A Fraction Enriched in a Novel Glucocorticoid Receptor-interacting Protein Stimulates Receptor-dependent Transcription in Vitro*

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Glucocorticoids influence numerous cell functions by regulating gene activity. The glucocorticoid receptor (GR) is a ligand-activated transcription factor and, like any other transcription factor, does not modulate gene activity just by binding to DNA. Interaction with other proteins is probably required to enhance the establishment of a functional transcription initiation complex. To identify such proteins, we analyzed the in vitro interaction of the glucocorticoid receptor bound to a double glucocorticoid response element with nuclear proteins and describe here three interacting proteins with different molecular weights. One of them, which we named GRIP 170 (GR-interacting protein), was purified and microsequenced, and it turned out to be an unknown protein. When tested in a cell-free transcription assay, the fraction highly enriched for GRIP 170 does not influence basal promoter activity but does enhance GR induction.

Glucocorticoids are active in inducing gluconeogenesis in liver, promote the development of various organs, cause apoptosis of lymphoid cells, and are necessary for the growth of many cell types in vitro. The glucocorticoid receptor (GR)1 belongs to the superfamily of steroid hormone receptors and was the first transcription factor to be isolated and studied in detail (1, 2). Most glucocorticoid-regulated genes contain a short palindromic DNA sequence known as glucocorticoid response element (GRE). Depending on the type of GRE and on the promoter context, binding of GR to these elements can result in either gene induction or repression.

For regulating gene expression, the glucocorticoid receptor, like any other transcription factor, has to interact either directly with the transcription/initiation complex or indirectly via adapters or intermediary factors (3, 4). Nuclear receptors have been demonstrated to interact with both types of factors. For some nuclear receptors, but not for GR, interaction with TFII B has been shown (5–7). The estrogen receptor was demonstrated to interact with TAFI D complex (8) and with transcription intermediary factor 1, a putative mediator of the ligand-dependent activation function (9). Other, unknown factors have been demonstrated to interact with nuclear receptors in vitro (10, 11), or in vivo (12) or to enhance receptor-DNA binding in vitro (13). In addition, receptor interaction with proteins involved in alterations of chromatin structure or function, Swi3 and Spt6, has been shown (14, 15).

We have been interested in analyzing mechanisms for a synergistically increased steroid response mediated by multiple binding sites for different or identical transcription factors. Cooperative DNA binding has been shown for the glucocorticoid and the progesterone receptor in the case of duplicated binding sites (16–19). GR can also synergize with various transcription factors (20). Synergistic activation with the ubiquitous transcription factor OTF-1/Oct-1 is found in the context of the MMTV promoter, and this effect seems to be mediated by cooperative DNA binding (21).

Replacement of the DNA binding domain of GR with the GAL4-DNA binding domain abolished binding to duplicated upstream activating sequence elements in contrast to functional synergy, which could still be observed (19). Functional synergy may involve two transcription factors simultaneously (and cooperatively) touching a target protein. Therefore, we designed a strategy for the search of GR-interacting factors, which upon isolation should enhance GR mediated in vitro transcription. Our strategy is based on the use of DNA-bound GRs to a double GRE to avoid interactions irrelevant to transcriptional control and to possibly increase the binding affinity to the target protein. Using this procedure we identified three GR-interacting proteins (GRIPs).

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides 53/54 and 51/52 containing two GREs or a single GRE, respectively (20) were purchased from Eurogentec (Belgium). Endoproteinase Lys-C was from Boehringer Mannheim; [α-32P]ATP was from Amersham Corp. Fast protein liquid chromatography system and columns for protein separation were purchased from Pharmacia Biotech Inc. Sykam HPLC (München, Germany) was used for peptide separation. HPLC columns and all solvents for automatic protein sequencing were from Applied Biosystems (Wertesbad, Germany). PVDF Immobilon membrane was from Millipore (Eschborn, Germany). Dexamethasone was obtained from Sigma. All other chemicals were purchased from Merck.

Cell Culture—Human HeLa S cells were grown in spinner flasks in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn calf serum, sodium pyruvate, glutamine, nonessential amino acids, vitamins, and antibiotics. Dexamethasone was added 45 min prior harvesting to a final concentration of 10−7 M at a density of 8 × 105 cells/ml.

Preparation of Nuclear Extracts for Protein Purification—Nuclear extracts were prepared by the method of Shapiro et al. (22) with minor modifications. The ammoniumsulfate precipitated proteins were resuspended in buffer D (20 mM Hepes, pH 7.9, 100 mM KCl, 0.2 mM EDTA,
1 mM DTT, 20% (v/v) glycerol) (23). After extended dialysis against buffer D the dialysate was centrifuged at 100,000 x g for 30 min at 4 °C, and the supernatant was stored at –80 °C.

Electrophoresis and Electrophoretic Mobility Shift Assay (EMSA) and Western Blotting Analysis—Electrophoretic Mobility Shift Assay (EMSA) was performed according to SivaRaman et al. (26). The double-stranded oligonucleotide containing a single GRE was labeled with [γ-32P]ATP using Klenow polymerase (Boehringer Mannheim) according to the supplier’s directions.

The Western blot analysis was carried out as follows. After separation of nuclear proteins by SDS-PAGE using a 7% polyacrylamide gel, the proteins were electroblotted to a PVDF membrane (Immobilon P, Millipore). After blocking, membranes were incubated either with a 1:100 dilution of a monoclonal antibody recognizing GR, or with a 1:200 dilution of an affinity-purified polyclonal antibody raised against Hbrm. Purified goat anti rabbit antibody was used as secondary antibody (Aurion, Germany). The immune complexes were visualized by the Immuno Gold detection kit according to the manufacturer (Aurion, Germany).

Renaturation and Blocking—For renaturation the PVDF membrane was transferred to HEMN buffer (20 mM Hepes, pH 7.4, 0.5 mM EDTA, 5 mM MgCl₂, 150 mM NaCl) plus 0.1% Nonidet P-40 and 3 mM DTT, containing 6 mM guanidine hydrochloride for denaturation. The guanidine hydrochloride was diluted out step-wise by adding same buffer without guanidine hydrochloride, allowing the proteins to partially renature. After renaturation the PVDF membrane was blocked at 4 °C overnight in HEMN buffer + 5% nonfat dry milk (Carnation).

d-FSW Assay—The cross-linked far Southwestern analysis was performed as follows. 10 μg of partially purified GR fraction was incubated with 0.5 pmol of 32P-labeled double GRE oligonucleotide (dGRE) in the presence of 5-1 μg of poly(dI-dC) for 10 h at 4 °C in binding buffer (10 mM Tris, pH 7.5, 60 mM KCl, 2.5 mM MgCl₂, 1 mM EDTA, 5% (v/v) glycerol), followed by UV-cross-link at 286 nm for 5 min at room temperature. The cross-linked mixture had been diluted in 3 ml of HEMN buffer containing 1 μg/ml poly(dI-dC), 0.1 mM dexamethasone, 2 mM ATP, and 3 mM DTT. This solution was used as a probe for the renatured proteins on the milk powder-blocked PVDF membrane. The PVDF membrane was carried out for 3 h at 20 °C and then stored at −30 °C in a plastic bag. Afterwards the PVDF membrane was washed twice for 10 min in HEMN buffer containing 0.1% Tween 20 and 3 mM DTT. The dry membrane was autoradiographed overnight.

Protein Purification—Crude nuclear protein extracts were separated first on an anion exchange chromatography by loading 100–150 μg of protein on a Q-Sepharose column. The proteins were eluted by applying a linear gradient of 0.1 mM NaCl to 1 mM NaCl in QS buffer (20 mM Tris, pH 7.4, 20 mM KCl, 2 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 10% (v/v) glycerol) for 90 min at a flow rate of 2 ml/min. The GR-binding activity of the fractions was assayed in d-FSW experiments. Positive fractions containing GRIP 170 were pooled (in QS), diluted, adjusted to pH 6, and loaded on Mono S columns for cation exchange chromatography. The proteins were separated using a linear gradient from 0 mM NaCl to 1 mM NaCl in MS buffer (20 mM MES, pH 6.0, 20 mM KCl, 2 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 10% (v/v) glycerol) for 50 min at a flow rate of 0.8 ml/min. The fractions were assayed with the d-FSW, and the fractions expressing GRIP 170 activity were pooled, diluted, adjusted to pH 7.4, and applied on a heparin-Sepharose column. Protein separation was carried out with a linear gradient between 0 mM NaCl and 0.5 mM NaCl in HS buffer (25 mM Hepes, pH 7.6, 5 mM MgCl₂, 1 mM EGTA, 10% (v/v) glycerol) for 80 min at a flow rate of 2 ml/min. This gradient was followed by a one-step elution with 1.5 mM salt under the same conditions. Fractions containing GRIP 170, as determined by d-FSW, were pooled, dialyzed, and concentrated to a 1 ml volume by an exchange chromatography. Therefore, the proteins were eluted by applying a step linear gradient of 0.1–0.8 mM NaCl in HS buffer for 40 min at a flow rate of 1 ml/min. This resulted in three positive fractions containing GRIP 170 as shown by d-FSW.

Protein Digestion, Reversed Phase HPLC, and Microsequencing—Protein digestion, reversed phase HPLC, and microsequencing of peptides were carried out as described by Eggert et al. (27). N-terminal microsequencing was performed by running a preparative SDS-PAGE. The gel was blotted to Immobilon membrane, which was stained with Coomassie Blue solution (0.2% Coomassie Blue, 0.5% acetic acid, 20% (v/v) methanol) and washed extensively (30% (v/v) methanol). The GRIP 170 bands were excised and analyzed by microsequencing.

In Vitro Transcription—HeLa cell nuclear extract was prepared essentially after the method of Dignam et al. (23). Recombinant full-length human GR was expressed in Spodoptera frugiperda SF9-cells by an ITM vector containing human GR cDNA and the cytomegalovirus promoter (25). DNA templates were derived from pC(AT)50 and contain either the full-length MMTV promoter from Srinivasan and Thompson (28). DNA templates were derived from pC(AT)50 and contain either the full-length MMTV promoter from Srinivasan and Thompson (28). DNA templates were derived from pC(AT)50 and contain either the full-length MMTV promoter from Srinivasan and Thompson (28). DNA templates were derived from pC(AT)50 and contain either the full-length MMTV promoter from Srinivasan and Thompson (28). DNA templates were derived from pC(AT)50 and contain either the full-length MMTV promoter from Srinivasan and Thompson (28).

Template DNAs were preincubated with GR, GRIP 170, and 100 ml in each aliquot of the GRIP 170 buffer 10 min on ice in a volume of 12.5 μl. Then HeLa cell nuclear extract (–40 μg), nucleotides were added to make a final volume of 25 μl, and transcription was carried out for 45 min at 30 °C. Transcripts were treated as described (29), separated by 6.5% denaturing polyacrylamide gel electrophoresis, and visualized by autoradiography. Quantitation was made with a Phosphorimager (Molecular Dynamics), and relative transcription was determined with respect to the control template.

RESULTS

Identification of Proteins Interacting with DNA-bound GR—To identify proteins that interact with GR molecules bound to two adjacent GRE sequences, we generated a probe consisting of a 32P-labeled dGRE UV-cross-linked to GR molecules. For this purpose we enriched GR from HeLa nuclear extracts by Q-Sepharose fractionation. This results in a 30-fold enriched GR-fraction compared with the crude extract. The 97-kDa hGR was identified by Western blotting with a rabbit polyclonal antibody directed against a synthetic hGR peptide (amino acids 346–367). This fraction generated a strong band shift in an electrophoretic mobility shift assay (not shown), and GR could be UV-cross-linked to 32P-labeled GRE sequences. Binding and UV-cross-linking was GRE-specific since it was comparable with unlabeled GRE DNA (not shown). This GR-32P-dGRE complex was used as a probe against HeLa nuclear proteins or, as a control, against E.scherichia coli proteins, which were separated by SDS-PAGE and blotted on an Immobilon membrane. Fig. 1 shows an autoradiograph of such an experiment. Four bands migrating according to molecular masses of about 95, 115, 120, and 170 kDa can be detected. The 120-kDa band is difficult to resolve from the 115-kDa band, but transcription fractionation (see below) clearly identifies and separates the 115- and 120-kDa bands. The 115-kDa band is seen in

![Fig. 1. Direct interaction of GR-32P-dGRE complex with several nuclear proteins. 100 μg of nuclear extract of desamethasone-treated HeLa-cells (N, oval numbered lanes) and 100 μg of bacterial extract (B, odd-numbered lanes) were separated by SDS-PAGE and blotted on an Immobilon membrane. 32P-labeled dGRE was cross-linked to a protein fraction in the presence of poly(dI-dC) for 5 min at 280 nm. Coomassie blue protein fractions were resolved as follows: 10 μg of GR fraction (lanes 1-4), 10 μg of nuclear extract (lanes 5 and 6), 10 μg of bacterial extract (B, lanes 7 and 8), and no protein (lanes 9 and 10). Competition of the cross-link reaction with 25-fold excess of unlabeled GRE-DNA reduces intensities or completely inhibits labeling of the 170-, 120-, and 95-kDa bands (lane 4). The bands were visualized by autoradiography and labeled according to their apparent molecular masses (170, 120, 115, and 95 kDa).](http://www.jbc.org/)

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glucocorticoid receptor-interacting protein

Characterization and Purification of GRIP 170—Previously, a protein in the range of 170 kDa has been shown to enhance the GR-mediated gene induction. This protein, Swi2 in yeast or Hbrm in humans, with a molecular mass of 180 kDa (30) is not the 170-kDa protein found here, since a Western blot with an antibody against Hbrm (α Hbrm) or against hGR (α GR), and the antibody complexes were visualized by Immuno Gold detection. C, nuclear extract (N) and 40 μg of a GRIP 170-enriched Q-Sepharose fraction (Q 35) were SDS-PAGE-separated and blotted. The blot was incubated with GRbv-32P-dGRE in the absence of competitor (lanes 1 and 2) or in the presence of 25-fold excess unlabeled GRbv-dGRE (lanes 3 and 4) or with GRbx-32P-sGRE (lanes 5 and 6). Southwestern experiments with the labeled GRE in the absence of proteins in the probe (Fig. 1, lane 10) and with almost any DNA sequence tested (not shown) and, therefore, seems to be an unspecific DNA-binding protein. The other bands, however, were specific as demonstrated by the fact that (i) bacterial proteins on the blot are not visualized (Fig. 1, odd-numbered lanes), (ii) specific GRE competition during probe cross-linking results in reduced 170-, 120-, and 95-kDa bands (Fig. 1, lane4), (iii) whole nuclear extract, which contains low amounts of GR, labels the 110- and 95-kDa bands only, and (iv) bacterial extract cross-linked to DNA visualizes a single band (115 kDa), which is seen with the protein-free DNA probe alone (Fig. 1, lanes 8 and 10). Since the 170-kDa band most clearly showed specificity for the GR-32P-dGRE complex, we analyzed this protein. Although from our competition tests in the cross-linking reaction we knew that HeLa-GR was bound to the double GRE, we wanted to confirm this result with a glucocorticoid receptor from another source. We used human GR expressed with a baculovirus vector in insect cells. After purification by DNA affinity column (28) we used this protein to generate a GR-32P-dGRE probe. As seen in Fig. 2A, the baculovirus-expressed GR identifies nearly the same bands as the HeLa-GR including the 170-kDa band.

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A Fraction Highly Enriched for GRIP 170 Stimulates GR-Induced Transcription in Vitro—Since enhancement of receptor binding to DNA by receptor-interacting proteins has been demonstrated (13), we tested the purified GRIP 170 fraction in an EMSA with baculovirus-expressed GR (Fig. 4C). The addition of GRIP 170 neither causes a retarded band nor changes the intensity or position of the GR/DNA complex.

Another step toward the functional analysis of a protein interacting with a transcription factor would be the use of an in vitro transcription system. Therefore, we prepared nuclear extracts from HeLa cells (23) that efficiently mediate induction by purified glucocorticoid receptor (29). Human GR was expressed in insect cells with a baculovirus expression system and purified (28). DNA templates used contain the full-length MMTV promoter up to position –240 bp of the transcriptional start site. This promoter region encompasses the natural GRE sequences (31) and was fused to a G-less cassette of 380 bp in length. As an internal control the reaction contained a second template with a truncated MMTV promoter, lacking the GRE sequences but containing the nuclear factor I and octamer transcription factor I binding sites. This promoter up to –80 bp was fused to a G-less cassette of 280 bp in length and provides a standard for basal gene transcription. In vitro transcription in the presence of radioactive nucleotides yielded a specific transcript for both templates (Fig. 5A). The upper band in each pair of bands represents read-through transcripts, which are almost not affected by the presence of GR or GRIP 170 fraction, whereas the lower band represents the correctly initiated transcripts for both DNA templates and is enhanced in the presence of GR with the full-length MMTV template. Phosphor imager quantitation was used to determine the relative transcription activity. Addition of the GRIP 170 fraction to the basal transcription reaction results, if in anything, in a marginal increase in transcription on both promoters (Fig. 5, A, lanes 2 and 4, and B). Similarly, no effect was seen with an adenovirus major late promoter as template (data not shown). Addition of baculovirus-expressed GR generates a transcriptional induction as expected (29). The combined addition of GR and GRIP 170 fraction shows a synergistic increase in transcription (Fig. 5, A, lane 6, and B). This induction very likely was mediated by GRIP 170 because all of the reactions contained the same amount of GRIP 170 buffer, since a different fraction not containing GRIP 170 did not change transcription efficiency, and finally GRIP 170 “shoulder fractions” are less effective as the peak fraction (Table I).

DISCUSSION

Here we identified three proteins (170, 120, and 95 kDa) that interact with a specifically designed glucocorticoid receptor probe. The probe consists of a 32P-labeled DNA harboring a double GRE with UV-cross-linked GR molecules. This probe serves several requirements. First, the GR can be easily and efficiently labeled. Second, the double GRE arrangement provides several DNA bound GR molecules, which increase the binding affinity to the target protein. This assumption is based...
the estrogen receptor only in the presence of estrogen. For GR such a ligand-dependent binding would not be detectable, since GR will be activated through the purification procedure (39, 40). Thus, based on the molecular weight, GRIP 170 could be similar or identical to ERAP 160/RIP 160. For the GRIP 170 fraction we found a GR-dependent transcriptional increase in the in vitro transcription assay, an activity not identified for previously described receptor-associated or interacting factors (10–13). Although in vitro binding to GR depends on the presence of two GREs, it remains to be shown, whether GRIP 170 is required to mediate synergy or to mediate the intrinsic induction from a single GRE in vivo.

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