++b | ++ |

| a | , white color, no β-galactosidase activity. |
| b | , strong blue color appeared within 1–2 h. |
| | , faint blue color indicating β-galactosidase activity appeared after 6–12 h incubation. |
| | NRs and hormones used as positive controls for the reporter genes: for ERE, ER with 100 nM estradiol; for GRE, glucocorticoid receptor with 10 μM deoxy cortisol, for TRE, thyroid hormone receptor β1 with 10 μM triiodothyronine. |
ERR3 (data not shown). These results suggested that ERR3 was able to function in a ligand-independent manner.

To localize the transcriptional activation domain(s) of ERR3, deletion mutants of ERR3 lacking the N-terminal domain or the C-terminal HBD were tested in mammalian cells for their ability to activate reporter genes. When the C-terminal region homologous to NR HBDs was deleted, the N-terminal region (the putative AF-1 domain) of ERR3 showed only very weak transcriptional activation activity compared with the control of reporter gene alone (Fig. 4B). In contrast, after deletion of the N-terminal region, the remaining DBD/HBD fragment of ERR3 was still very active. Sequence analysis indicated that all three ERR family members share a highly conserved region near their C termini, which is also highly homologous with the typical AF-2 activation domain found in the analogous location in essentially all NRs that function as transcriptional activators (Fig. 4A). In order to test whether the putative AF-2 domain of ERR3 is also responsible for the transcriptional activation activity in its HBD, we altered that region by making alanine substitutions in pairs of conserved hydrophobic residues (L449A/F450A and M453A/L454A) or by deleting amino acids 449–458 (ΔAF-2). Similar mutations in ER as well as other NRs eliminated transcriptional activation activity but did not disrupt other functions of the HBD such as hormone binding (9, 15). Either the alanine substitutions or the deletion essentially eliminated the transcriptional activation function of the ERR3 HBD (Fig. 4B), indicating that this highly conserved region in ERR3 also represents a functional AF-2 activation domain. Immunoblot analyses indicated that the mutant ERR3 proteins were expressed at levels about one-third to one-half that of wild type ERR3 (data not shown). The almost complete loss of activity observed cannot be explained by this moderate reduction in expression and indicates that the mutations caused severe loss of protein function.

Ligand-independent and AF-2 Domain-dependent Binding of Transcriptional Coactivators by ERR3—Transcriptional coactivators, including GRIP1 and other p160 coactivators, play key roles in the normal function of NRs, and such roles are based on the physical interaction between NRs and coactivators (18, 19, 23, 30). ERR3 was isolated through its ability to interact with GRIP1. To confirm the specificity of this interaction, the isolated Gal4AD/ERR3 clone was coexpressed with Gal4DBD/GRIP15–765 and other control proteins in yeast two-hybrid assays. Gal4AD/ERR3 interacted with Gal4DBD itself or its fusion protein with an irrelevant protein, p53 (Fig. 5A). The interaction between ERR3 and GRIP1 in the yeast two-hybrid assays occurred in the absence of any exogenously added hormones, whereas classical nuclear hormone receptors interact with GRIP1 in a strictly hormone-dependent manner (21, 23, 24, 30). The ligand-independent interaction between GRIP1 and ERR3 was also observed in vitro by GST pull-down assays. While bead-bound GST did not bind ERR3 synthesized in vitro, the bead-bound GST-GRIP1Δ63–1121 bound ERR3 (Fig. 5B). No hormone was added either in the synthesis of the proteins or in the

FIG. 3. Activation of reporter genes by ERR3 in mammalian cells. A, different amounts of pSG5.HA-ERR3 were transiently transfected into CV-1 cells with 0.5 µg of luciferase reporter gene EREII-LUC. CV-1 cells were grown in charcoal/dextran-treated serum with no exogenously added hormone. B, CV-1 cells were transiently transfected with 0.5 µg of pSG5.HA-ERR3 (ERR3) or empty pSG5.HA vector (vector), together with 0.5 µg of a luciferase reporter gene controlled by either MTV(ERE), native MMTV (containing GREs) or MTV(TRE) promoter.
yeast strain SFY526, which contains an integrated b-galactosidase reporter gene controlled by Gal4-binding sites, was transformed with pGAD10. ERR3 encoding Gal4AD/ERR328–458 and pGBT9 plasmids encoding Gal4DBD or Gal4DBD fused with GRIP115–765 or p5372–390. Yeast two-hybrid assays were performed with no added ligand, and two-hybrid assays were performed with no added ligand, and b-galactosidase activity from the yeast cell extracts indicated protein-protein interactions. ERR3 flanking DBD, Gal4DBD; AD, Gal4AD; u, units. B, full-length ERR3 (ERR3 fl) or its fragments or mutants (as described in Fig. 4B) were synthesized in vitro with [35S]methionine and then incubated with Sepharose bead-bound GST or GST/GRIP1505–1121, in the absence of any added hormone. After washing, the bead-bound ERR3 proteins were eluted and analyzed by SDS-PAGE and autoradiography. For reference a sample equivalent to 1/10 of the labeled protein in the binding assay is shown along with the total amount of labeled protein bound to the beads. C, Gal4DBD fused with wild type or mutant ERR3 HBDB (amino acids 214–458) and Gal4AD or Gal4AD fused to full-length GRIP1 were co-expressed in yeast two-hybrid assays as in A.

Binding reactions. GST pull-down assays also indicated that the HBD of ERR3 was responsible for the interaction with GRIP1, while its N-terminal domain (AF-1) did not interact with GRIP1 (Fig. 5B). The same mutations in the AF-2 domain of ERR3 that eliminated its ability to activate a reporter gene (data not shown). Thus the orphan receptor ERR3, like the classical nuclear hormone receptors, can utilize GRIP1 as a transcriptional coactivator for a wide group of NRs, both in yeast and mammalian cells (23, 30, 54). Since ERR3 can interact with GRIP1 and this interaction closely correlates with the transcriptional activation activity of ERR3, we predicted that GRIP1 would also serve as a transcriptional coactivator for ERR3. In transiently transfected CV-1 cells the activation of an ERR controlled reporter gene by ERR3 was enhanced by wild type GRIP1, but not by GRIP1 containing mutations in NR Boxes II and III (GRIP1mt), SRC-1a, or ACTR were synthesized with [35S]methionine in vitro and then incubated with Sepharose bead-bound GST or GST/ERR328–458 in the absence of any added hormone. After washing, the bead-bound ERR3 proteins were eluted and analyzed by SDS-PAGE and autoradiography.

GRIP1 Functions as a Transcriptional Coactivator for ERR3 in Both Yeast and Mammalian Cells—GRIP1 can function as a transcriptional coactivator for both two-predicted coactivator family members, SRC-1 and ACTR, in vitro in addition to GRIP1 (Fig. 6C), thus indicating a conserved interaction of ERR3 with all three p160 coactivator family members.

GRIP1 and wild type GRIP1 fusion proteins were all able to interact with p300. The combined NR Boxes II and III mutations of GRIP1 also drastically reduced binding of GRIP1 to GST/ERR3 in vitro (Fig. 6C). ERR3 bound the other two p160 coactivator family members, SRC-1 and ACTR, in vitro in addition to GRIP1 (Fig. 6C), thus indicating a conserved interaction of ERR3 with all three p160 coactivator family members.

Relating the results of in vitro assays to those obtained in yeast, it is clear that the binding properties of ERR3 and GRIP1 alone reflect the interaction of the two proteins in vivo. Thus, the binding properties of ERR3 and GRIP1 alone reflect the interaction of the two proteins in vivo.
not activated by a Gal4DBD/ERR3(28–458) fusion protein above the level achieved with Gal4DBD alone (Fig. 7B), although ERR3 did show transcriptional activation in CV-1 cells (Fig. 3). The discrepancy between the activities of ERR3 in yeast and mammalian cells was consistent with our earlier findings that yeast cells do not have transcriptional coactivators to support the NR AF-2 activation functions (22, 23). Since ERR3 has a very weak AF-1 domain, its transcriptional activation activity is almost entirely due to its AF-2 domain (Fig. 5). The similar behaviors of ERR3 and other NRs in yeast and mammalian cells indicated that ERR3 and other NRs utilize similar mechanisms for transcriptional activation.

**DISCUSSION**

Orphan NRs have recently been a very fast growing family of receptors; during the last 10 years 19 subfamilies of orphan receptors have been identified, compared with only 10 subfamilies of NRs with known ligands (5, 6). Given the well studied paradigm of the classical nuclear hormone receptors as a guide, the large number of currently known orphan NRs represent a valuable reservoir of research tools that can be used to elucidate new aspects of developmental and regulatory biology. Here we report the identification of a novel mouse orphan receptor ERR3, which we have assigned to the ERR family, because of its extensive sequence homology with the two earlier members of the family, ERR1 and ERR2 (37). ERR1 and ERR2 were originally isolated by low-stringency screening of cDNA libraries with the human ER DBD as a probe, and were the first two orphan receptors identified. The high degree of homology between the N-terminal domains of ERR2 and ERR3 is unusual among non-allelic NRs, even within the same subfamily, and suggests that these two genes may be the result of a recent gene duplication event or that their respective physiological roles require a conserved AF-1 function.

In many respects ERR3 functions like a classical NR. While its natural target binding sites on DNA remain to be determined, ERR3 selectively recognized EREs and selectively activated transcription of ERE controlled reporter genes in yeast and mammalian cells. ERR3 bound GRIP1 and other p160 coactivator proteins, and these transcriptional coactivators enhanced ERR3 function in yeast and mammalian cells. Both coactivator binding and transcriptional activation by ERR3 required an intact AF-2 domain, which is structurally and functionally homologous to those in classical hormonally regulated NRs. While the AF-2 activation domain and coactivator binding capabilities of ERR1 and ERR2 have not yet been defined, the degree of sequence homology among these three proteins (Fig. 1) and their similar abilities to exhibit transcriptional activation activity (55) suggest that the results reported here for ERR3 may be representative of the other two ERRs too.

In contrast to the actions of the classical nuclear hormone receptors, all of the activities of ERR3 were observed in the absence of any added ligand. The transcriptional activation by ERR3 in yeast and mammalian cells and the coactivator binding to ERR3 in yeast and *in vitro* were all observed in the absence of any added ligand. We propose two models to explain the apparently ligand independent function of ERR3 in the various studies presented here. Model 1, in the absence of any ligand ERR3 is a constitutive transcriptional activator protein. Along with the ligand independent basal activity, this model allows three possible scenarios for an effect of ligand: (a) there is no ligand; (b) binding of a currently unknown ligand further stimulates transcriptional activation by ERR3; (c) binding of a currently unknown ligand inhibits transcriptional activation. Model 2, ERR3 is a ligand-dependent transcriptional activator, and the ligand is ubiquitous in the mammalian, yeast, and *in vitro* experimental systems used in our studies. Model 2 seems somewhat unlikely, since the regulatory purpose of a ubiquitous ligand is difficult to envision. It is also possible that ERR3 is activated by a post-translational modification such as phosphorylation. However, when ERR3 was synthesized in a reticulocyte lysate in the presence of [γ-32P]ATP, we detected no phosphorylation of ERR3 (data not shown). The fact that ERR3 synthesized in bacteria (Fig. 6), yeast (Fig. 5A), mammalian cells (Fig. 7A), or reticulocyte lysates (Fig. 5B) bound GRIP1 efficiently suggests that this binding does not require ligands or post-translational modifications. Nevertheless, the possibility that the basal level of GRIP1 binding and transcriptional activation by ERR3 could be further regulated by post-translational modifications of ERR3 remains to be tested.

No easy formula has been developed for finding ligands for orphan receptors or excluding the possibility that a ligand exists. Steroidogenic factor 1 and hepatic nuclear factor-4,

**Fig. 7.** GRIP1 functioned as a coactivator for ERR3 in mammalian cells and yeast. A, CV-1 cells were transiently transfected with MTV/ERE-LUC reporter gene (0.5 µg), pSG5.HA-ERR3 (0.5 µg), and the indicated amount of pSG5.GRIP1/wt (GRIP1wt) or the same vector containing mutations in NR boxes II and III (GRIP1mt). Cells were grown in charcoal/dextran-treated serum with no added hormone. B, the indicated proteins were expressed in yeast SFY526 cells in the absence of hormone; the activity of the integrated β-galactosidase reporter gene controlled by Gal4-binding sites was measured in cell extracts. DBD, Gal4DBD; ERR3, ERR328–458; GRIP1, full-length GRIP1. C, ERR3 HBD (wild-type or AF-2 mutants) fused to Gal4DBD (as in Fig. 5) was expressed in yeast SFY526 cells in the presence or absence of full-length GRIP1. Cell extracts were tested for β-galactosidase activity.
which activate transcription of target genes in the absence of any added ligand, were originally thought to be ligand independent, but recent reports of putative ligands have re-opened this question (56, 57). CARδ is an example of Model 1c (36). We propose the simplest model, Model 1a, as a default model for ERR3 until evidence for the existence of a ligand is found. Classical NRs undergo a conformational change after binding hormone, and the resulting conformation allows them to interact with their transcriptional coactivators (30, 31, 34, 58). If ERR3 is indeed active in the absence of ligand, it will be interesting in the future to see what kind of structural features of ERR3 allow the constitutive interaction with coactivators.

The existence of all three ERRs in humans as well as mice implies distinct and important roles for each ERR protein. Although we have demonstrated that ERR3 is a functional transcriptional activator, its true physiological roles remain to be determined. The extensive structural homology between ERR2 and ERR3, and their similar abilities to activate ERE-controlled genes, suggest the possibility that they could have redundant functions. However, the genes for these two proteins have different temporal and spatial patterns of expression, indicating different developmental and/or regulatory roles. In adult mouse tissues, ERR2 is expressed at low levels and in a restricted pattern (37). In mouse embryo, ERR2 expression is restricted to trophoblastic progenitor cells between days 6.5 and 7.5 post-coitum, and its role was found to be in the early stages of chorion formation during mouse embryogenesis (59). Homozygous mutant embryos generated by targeted disruption of the ERR2 gene have severely impaired placental formation and died at 10.5 days post-coitum (60). In contrast, the highest expression of ERR3 occurs around days 11–15 of mouse embryonic development, a period of very active organogenesis. ERR3 was also expressed in selected adult tissues. Thus ERR3's roles in mouse development are temporally distinct from that of ERR2. From our studies on ERR3 and the previously published studies of ERR1, it appears that ERR1 and ERR3 have roughly somewhat similar although not identical distributions in adult tissues (37, 61, 62). ERR1 was widely distributed in later stages of mouse embryonic development, a period of very active organogenesis. ERR1 and ERR2, were identified by screening cDNA libraries for clones with homology to known NRs. The continuing rapid rate of identification of new orphan NRs suggests that more members of this subclass of NRs remain to be discovered. Our findings on the interaction of ERR3 with GRIP1 extends the paradigm established from studies with classical nuclear hormone receptors, that the interaction between the NR and p160 coactivator is highly conserved. The current study demonstrates that this apparent universality of interaction between the AF-2 domains of classical hormone binding and orphan NRs and the LXXLL motifs of p160 coactivators provides a novel approach for the identification of more new orphan NRs.

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