Communication

Interaction of the Ligand-activated Glucocorticoid Receptor with the 14-3-3η Protein*

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The glucocorticoid receptor (GR) is a ligand-activated transcription factor. In this study, we used the yeast two-hybrid system to isolate cDNAs encoding proteins that interact with the human GR ligand-binding domain (LBD) in a ligand-dependent manner. One isolated cDNA from a HeLa cell library encoded the COOH-terminal portion of the η isoform of the 14-3-3 protein (residues 187–246). Glucocorticoid agonists, triamcinolone acetonide and dexamethasone, induced the GR LBD/14-3-3η protein fragment interaction, but an antagonist, RU486, did not. Glutathione S-transferase pull-down experiments in vitro showed that full-length 14-3-3η protein also interacted with the activated GR. Transient transfection studies using COS-7 cells revealed a stimulatory effect of 14-3-3η protein on transcriptional activation by the GR. The 14-3-3 family members have recently been found to associate with a number of important signaling proteins, such as protein kinase C and Raf-1, as functional modulators. Our findings suggest a novel regulatory role of 14-3-3η protein in GR-mediated signaling pathways and also point to a mechanism whereby GR may cross-talk with other signal transduction systems.

The human glucocorticoid receptor (hGR),† a member of the nuclear receptor superfamily, mediates the effects of glucocorticoids by regulating the transcription of target genes (for review, see Ref. 1). The GR contains three major structural domains. The η transactivation domain in the NH2-terminal portion is important for gene activation by DNA-bound GR and interacts with components of the transcriptional machinery (2). The DNA-binding domain (DBD), in the central part of the receptor protein, associates with specific glucocorticoid response elements (GREs) within the target genes (reviewed in Ref. 3). The COOH-terminal ligand-binding domain (LBD) contains overlapping functional domains responsible for ligand binding (4), nuclear translocation (5), dimerization (6), transactivation (e.g., the τ domain) (7), and binding of 90-kDa heat-shock protein (HSP90) (8). The unliganded GR in the cytoplasm forms a complex with HSP chaperones to keep the GR in an inactive, yet ligand-activatable state. Upon ligand binding, HSP90 dissociates from the activated GR, which can enter the nucleus and act as a transcription factor (see Refs. 1 and 9 and references therein).

Using advanced screening methods for the protein-protein interaction, recent studies have shown that the transcriptional activity of nuclear hormone receptors can be modulated by the interaction with other proteins, such as coactivators or corepressors of transcription and coinTEGRATORS of diverse signal transduction pathways (for reviews, see Refs. 10 and 11).

In our yeast two-hybrid screening, the COOH-terminal portion of the human η isoform of the 14-3-3 protein family was found to interact with the hGR-LBD in a ligand-dependent manner. The interaction between full-length 14-3-3η protein and the activated GR was also confirmed in in vitro experiments. Moreover, the expression of 14-3-3η protein in mammalian cells showed a stimulatory effect on transcriptional activation by GR. 14-3-3 proteins have recently been the subjects of considerable attention, since they can interact with a wide variety of signaling proteins, including those in the Ca2+ signaling and mitogen-activated protein kinase cascades (for review, see Ref. 12). Our observations indicate that 14-3-3η protein may also play a role in GR-mediated signaling pathways.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid System—A LexA-based yeast two-hybrid screening was performed according to protocols from the Brent Laboratory, Massachusetts General Hospital, Boston, MA (13). The following plasmids were used: pEG202 (HIS3+), expressing LexA-fused baits, pRFPHM1 (HIS3+) expressing a LexA-fused bioid protein, pSH18-34 (URA+) expressing a lacZ reporter gene with LexA-binding sites, and pJG4-5 (TRP1+) expressing B42 transactivation domain fused to HeLa cell cDNA library-encoded proteins. The LexA-fused baits were expressed from plasmids, pEG202/GR-LBD (residues 485–777) and pEG202/GR-LBD DBD (418–503).

Yeast strain EGY188 (MATa, his3, trp1, ura3, 2LexAop-LacL2) was transformed with pSH18–34, pEG202/GR-LBD, and a HeLa cell cDNA library cloned in pJG4-5, and transformants were selected on galactose-Ura-Trp-Leu plates. The library plasmids from LEU2 colonies were further tested for LacZ activity on Ura-Trp-Leu plates containing 50 μg/ml tricinomilone acetonide (TA). LEU2+ colonies were further tested for LacZ activity on Ura-Trp-Leu plates containing 50 μg/ml 5-bromo-4-chlor-3-indolyl β-D-galactoside (X-Gal) and 0.5 μM TA. The library plasmids from LEU2+ and LacZ+ colonies were recovered, and sequences of nonidentical clones were determined.

To verify the specificity, the empty pJG4-5 plasmid or isolated pJG4-5 cDNA plasmid was introduced either into the original yeast strain expressing the LexA-GR-LBD bait or strains expressing LexA-fused GR DBD or bioid protein. The LEU2+ and LacZ+ activities of these strains were tested on plates and by β-galactosidase assays (14) in the absence or presence of various steroids.

Full-length cDNA of Human 14-3-3η Protein—A cDNA encoding full-length 14-3-3η protein was isolated from human liver cDNA library (Clontech, Palo Alto, CA), using specific polymerase chain reaction primers, and inserted into the EcoRI/XhoI sites of the pHCMV expression vector (Stratagene, La Jolla, CA).

Protein Expression—[35S]Methionine-labeled full-length hGR and 14-3-3η proteins were translated in vitro using an in vitro translation kit (Promega, Madison, WI) at 30 °C for 90 min.
The EcoRI/XhoI fragment from pBKCMV-14-3-3b was subcloned into the glutathione S-transferase (GST) fusion vector pGEX-4T-1 (Pharmacia Biotech Inc., Uppsala, Sweden). The original and modified pGEX vectors were transformed into *Escherichia coli*, strain BL21 (DE3)pLysS. The expression of GST and GST-fused proteins was induced by adding isopropyl 1-thio-β-D-galactopyranoside to a final concentration of 0.1 mM into the bacterial cultures. The induced proteins were purified from the bacterial extracts using glutathione-agarose beads (Sigma) by standard procedures. GST-fused hGR γ domain was a gift from Dr. Jacqueline Ford (Karolinska Institute, Sweden).

GST Pull-down Experiments—Purified GST and GST fusion proteins were bound to glutathione beads at an approximate concentration of 1 mg of protein/ml of beads. Immediately after the *in vitro* translation, 10 μl of the 35S-labeled GR translation mix was diluted either into 400 μl of ice-cold GST pull-down buffer (20 mM Hepes-KOH, pH 7.9, 10% (v/v) glycerol, 100 mM KCl, 5 mM MgCl2, 0.2 mM EDTA, 0.01% (v/v) Nonidet P-40, 1 mg/ml bovine serum albumin (BSA), 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) containing 0.2 μM TA, 1 μM dexamethasone (Dex), or 1 μM RU486, and incubated at 4 °C for 2 h. To stabilize the GR-HSP90 complex in the translation mixture, the buffer containing 20 mM sodium molybdate (15) was also used. 200 μl of each sample was then mixed either with 25 μl of GST- or GST/14-3-3-glutathione beads at 4 °C overnight. The beads were recovered by centrifugation. After washing the beads, bound proteins were eluted by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, analyzed on SDS-PAGE, and visualized by autoradiography.

In experiments using *in vitro* translated 14-3-3 protein, 25 μl of the 35S-labeled 14-3-3 protein translation mix was diluted either into 600 μl of the GST pull-down buffer or the same buffer without Nonidet P-40 and containing 0.1 mg/ml BSA. 200 μl of the sample was then mixed either with 25 μl of GST-, GST/14-3-3-, or GST/GR γ domain-glutathione beads at 4 °C overnight.

**RESULTS AND DISCUSSION**

Using a LexA-based yeast two-hybrid screening, we isolated cDNAs from a HeLa cell library encoding proteins that interact with the hGR LBD in a ligand-dependent manner. Sequence analysis revealed that one of the clones encoded the COOH-terminal portion of human 14-3-3 protein (residues 187–246) (17).

We next verified the interaction of the COOH-terminal 14-3-3 protein fragment with the ligand-activated hGR LBD. The plasmid expressing the truncated 14-3-3 protein fused to the B42 transactivation domain or the control plasmid expressing only the transactivation domain was re-transformed either into the original yeast strain expressing LexA-fused GR LBD or other strains expressing LexA-fused GR DBD or the bicoid protein. The 14-3-3 protein fragment interacted specifically with GR LBD, since only the strain coexpressing LexA-GR LBD and the 14-3-3 protein fragment grew on LEU- plates (not shown). Furthermore, the interaction was ligand-dependent, since the cell growth was only observed on plates containing a GR ligand, TA (not shown). The interaction was studied in more detail by determining the levels of the lacZ reporter expressed in extracts prepared from the yeast strains using β-galactosidase assays. The strain coexpressing the 14-3-3 protein fragment and LexA-fused GR LBD showed a strong β-galactosidase expression in the presence of TA (Fig. 1A).

![Fig. 1. Ligand dependence and ligand specificity of the interaction between hGR LBD and COOH-terminal fragment of 14-3-3 protein in the yeast two-hybrid system. A, the EGY188 yeast strain containing the lacZ reporter gene under the control of LexA-binding sites was transformed either with pEG202/GR LBD, pEG202/GR DBD, or pFHJM1 to express LexA-fused GR LBD, GR DBD, and the bicoid protein, respectively. The empty pJG4-5 plasmid or pJG4-5 expressing the COOH-terminal fragment of 14-3-3 protein (C-14-3-3) was introduced into each strain. Yeast cells were grown in selective media in the presence or absence of 10 μM TA. B, the EGY188 yeast strain expressing LexA-fused GR LBD was transformed with either the empty pJG4-5 plasmid or pJG4-5 expressing the 14-3-3 protein fragment. Yeast cells were grown in selective media containing 10 μM TA, 50 μM Dex, or 50 μM RU486. β-Galactosidase activity in each yeast extract was measured in triplicate, 5 h after the steroid treatments. The activity is expressed as described previously (14). The values represent the mean ± standard deviation.](image-url)

In accordance with the results on LEU- plates, the 14-3-3 protein fragment did not interact with GR DBD or bicoid protein (Fig. 1A). We also tested the 14-3-3 protein fragment/GR LBD interaction in the presence of various steroids. The results showed that the two GR agonists TA and Dex induced the protein-protein interaction, but the antagonist RU486 did not (Fig. 1B).

We next isolated a full-length cDNA of human 14-3-3 protein and constructed a plasmid for expression of a full-length 14-3-3 protein. However, the expression of this protein in the EGY188 yeast strain resulted in a strong toxic effect on cell growth, and thus we could not examine the GR LBD/full-length 14-3-3 protein interaction in the yeast two-hybrid system. Similar toxic effects were also observed in yeast strains overexpressing the yeast 14-3-3 gene (18).

To determine whether the interaction between 14-3-3 protein and GR LBD could also occur *in vitro*, as well as *in vivo* in yeast cells, we performed GST pull-down experiments. In these experiments, full-length proteins of both 14-3-3 protein and GR were used. It is known that the *in vitro* translated GR forms a

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FIG. 2. Interaction between hGR and 14-3-3η protein and dimer formation of 14-3-3η protein in vitro. A, 35S-labeled GR was translated in vitro. The translation mixture was incubated at 4°C for 2 h, in the presence of 20 mM molybdate (Mo) or steroids (0.2 μM TA, 1 μM Dex, or 1 μM RU486), and mixed with GST (a) or GST/14-3-3η protein (b) glutathione beads. B, 35S-labeled 14-3-3η protein was incubated with either GST (a), GST/14-3-3η protein (b), or GST/GR (c) τ1 domain glutathione beads. Incubation was performed either at higher (H) or lower (L) stringency as described in the text. The input and eluted samples from washed beads were analyzed on SDS-PAGE and visualized by autoradiography. Molecular mass markers (Pharmacia) are shown in kilodaltons. The arrowheads in A and B indicate the GR (94 kDa) and 14-3-3η protein (28 kDa) bands, respectively.

complex with HSP90 from the reticulocyte lysate and that this GR-HSP90 complex can be stabilized by molybdate (15). In vitro translated and molybdate-treated GR showed no significant binding to GST protein or GST-fused 14-3-3η protein (Fig. 2A, lanes 2 and 3). On the other hand, the GR treated with the GR agonists TA and Dex preferentially bound to GST-fused 14-3-3η protein (Fig. 2A, lanes 5 and 7), compared with GST protein alone (Fig. 2A, lanes 4 and 6). In addition, treatment with the antagonist, RU486, resulted in a lower level of receptor interaction with the 14-3-3η protein (Fig. 2A, lane 9). Since molybdate and RU486 are known to stabilize the GR-HSP90 complex (15, 19), these results suggest that prior dissociation of the GR from HSP90 is necessary for the interaction with 14-3-3η protein in vitro.

14-3-3 protein isoforms have a molecular mass of around 30 kDa and can form homo- and heterodimers (20). Crystallization of the proteins revealed that the dimeric molecule has a large negatively charged groove (21, 22). This groove is likely to represent the binding surface of 14-3-3 proteins with their target proteins, and consistent with our findings, it is largely constructed from the COOH-terminal part of 14-3-3 proteins. In our experiments, in vitro translated 14-3-3η protein could bind to GST-fused 14-3-3η protein (Fig. 2B, lane 3), indicating that our expressed proteins dimerize and that they could interact with the GR as dimers.

Recently, 14-3-3 protein-binding motifs in two target proteins, Raf-1 and the platelet adhesion receptor, have been identified as (RSXSXP) and (XXXSXXSSXXXSSXXS), respectively (23, 24). Furthermore, the (RSXSXP) motif has been found in many other known 14-3-3 protein-binding molecules (23), but some evidence suggests that there are alternative protein contacts with 14-3-3 proteins in the case of A20, an inhibitor of tumor necrosis factor-induced apoptosis (25). This is also true for the GR LBD protein, which does not contain either motif but interacts strongly with the 14-3-3η protein fragment in the yeast two-hybrid system. However, the (RSXSXP) motif is found in the τ1 region of the GR NH2-terminal domain (23). To examine whether the 14-3-3η protein could interact with the hGR τ1 domain, we used GST-fused GR τ1 domain. In vitro translated 14-3-3η protein did not bind to GST-fused GR τ1 domain under normal stringency conditions (Fig. 2B, lane 4), but a weak interaction was seen at lower stringency (Fig. 2B, lane 6). Since our results showed that the interaction between 14-3-3η protein and the GR τ1 domain is relatively weak, we suggest that the interaction we observe with the intact GR in vitro is mainly via the GR LBD.

To determine whether the interaction between the ligand-activated GR and 14-3-3η protein had any effect on transcriptional activation by the GR, we performed transient transfections of COS-7 cells. In cotransfection experiments, 14-3-3η protein enhanced the response to Dex in a dose-dependent manner. At the highest levels of 14-3-3η tested, Dex-induced transactivation was enhanced by 7-fold (Fig. 3A, left panel).
Although the levels of transactivation were also increased in the absence of Dex (Fig. 3A, left panel), this was largely dependent on expression of the GR, because no significant increase was seen in control cells transfected with the empty expression vector (Fig. 3A, right panel). It is possible that small amounts of hormone might have been present in media components or that GR overexpression resulted in a small proportion of GR that was active in the absence of ligand. Even if these effects are disregarded, Dex-induced transactivation was still increased from 16-fold in the absence of 14-3-3p to 28-fold in its presence (35 μmol; Fig. 3A, left panel). To determine whether 14-3-3p protein affected steroid sensitivity, a dose-response curve was performed (Fig. 3B). In cells cotransfected with the 14-3-3p expression vector, the amount of Dex needed to induce half maximal activity was marginally reduced. This indicates that 14-3-3p protein might facilitate receptor activation by hormone in addition to any effects at other levels, such as transcriptional activation.

Highly conserved 14-3-3 protein family members have been identified in mammalian cells (at least seven isoforms) and nonmammalian eukaryotic cells. These proteins have been found to associate with a number of key signaling proteins and cell cycle regulators (see Ref. 12 and references therein and Refs. 24 and 26), such as protein kinase C, Raf-1, phosphatidylinositol 3-kinase, polyomavirus middle tumor antigen, Bcr and Bcr-Abl, platelet adhesion receptor, and Cdc25 phosphatase. Although the functional role of 14-3-3p proteins in these interactions is still poorly defined, 14-3-3p proteins apparently modulate the functions of protein kinase C and Raf-1. Recently, a model for the Raf-1 activation by 14-3-3p proteins has been proposed, suggesting that 14-3-3p protein dimers mediate Raf-1 oligomerization and that this is an essential step in the activation of Raf-1 (27).

It has been shown that 14-3-3p protein dimers can serve as adaptors, bringing together different target proteins (25, 28). 14-3-3p proteins are also known to have chaperone functions in mitochondrial protein import (29) and in solubilizing A20 (25), and they act as a solubility cofactor for keratins in a cell cycle-dependent manner (30). Moreover, nonmammalian 14-3-3p protein family members have been shown to associate with transcriptional regulatory elements (31) and to be required for DNA checkpoints during the cell cycle (32).

Considering the characteristic structure and known features of 14-3-3p proteins, several possible mechanisms could account for the role of 14-3-3p in stimulating the transactivation capacity of the GR. An attractive model would envisage a role as a chaperone that accepts the monomeric GR released by the HSP90 complex and facilitates its dimerization and/or translocation to the nucleus. This would be consistent with the recent proposal that 14-3-3 proteins mediate dimerization of the Raf-1 protein (27) and with a previous report of a 6 S complex that potentiates DNA binding by the GR (33). Other models, including roles as a transcriptional coactivator or as a mediator of cross-talk mechanisms with other signal transduction pathways, are also possible.

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