Nuclear Orphan Receptor as a Repressor of Glucocorticoid Receptor Transcriptional Activity*

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Nuclear orphan receptors belong to the superfamily of ligand-activated transcription factors that show a close structural relationship and sequence homology. Ligands and functions of most of the orphan receptors have not yet been identified. The first nuclear orphan receptors that were cloned displayed a high degree of amino acid identity with the human estrogen receptor and were termed estrogen receptor-related (ERR) 1 and 2. In the present study, we show that ERR2 functions as a potent repressor of transcriptional activity mediated by the glucocorticoid receptor (GR). Transient transfection of different cell lines with a steroid-responsive reporter plasmid and receptor expression plasmids revealed that transcriptional activity mediated by GR in response to agonists was strongly suppressed by coexpression of ERR2. The orphan receptor displayed no promoter activity when expressed without GR. The inhibitory activity of ERR2 is cell-specific and also receptor-specific because transactivation mediated by the progesterone receptor is unaffected by ERR2. Our observations provide evidence that the nuclear orphan receptor ERR2 acts as an endogenous modulator of GR transcriptional activity.

The nuclear receptor superfamily is composed of ligand-activated transcription factors including receptors for glucocorticoids, mineralocorticoids, gonadal steroids, retinoids, thyroid hormone, and vitamin D. These receptors have a major influence on differentiation, cell proliferation, development, and maintenance of homeostasis. The action of nuclear receptors is mediated by binding of the ligand-activated receptor to cis-acting sequences regulating the expression of target genes in cooperation with proteins of the basic transcriptional machinery or additional transcription factors. Molecular cDNA cloning and comparison of the amino acid sequences deduced have shown that all members of the nuclear receptor superfamily are structured in a similar way and organized into different domains. Moreover, they show a particularly high degree of sequence homology, mainly in the DNA-binding domain (1–4). Based on structural similarities and sequence homology in comparison with members of the nuclear receptor superfamily, a number of putative receptors, designated nuclear orphan receptors, have been cloned, whose functions and ligands have not yet been identified (3–5). Furthermore, the nature of action of the orphan receptors that were first cloned and termed estrogen receptor-related (ERR) 1 and 2 remains unknown to date (5). Since interactions between members of the nuclear receptor superfamily are not uncommon (4, 6), we investigated the functional consequence of coexpression of these orphan receptors with the more traditional members of the nuclear receptor superfamily with regard to transcriptional activity. Our findings indicate that ERR2 acts as a cell-specific inhibitor of glucocorticoid receptor (GR)-mediated gene expression.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfections, and Assays for Luciferase and β-Galactosidase Activity—Cells (CV-1, monkey kidney, COS-1, monkey kidney, SK-N-MC, human neuroblastoma, HeLa, human cervix carcinoma) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% charcoal-stripped serum-free fetal calf serum (FCS) (7). Transfections were performed using an electroporation system (Bio-technologies and Experimental Research, Inc., San Diego, CA) after determination of the optimal electric field strength (8, 9). Cells were cotransfected with 5 μg of MTV-LUC reporter plasmid, 5 μg of β-galactosidase expression vector (pCH110; Pharmacia LKB, Freiburg, Germany), and either 2 μg of GR, progesterone receptor (PR), or luciferase (LUC) or 4 μg of ERR2 expression plasmid either alone or in combination with 1, 2, or 4 μg of ERR2 expression plasmid as indicated in the figures. prSV-CAT up to 16 μg was included to keep the total amount of Rous sarcoma virus promoter-driven expression plasmid constant. COS-1 cells were transfected with 5 μg of MTV-LUC, 5 μg of pCH110, and 5 μg of expression vector for either GR, ERR2, or the two together. prSV-CAT up to 20 μg was included to keep the total amount of Rous sarcoma virus promoter-driven expression plasmid constant. Electroporated cells were replated in DMEM supplemented with 10% charcoal-stripped serum-free FCS and incubated immediately with vehicle or various concentrations of endogenous or synthetic agonists as indicated. Phendin red-free DMEM supplemented with 10% charcoal-stripped serum-free FCS was used when transcriptional activity of PR was investigated. After 24 h, cells were harvested and extracts were assayed for luciferase (10, 11) and β-galactosidase (12) activity as a control for transfection efficiency. Cell extract (10 μl) and 25 μl of o-nitrophenyl-β-D-galactopyranoside dissolved at a concentration of 2 mM were added to 100 μl of assay buffer containing 60 mM NaH2PO4, 40 mM NaHPO4, 10 mM KCl, 1 mM MgCl2, and 50 mM β-mercaptoethanol. After 15 min of incubation at 37°C, the reaction was stopped by adding 50 μl of 1 M Na2CO3. The optical density ranged from 0.1 to 0.8 at 410 nm. To standardize for transfection efficiency, the relative light units obtained in the luciferase assay were divided by the optical density obtained in the β-galactosidase assay. Results are shown in arbitrary units of LUC activity corrected for transfection efficiency by the corresponding β-galactosidase activity and are presented as the average of at least five independent transfection experiments with a variation from the mean of less than 25%. The IC50 values (the concentration needed to obtain half-maximal response) were determined graphically from the dose-response curves. Whole-cell extract supernatant used for electrophoretic mobility shift assay was made of COS-1 cells that were transfected with 5 μg of pGEM4 (Promega Corporation, Madison, WI), 5 μg of GR or ERR2 expression vector, or the two

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together and up to 15 µg of pRSV-CAT.

Construction of Plasmids—The construction of the reporter plasmid MTV-LUC and of the plasmids encoding human GRα, human ERR2, human PRα, and LUC has been described previously (5, 13–15).

Electrophoretic Mobility Shift Assay—³²P-5'-End-labeled glucocorticoid response element (GRE) oligomer was constructed by annealing overlapping oligonucleotides (5'-AGCTTGAGCTAGAAACATGTG-3') and filling in the recessed ends using Klenow fragment (Boehringer Mannheim, Mannheim, Germany) and incorporating [³²P]dCTP (DuPont NEN, Dreieich, Germany). Whole-cell extract supernatant from transfected COS-1 cells was prepared by homogenization in ice-cold buffer containing 20 mM Tris-HCl, pH 7.5, 600 mM KCl, 20% glycerol, and 2 mM dithiothreitol and subsequent centrifugation (100,000 x g at 2°C) for 60 min. Electrophoretic mobility shift assays consisted of 10 µg Tris-HCl, pH 7.5, 80 µg KCl, 10% glycerol, 2 µM dithiothreitol, 1 µg of poly(dI-dC), and 3 µl of whole-cell extract supernatant (6 µg of protein) prepared from transfected COS-1 cells in a total volume of 20 µl (6, 16). 500 ng of a polyclonal GR antibody (17) were included in the reaction where indicated. After incubation on ice for 10 min, 0.1 ng of [³²P]dCTP was included in the reaction where indicated. After incubation on ice for 10 min, DNA-protein complexes were resolved on a 4% nondenaturing polyacrylamide gel with buffer recirculation in 6.7 mM Tris-HCl, pH 7.5, 33 mM sodium acetate, and 1 mM EDTA. The gel was dried under vacuum and autoradiographed at −70°C.

RESULTS AND DISCUSSION

CV-1 cells lacking endogenous GRs were transfected transiently with MTV-LUC, a reporter plasmid encoding the mouse mammary tumor virus promoter upstream of the luciferase gene (MTV-LUC) and expression plasmids encoding the human GR and/or the human ERR2. Stimulation of GR-transfected cells with agonists revealed a remarkable, dose-dependent reporter gene expression (Fig. 1), whereas the orphan receptor displayed no promoter activity (Fig. 1). However, coexpression of ERR2 drastically repressed transcriptional activity mediated by GR in response to a physiological concentration of cortisol (Fig. 1A). Remarkable inhibition of GR activity by ERR2 was also obtained when different amounts of GR expression plasmid were transfected and the GR/ERR2 ratio varied between 2 and 0.5. ERR2-mediated repression of glucocorticoid-induced transcriptional activity was obtained when transfected cells were stimulated with low to high nanomolar concentrations of cortisol or dexamethasone. However, the dose-response curve of GR-activated reporter gene expression in response to increasing concentrations of endogenous or synthetic glucocorticoids was not only suppressed but also right-shifted when ERR2 was coexpressed (Fig. 1, B and C). The concentration of cortisol and dexamethasone needed to obtain half-maximal response was 21 and 2 nm, respectively, when GR was expressed alone and 104 and 11 nm, respectively, when ERR2 was coexpressed. Antagonism toward glucocorticoid activity by ERR2 was also obtained when SK-N-MC cells (Fig. 2) or COS-1 cells (Fig. 6A) were transfected, whereas transcriptional activity of cotransfected as well as endogenous GR in HeLa cells was nearly unaffected by ERR2 (Fig. 3). Therefore, the inhibitory activity of the orphan receptor depends on the cell type. We next investigated whether the orphan receptor is a general repressor of nuclear receptor transcriptional activity. As shown in Fig. 4, coexpression of ERR2 in CV-1 cells does not inhibit transcriptional activity mediated by the human PR in response to progesterone. Similar results were obtained when SK-N-MC cells were transfected. These data indicate that repression of GR-activated reporter gene expression is not mediated via an influence of ERR2 on the basic transcriptional or translational machinery. It also makes clear that the orphan receptor does not suppress transcriptional activity by competing for functionally limiting factors generally needed for steroid receptor activity (18). To exclude the possibility that repression of GR transcriptional activity is caused by a decrease in GR expression, we tested whether coexpression of ERR2 has an effect on the promoter that regulates GR expression. As shown in Fig. 5, expression of pRSV-LUC is unaffected by ERR2, which indicates that the Rous sarcoma virus promoter is not influenced by the orphan receptor. Western blot analysis also confirmed that ERR2 has no effect on the expression of cotransfected GR. Investigation of DNA binding of ERR2 expressed in COS-1 cells using an electrophoretic mobility shift assay showed that the orphan receptor was not able to bind to a common GR binding site (19). In conclusion, it can be concluded that ERR2 is a potent inhibitor of GR transcriptional activity.

FIG. 1. Transactivation properties of GR, ERR2, and coexpressed GR and ERR2 in CV-1 cells. A, CV-1 cells were transfected with expression vector for either GR (2 µg) or ERR2 (4 µg), or 2 µg of GR and 4 µg of ERR2 expression plasmid was transfected in combination with 1, 2, or 4 µg of ERR2 expression plasmid. Transfected cells were incubated with 10 nM cortisol (gray bars) or with vehicle (black bars). B and C, CV-1 cells were transfected with either 2 µg of GR expression plasmid (circles) alone or with 2 µg of GR and 4 µg of ERR2 expression plasmid together (squares). After transfection, cells were incubated with cortisol (B) or dexamethasone (C) concentrations as indicated.

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labeled GRE under these conditions (Fig. 6B, lane 2), whereas the GR was able to bind (Fig. 6B, lane 3) as has been shown previously (6, 19). The specificity of this DNA binding complex was confirmed by supershift with a polyclonal GR antibody (Fig. 6B, lane 4). Next, we asked whether the orphan receptor affects DNA binding of GR. As shown in Fig. 6B (lane 5), coexpression of GR and ERR2 in COS-1 cells does not influence binding of the GR to the GRE. These data are also another proof that ERR2 has no influence on the expression of GR and provide evidence that repression of GR activity by ERR2 is not caused by a competition for response element binding.

Our findings indicate that the nuclear orphan receptor ERR2 acts as a cell-specific repressor of transcriptional activity mediated by GR. The inhibitory activity of ERR2 appears to be constitutive because cotransfection of ERR2 expression plasmid is sufficient to suppress GR activity. Therefore, either no ligand is needed by the orphan to inhibit transcription or an endogenous, activating ligand is present in a sufficient amount. The possibility that ERR2 acts via an influence on the basic transcriptional or translational machinery can be excluded because expression of luciferase is unaltered by coexpression of ERR2 when the luciferase gene is under control of a constitutively active (Fig. 5) or PR-regulated promoter (Fig. 4). Moreover, the fact that PR-regulated gene expression is not influenced by ERR2 provides evidence that the suppressive activity does not refer to all members of the steroid receptor family. This excludes that the orphan receptor acts via competing for a basic factor needed by steroid receptors to regulate expression of genes (18). Because ERR2 is not able to bind to a GRE (Fig. 6B) the suppressive action does not involve competition for response element binding, which may be the mechanism of action of GRβ-mediated repression of GR activity (20). The inhibitory effect of the orphan receptor is also not caused by the formation of an ERR2-GR complex that displays altered DNA binding properties, as shown for other inhibitors of GR action such as c-j un (21, 22), calreticulin (23, 24), and the p65 subunit of NF-κ B (25). This can be excluded because GRE binding of GR is completely unaffected by coexpression of ERR2 (Fig. 6B).

The exact mechanism of ERR2-mediated suppression of GR transcriptional activity remains unknown, but our data correspond to a model of ERR2 action in which the orphan receptor acts as a trans-repressor of glucocorticoid activity by competing with high affinity for a factor specifically required by the GR to act as an activator of transcription. This factor may be expressed or be functionally available in limited amounts in some cell types. A similar mode of action has been described for the inhibitory action of the A form of the PR on the activity of the
PRB form or the mineralocorticoid receptor (26, 27).

In this respect it is interesting that in a study recently published (28) it could be demonstrated that rat ERR2 is expressed in tissues that are also known to be enriched with GR, e.g., the pituitary and the paraventricular nucleus.

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Fig. 6. Transactivation and DNA binding properties of GR, ERR2, and coexpressed GR and ERR2 in COS-1 cells. A, COS-1 cells were transfected with expression vector for either GR (5 μg) or ERR2 (5 μg), or 5 μg of GR expression plasmid was transfected in combination with 5 μg of ERR2 expression plasmid. Transfected cells were incubated with 10 nM cortisol (gray bars) or with vehicle (black bars). B, extracts of COS-1 cells transfected with pRSV-CAT either alone (lane 1) or together with expression plasmid for ERR2 (lanes 2 and 5) and/or GR (lanes 3, 4, and 5) were incubated with labeled GRE, and electrophoresis was performed on a native polyacrylamide gel. Anti-GR polyclonal antibody was included in the reaction as indicated (lane 4).
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