Communication

Hormone-dependent Transactivation by Estrogen Receptor Chimeras That Do Not Interact with hsp90

EVIDENCE FOR TRANSCRIPTIONAL REPRESSORS

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The ligand-free estrogen receptor (ER), like other steroid receptors, interacts with the 90-kDa heat shock protein hsp90 in vitro. Analysis of the effect of potential ER-hsp90 interactions in vitro on receptor function is complicated by the fact that hsp90 binds to ER domains required for hormone binding and stable DNA binding. ER chimeras were therefore created by replacing the ER DNA binding domain with that of GAL4. In addition, the N-terminal AF-1 domain of the ER was replaced with the strong constitutive activation domain of VP16 to create VP16-GAL-ERs. These chimeras bind DNA in a ligand-independent manner, but, importantly, are ligand-dependent transactivators, unlike VP16-GAL, which displays strong constitutive activity under the same conditions. Hormone induces transactivation by VP16-GAL-ERs to levels similar to the constitutive activity of VP16-GAL. Glycerol gradient and coimmunoprecipitation experiments showed that, unlike the wild-type ER, VP16-GAL-ER chimeras do not interact with hsp90. Deletion analyses indicate that a region of the ER, primarily between amino acids 370 and 470, is responsible for repressed transcription. Our results suggest that interaction with hsp90 is not necessary for controlling hormone-dependent transcription by the ER and, moreover, provide evidence for transcriptional repressors that interact with the N-terminal portion of the receptor’s ligand binding domain in the absence of hormone.

The estrogen receptor (ER) is a ligand-activated transcriptional regulator (1–4). Ligand-free (apo) steroid receptors can be isolated from cell extracts associated with complexes composed of a number of heat shock and immunophilin proteins (5–7). The major nonreceptor constituent of these complexes is a dimer of the 90-kDa heat shock protein, hsp90. Addition of hormone leads to complex dissociation and receptor homodimerization. These in vitro experiments would be consistent with a model where steroid receptors are in a multisubunit cytoplasmic complex in the absence of ligand and that hormone binding leads to complex dissociation, receptor homodimerization, and transfer of cytoplasmic receptor to the nucleus.

A potential role for hsp90 in vivo in controlling ligand-inducible transactivation by the glucocorticoid receptor (GR) has been well supported by genetic studies in Saccharomyces cerevisiae (8). Reduced expression of the hsp90 gene strongly inhibited GR-dependent transactivation, suggesting that hsp90 stabilized the ligand-free GR. However, the ER was less affected in similar experiments (8), suggesting that hsp90 may not be necessary for regulating ligand-inducible transcription by ER. Moreover, several immunocytochemical studies have suggested that the hormone-free ER is at least partially nuclear (9–12). Gene transfer experiments have shown that the receptor can be nuclear in the absence of hormone, and can bind DNA, providing evidence for the presence of ligand-free ER homodimers (13, 14).

Stable ER-hsp90 interactions in vitro require portions of domains essential for ligand binding and stable DNA binding (5, 15), thus complicating analysis of potential interactions in vivo. Here, we have created ER chimeras that are functional in vivo, and that do not interact with hsp90 in vitro, by replacing the ER DNA binding domain with that of the yeast transactivator GAL4. Our results suggest that interaction with hsp90 is not necessary for controlling hormone-dependent transcription by the ER, and, moreover, provide evidence for transcriptional repressors that interact with the ligand binding domain of the receptor in the absence of hormone.

MATERIALS AND METHODS

Recombinants—All chimeras were constructed in the pSG5 expression vector (16) by polymerase chain reaction amplification of appropriate regions of VP16, GAL4, and the wild-type ER HEG0. Duplicates of each recombinant were tested for transactivation and verified by DNA sequencing.

Cell Culture—COS-7 cells were grown in 3.5-cm dishes in Dulbecco’s modified Eagle’s medium containing charcoal stripped 5% fetal bovine serum. Lipofections were performed according to manufacturer’s instructions (Life Technologies, Inc.). For luciferase assays, 100 ng of chimera expression vector was used along with 500 ng of 17M5TATA-luc and 1 µg of p610AZ β-galactosidase expression vector for standardization. Cells were lysed in 250 µl of lysis buffer (Promega). 50- and 45-µl aliquots were used for β-galactosidase and luciferase assays, respectively. For Western and gel retardation analyses, 1.0 µg of ER expression vector was lipofected along with 1.0 µg of p610AZ.

Glycerol and coimmunoprecipitation experiments showed that, unlike the wild-type ER, VP16-GAL-ER chimeras do not interact with hsp90. Deletion analyses indicate that a region of the ER, primarily between amino acids 370 and 470, is responsible for repressed transcription. Our results suggest that interaction with hsp90 is not necessary for controlling hormone-dependent transcription by the ER and, moreover, provide evidence for transcriptional repressors that interact with the ligand binding domain of the receptor in the absence of hormone.

Immunoprecipitation—Immunoprecipitations were performed essentially as described by Scherrer et al. (18). Cytosol was prepared from transfected or untransfected COS-7 cells in HEPES buffer (10 mM HEPES, 1 mM EDTA, 20 mM sodium molybdate, 50 mM NaCl, and protease inhibitors). Clarified lysates were diluted in TEGM buffer (20 mM Tris, 4 mM EDTA, 10% glycerol, 20 mM sodium molybdate, 50 mM NaCl, and protease inhibitors) and incubated overnight on ice with anti-GAL4 antibodies 2G13 and 3G12 or with anti-ER antibody F3 (20). Glycerol gradient fractions from 4 to 8 peaks were pooled and diluted in TEGM prior to immunoprecipitation. Immune complexes were absorbed to protein G-Sepharose, washed four times, and analyzed by Western blotting.

Western Analysis—48 h after transfection, cells were harvested in
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FIG. 1. Characterization of VP16-GAL-ER chimeras. A, the luciferase reporter plasmid containing five GAL4 DBD binding sites, 17M5-TATA-luc is shown at the top. A schematic representation (not to scale) of the wild-type human ER HEG0 is shown below, indicating the DNA and hormone binding domains, along with the N- and C-terminal transactivation domains, AF-1 and AF-2. The structures of the chimeric activators are indicated below. B, transactivation by chimeras. Luciferase activity of three to five independent experiments from extracts of COS-7 cells transfected with 17M5-TATA-luc, p610A2, and parental expression vector (no expression) or expression vector GAL-ER (G-ER), VP16-GAL (V-G), or VP16-GAL-ER (V-G-ER) as indicated, in the absence (shaded bars), or presence (black bars) of 100 nM estradiol. Fold induction by hormone varied by a maximum of ±20%. Inset, Western analysis of extract of COS-7 cells expressing VP16-GAL-ER258 (258) or VP16-GAL-ER300 (300). Cells were untreated (—) or treated with 10 nM estradiol (+) for 24 h prior to extraction. C, Western analysis of extracts of COS-7 cells transfected with VP16-GAL or VP16-GAL-ER expression vectors. D, gel retardation of COS-7 extracts from cells transfected with parental expression vector (—), VP16-GAL, VP16-GAL-ER258, or VP16-GAL-ER300, using a 17-mer binding site. Phosphate-buffered saline and divided in half. One aliquot was lysed in lysis buffer (Promega), and b-galactosidase assays were performed (21) to assess transfection efficiency. The other aliquot was lysed directly in SDS-polyacrylamide gel electrophoresis sample buffer and used for Western analysis. Blots were incubated with a combination of anti-GAL4 DBD monoclonal antibodies 3GV3 and 3GV2 (19) diluted 1/1000 in Tris-buffered saline-Tween and 1% milk powder. Western analysis of hsp90 was performed using anti-hsp90 monoclonal antibody SPA-835 (Stressgen). Blots were developed using the ECL detection system (DuPont NEN). Glycerol Gradient Analysis—Glycerol gradients were used to analyze molybdate-stabilized extracts of cells transfected with the wild-type ER or VP16-GAL-ER chimeras for interaction with hsp90. The wild-type ER displayed a characteristic salt-dependent shift in sedimentation coefficient (5) from the 8 S hsp90-containing complex, to the hsp90-free 4 S form (Fig. 2A). In contrast, 4 S, but no 8 S complex formation, was observed in extracts of cells transfected with VP16-GAL-ER258 (Fig. 2B) or VP16-GAL-ER300 (not shown), indicating that they did not interact with hsp90. Western analysis of gradient fractions showed that peak hormone binding corresponds to peaks of intact protein, suggesting that no significant proteolysis of chimeras occurred (Fig. 2B). No interaction of VP16-GAL-ER258 with hsp90 was detected by immunoprecipitation with anti-GAL antibodies of molybdate-stabilized whole cell extracts of transiently transfected COS-7 cells or of the 4 S peak from a glycerol gradient (Fig. 2C, lanes 1–6). Identical results were obtained using anti-ER antibody F3 (not shown). In contrast, immunoprecipitation of HEG0 expressed in COS-7 cells with F3 led to increased communoprecipitation of hsp90 (Fig. 2C, lanes 7–9). These results suggest that interaction with hsp90 does not control hormone-dependent transactivation by the chimeras. Gel retardation assays were also performed to test for hormone-dependent DNA binding. No significant effect of hormone on DNA binding by VP16-GAL-ER258 or VP16-GAL-ER300 was detected in assays performed with extracts made in the presence or absence of estradiol (Fig. 1D and data not shown). In this respect, the chimeras functioned similarly to the wild-type ER (22).

Deletion Analysis of the ER Ligand Binding Domain—Hor-
mone dependence of VP16-GAL-ER chimeras may be due to interaction with factors, other than hsp90, which repress transcription in the absence of hormone. A series of deletion mutants were created to test which portions of the ER LBD are responsible for the reduced transactivation in the absence of estradiol (Fig. 3A). C-terminal truncations beyond ER aa 553, which disrupt the integrity of the LBD, generated chimeras displaying low levels of constitutive transactivation (Fig. 3B). Significant constitutive activity is recovered, however, with C-terminal deletions beyond aa 430 to aa 370 and further to aa 302. Disruption of the LBD N terminus by deletion past aa 302 to aa 340, 370, or 430 generated chimeras exhibiting low levels of constitutive activity (Fig. 3B). Significant constitutive activation was only recovered by deletion of the N terminus to aa 470. Taken together, these data indicate that a portion of the LBD, primarily sequences between aa 370 and 470, is required for repressed transcription observed in the absence of hormone.

**DISCUSSION**

Our results suggest that the mechanism of action of the ER is intermediate between that of the GR subfamily of steroid receptors and those of the thyroid hormone/retinoid/vitamin D3 nuclear receptors. The apo-GR is cytoplasmic, and ligand binding leads to its translocation to the nucleus where it binds palindromic DNA sequences as a homodimer (23). The cytoplasmic location of the apo-receptor would be consistent with its interaction in vitro with hsp90, which is predominantly cytoplasmic. In contrast, the thyroid hormone and related receptors do not interact with hsp90 in vitro (24), are nuclear in the absence of hormone and bind to response elements composed of directly repeated motifs as heterodimers with retinoid X receptors (25, 26).

The ER, like the GR, recognizes palindromic response elements as a homodimer (1–4). Numerous studies have indicated that the full-length ER interacts with hsp90 in vitro, suggesting that similar interactions may occur in vivo. Immunopre-
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Citation experiments by others have also indicated that the isolated ER LBD interacts weakly with hsp90 (18). However, the LBD used contains a Gly400→Val mutation, which destabilizes its structure (22, 27). Our glycerol gradient analyses with VP16-GAL-ER chimeras containing a Val400 mutation have shown that, unlike their Gly400 counterparts, these chimeras form salt-sensitive 8 S complexes in vitro (not shown). In vivo studies, including immunocytochemistry and gene transfer experiments, have provided evidence for the presence of homodimers of apo-ER in the nucleus (13, 14), which would be inconsistent with stable interaction with hsp90. Taken together, the above results suggest that if the ER interacts with hsp90 in vitro, this interaction is transient, and that a significant concentration of homodimeric aporeceptor is present in the nucleus.

Our results show that ER derivatives, which do not interact with hsp90 in vitro, can function as ligand-dependent transactivators in vivo. This occurs in spite of the fact that the VP16-GAL-ER chimeras contain an acidic activating domain which is strongly constitutively active when not tethered to the ER LBD. These results suggest that interaction with hsp90 in vivo is not essential for controlling ligand-dependent transactivation by the ER. It appears that the chimeras tested here are maintained in a transcriptionally repressed state in the absence of ligand, given that ligand induces transactivation to levels similar to those seen with the constitutive activator VP16-GAL. There are several potential candidates for a repressor. In yeast, HSP70 acts downstream of hsp90 to control ER and GR function (28). It is possible that molecules analogous to those that repress the thyroid hormone and retinoic acid receptors (29, 30) also act on the ER. A repressor may be specific or have a broad spectrum of effects on transcription similar to the yeast factor SSN6 (31). Deletion analyses of the ER suggest that a putative repressor would interact with a region of the LBD between aa 370 and 470 (Fig. 3).

The obvious function of a putative repressor would be to maintain DNA-bound apo-ER in a transcriptionally silent state. Hormone would stimulate dissociation of bound repressor, freeing the LBD for binding of transcriptional intermediary factors or coactivators (32–34). In this model, antagonists would either bind the ER and not stimulate repressor dissociation, or bind, repress repressor dissociation, but maintain the LBD in a conformation not recognized by transcriptional intermediary factors. Repression of the ER would not only act to block the activity of the LBD but also the AF-1 domain in the N terminus, which can be activated by phosphorylation in the presence of hormone (35) and which has the capacity to synergize with other classes of transactivators under certain conditions (21). The potential activity of AF-1 would be dampened in cells where truncated receptors lacking the LBD display significant levels of constitutive activity (36). It is also noteworthy that aa 370–470 are adjacent to the region of the LBD (aa 300–330) identified as being important for binding of TATA box-binding protein-associated factor TAFII30, which is required for transactivation by the ER (37).

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