The Non–Ligand Binding β-Isoform of the Human Glucocorticoid Receptor (hGRβ): Tissue Levels, Mechanism of Action, and Potential Physiologic Role

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ABSTRACT

Background: Alternative splicing of the transcripts of the human glucocorticoid receptor gene results in two mutually exclusive products, the classic, ligand-binding glucocorticoid receptor (hGRα), and a dominant negative non–ligand-binding isoform, hGRβ.

Materials and Methods: We examined the existence of and quantified both hGRα and hGRβ isoforms in a panel of human tissues, as well as in intact and fractionated HeLa cells, using specific quantitative Western blots and/or immunocytochemistry. We studied the potential interactions of hGRβ with heat shock protein (hsp) 90 and/or hGRα using cross-immunoadsorption/precipitation procedures followed by Western blots.

Results: For the first time, we demonstrated the natural existence of the hGRβ protein, which was widely expressed in human tissues. The ratio of immunoreactive hGRα to hGRβ varied from 0.2 to 1.0 among different tissues, and was approximately 0.2 in HeLa cells. In the latter, both isoforms were distributed in the cytoplasm and nucleus in the absence of the hormonal ligand, and translocated into the nucleus after addition of dexamethasone. The cytosolic and nuclear hGRα-to-hGRβ ratio remained the same before and after dexamethasone exposure, suggesting that upon activation the two isoforms translocated into the nucleus in equal proportions. hGRα- and hGRβ-specific antibodies cross-adsorbed and precipitated cytosolic and nuclear glucocorticoid hGRα and hGRβ, respectively, as well as hsp90, suggesting that hGRα and hGRβ are in complex with hsp90 and/or each other.

Conclusions: The hGRβ protein is widely expressed throughout the human body and present mostly in the cytoplasm of human cells, in complex with hsp90 and other proteins. In the presence of glucocorticoid, hGRβ probably heterodimerizes with ligand-bound hGRα and translocates into the nucleus to act as a dominant negative inhibitor of the classic receptor.

INTRODUCTION

The existence of two human glucocorticoid receptor (hGR) isoforms, the classic ligand-binding receptor hGRα, and a slightly smaller protein termed hGRβ, was predicted as early as 1985, based on the cDNA cloning experiments by Hollenberg et al. (1). Elucidation of the structure of the hGR gene provided further evidence for the alternative splicing of the two transcripts expressed from a single gene on chromosome 5 (2) (Fig. 1). The sequence of the two mRNA isoforms is identical for most of the coding region, which corresponds to the first eight exons of the hGR gene. The remainder is derived by alternative splicing of the nucleotide sequence encoded by the last exon of the hGR gene, previously referred to as exon 9α or 9β, respectively. Consequently, the two protein isoforms share the same first 727 amino acids, which include the τ1 and

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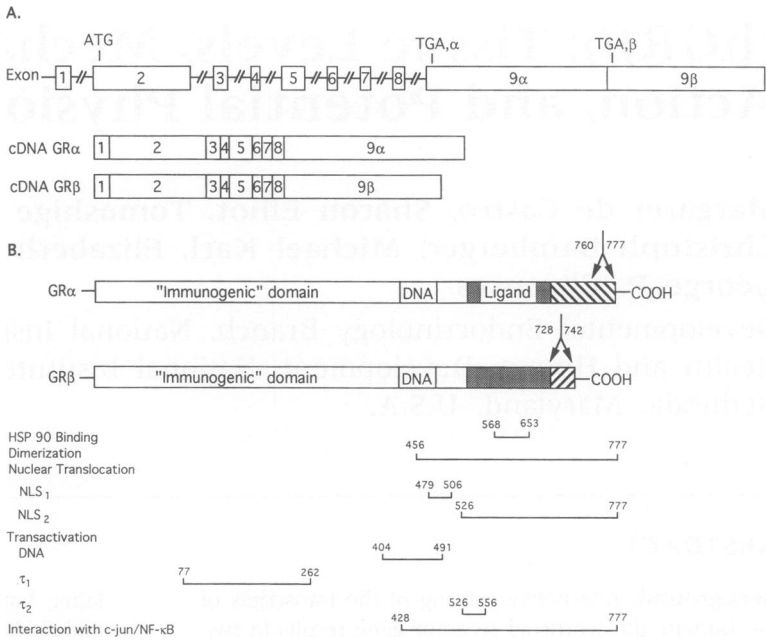

FIG. 1. Schematic structure of the gene, cDNA, and protein of the hGR isoforms α and β.

(A) Genomic structure (top) and cDNAs (bottom) of hGRα hGRβ isoforms, produced by alternative splicing. (B) Linearized structures of hGRα and hGRβ isoforms (top), identical up to residue 727. The amino acid sequences (shown between arrows) corresponding to residues 760–777 or 728–742 of hGRα and hGRβ, respectively, were used as antigenic epitopes for generation of specific antisera in rabbits. The various functional subdomains of hGRα are shown beneath the protein structures (bottom).

τ2 transactivation and DNA-binding domains but differ in their C terminus. The hGRβ has 15 distinct C-terminal amino acids, in lieu of the 50 C-terminal hGRα amino acids, which form the C terminal structure of the ligand-binding pocket. This difference renders hGRβ unable to bind glucocorticoids (1) and incapable of ligand-stimulated enhancement of the transcription rate of glucocorticoid-responsive genes (3–6).

As early as 1995, we demonstrated that hGRβ expressed from a cDNA clone bound specifically to glucocorticoid-responsive elements (GREs) and acted as a dominant negative inhibitor of hGRα activity in a heterologous COS-7 cell co-transfection system (7). Indeed, hGRβ inhibited approximately 50% of the maximum ligand-activated hGRα effect when transfected in 5-fold excess concentrations. These findings were recently replicated in COS-1, CV-1, and HeLa S3 cells, in the last case showing inhibition of endogenous hGRα action by hGRβ as well (8). In our earlier study, we also demonstrated that the hGRβ mRNA was expressed in virtually all human tissues examined, suggesting that this isoform might play an important role in defining tissue sensitivity to glucocorticoids (7). This was recently confirmed by quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) analysis, which allowed computation of a hGRβ-to-hGRα mRNA ratio ranging between 0.21 and 0.34 in human tissues (8). Two major questions were generated from these data: First, is the β-isoform *protein* indeed expressed in human tissues and, if so, are its quantities sufficient to allow a physiologic antagonistic role? Second, where is the β-isoform located, since it does not bind ligand but has been shown to react with nuclear GREs? Is it constitutively nuclear or mostly cytosolic, translocating into the nucleus with the assistance of another protein, such as hGRα (9–13)?

In this study, we examined the presence and quantities of the protein isoforms of hGRα and hGRβ in a panel of human tissues as well as in HeLa cells. This is a widely used human cancer cell line known to contain classic ligand binding glucocorticoid receptors. We then defined the subcellular localization of hGRβ in the basal state and after incubation of the cells with dexamethasone. Furthermore, we examined the interactions of the β-isoform with heat shock protein (hsp) 90 and hGRα.

MATERIALS AND METHODS

Human Tissues and Cell Cultures

Central nervous system and peripheral postmortem (8–14 hr) human tissues were obtained from NDRI (National Disease Research Interchange, Philadelphia, PA, U.S.A.) and kept at −70°C until assay. HeLa cells were obtained from the American Tissue Type Collection (ATTC, Rockville, MD, U.S.A.) and plated directly in culture flasks for protein analysis by immunoblotting or in multichamber slides

(Nunc, Napperville, IL, U.S.A.) for immunocytochemistry studies. In either case, they were grown to 80–90% confluence, in Dulbecco's modified Eagle's medium (DMEM) (Biofluids Inc., Rockville, MD, U.S.A.) supplemented with 10% charcoal/dextran-treated fetal bovine serum (HyClone, Logan, UT, U.S.A.), 100 U/ml penicillin/100 μg/ml streptomycin, and 2 mM glutamine (GIBCO, Gaithersburg, MD, U.S.A.), at 37°C, in a 5% CO_2 atmosphere.

Preparation of Human Tissues and Cytosolic and Nuclear Cell Fractions of HeLa Cells

Human tissues (0.5 g) were homogenized in 2 ml of 0.25 M sucrose, 3 mM imidazole, pH 7.5, and a combination of protease inhibitors (2 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, 50 μg/ml leupeptin) in an ice bath, with a Branson Sonifier cell disrupter 185 (Danbury, CT, U.S.A.) operated at maximum speed for 30 sec. HeLa cells were treated with 10^{-7} M dexamethasone (dex) or placebo for 30 min, as soon as they reached confluence. At the end of the incubation, they were harvested by trypsinization, centrifuged at 300 × *g* for 5 min, resuspended, and washed three times with phosphate-buffered saline (PBS). After a fourth 300 × *g* centrifugation, the cell pellets were frozen on dry ice for 30 min. They were then homogenized in 1 ml Taps buffer (25 mM Taps, 1 mM EDTA, 10% glycerol, pH 8.8, kept at 4°C) containing protease inhibitors (2 g/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, and 50 μg/ml leupeptin).

The tissue or HeLa cell homogenates were subsequently centrifuged at 300 × *g* for 10 min, and the pellets were discarded. The new supernatants were recentrifuged at 1000 × *g* for 10 min to obtain the nuclear fraction in the pellet, which was then dispersed in 0.3 ml of the same buffer and kept frozen at −70°C. The supernatants were ultracentrifuged at 105,000 × *g* for 1 hr, to obtain the cytosolic fraction, which was also kept frozen at −70°C, until assay. Protein determination was performed using BCA Protein Assay Reagent (Pierce, Rockford, IL, U.S.A.) and bovine serum albumin (BSA) as a standard.

Antisera Generation and Characterization

Anti-hGRα and -hGRβ polyclonal antisera were raised in female New Zealand white rabbits immunized with an 18-mer synthetic peptide (QIPKYSNGNIKKLLFHQK) or a 15-mer synthetic peptide (NVMWLKPESTSHTLI), derived from the amino acid sequences of the corresponding residues 760–777 or 728–742 of the hGRα and hGRβ proteins, respectively (Fig. 1). Since short peptides are unlikely to assume the native conformation in solution, we synthesized each oligopeptide in tandem with a "helper" 22-mer sequence (GPSLKLLSLIKGVIVHRLEGVE), which stabilized its conformation, as previously described (14). The resultant 40 amino acid–long peptides were given to rabbits intradermally at four to five sites in the dorsal flank, with 100 μg of peptide per site emulsified in complete Freund's adjuvant. Booster immunizations were performed every 4–5 weeks. Antibody titers were monitored by enzyme-linked immunosorbent assay (ELISA). We selected antisera with titers greater than 1:10,000. The antibodies were affinity-purified on a column prepared by coupling each of the synthetic hGRα- and hGRβ-specific oligopeptides to UltraLink (Pierce), as specified by the manufacturer.

The antisera dilutions were selected to provide specific binding with the least amount of nonspecific background. The specificity of the immunoreaction was controlled by substituting the primary antiserum with nonimmunized rabbit serum or by adding no primary antiserum. No immunopositivity was observed in these experiments. The specificity of the anti-hGRα versus anti-hGRβ antisera against the corresponding entire hGRα and hGRβ proteins was tested by expressing either protein in COS-7 cells using the pRShGRα and pRShGRβ expression vectors, generously provided by Dr. R. Evans (Salk Institute, CA). While both recombinant proteins interacted strongly with the commercial antibody, each reacted primarily with the appropriate isoform-specific antisera. Low-intensity hGRα- and hGRβ-immunoreactive bands, however, were observed in nontransfected COS-7 cells. As a positive control, we employed a commercially available polyclonal antibody raised in rabbits immunized with a 22-mer oligopeptide (amino acids 346–367 of the hGR), whose sequence is in the immunogenic amino terminal domain of the hGR, which is common in both the α- and β-isoforms (clone 57, cat PA1-511; Affinity Bioreagents, Inc., Golden, CO, U.S.A.).

We also employed an anti-hsp90 rabbit antiserum that reacts with hsp90 (15), a generous gift from Dr. Ettore Appella (National Cancer Institute, MD).

Protein Electrophoresis and Western Blots

The samples were treated with Laemmli buffer (16) containing 100 mM DTT and heated at 65°C for 15 min. Eight micrograms of protein from HeLa cells and 25 μg from human tissues were applied to an 8% precast polyacrylamide gel in a Novex miniature gel apparatus. Pre-stained markers (Novex), including myosin (205 kD), BSA (98 kD), glutamic dehydrogenase (64 kD), carbonic anhydrase (36 kD), myoglobin (30 kD), lysozyme (16 kD), aprotinin (6 kD), and insulin β-chain (4 kD), were electrophoresed in parallel to estimate the molecular weight. Electrotransfer of proteins from the gel to nitrocellulose was performed by electroelution at 30 V for 75 min. Nonspecific protein binding to the nitrocellulose paper was reduced by preincubation of the membranes in 5% milk block for 30 min. Immunoblotting was subsequently performed at 4°C, using our purified antibodies against the hGRα- and hGRβ-specific sequences at 5 and 4 μg/ml, respectively, and the previously characterized commercially available antibody at 1.75 μg/ml, all after an overnight incubation. The following morning, the blots were washed in wash buffer (50 mM sodium phosphate, 150 mM NaCl, and 0.05% Tween 20) for 60 min. The blots were then incubated at room temperature for 1 hr, with peroxidase-conjugated anti-rabbit immunoglobulin antibody (Dako, Carpinteria, CA, U.S.A.) employed at 1:3500, 1:4000, and 1:2500 dilution for our specific anti-hGRα and -hGRβ antibodies and the commercially available antibody, respectively. Washing was then done as described above. To detect immunoreactive bands, blots were exposed to chemiluminescence solution (LumiGlo chemiluminescence substrate; Kirkegard and Perry, Gaithersburg, MD, U.S.A.) for 1 min, followed by exposure to X-OMAT AR Film (Eastman Kodak Co., Rochester, NY, U.S.A.). After film development, the blots were washed with India ink (1 μl/ml) for 2 hr, to visualize the protein bands and confirm loading equivalency.

In addition, competition experiments were performed by preincubating the primary antisera overnight with increasing amounts of the corresponding immunogenic peptide. When used in Western analysis, signal intensity of the specific receptor bands diminished as the immunogenic peptide concentration increased, thereby demonstrating the specificity of the primary antisera.

Cross-Immunoadsorptions/Precipitations

Receptors were first immunoadsorbed from HeLa cell extracts containing 100 μg of total protein, by incubation with nonimmune rabbit serum, specific polyclonal anti-hGRα or -hGRβ sera in Taps buffer on ice for 1.5 hr, as described above. The reaction mixture was subsequently incubated with 10% Protein-A Sepharose CL-4B (Pharmacia, Uppsala, Sweden) on ice for 1.5 hr. After a 200 × *g* centrifugation for 1 min, the pellet was washed three times in dilution buffer (10 mM Tris-HCl, pH 8, 140 mM NaCl, 0.1% Triton X-100, 0.1% BSA, 0.025% sodium azide), followed by a fourth wash with 50 mM Tris-HCl, pH 6.8. The immunoprecipitates were pelleted by a 12,000 × *g* centrifugation at 4°C for 5 min, resuspended in SDS buffer, and boiled for 10 min before immunoblotting analysis to detect hGRα, hGRβ, or hsp90.

Measurement of hGRα and hGRβ Immunoreactive Bands by Quantitative Western Analysis

We determined the *relative* amount of GRα and GRβ proteins in human tissues and HeLa cells by quantitative Western analysis as previously described (17). Peptide standards were prepared by linking the antigenic hGRα or hGRβ immunizing peptides, modified by the addition of an N-terminal cysteine, to maleimide-activated BSA (Imject Activated Immunogen Conjugation Kits, Pierce). The peptide-BSA conjugate was then purified from the free peptide and other reaction impurities by gel filtration chromatography to be used as standards, as described in the manufacturer's instructions and reported previously (17). We determined the approximate amount of peptide-bovine albumin conjugate required to cover the range of hGRα and hGRβ proteins observed in human samples by examining a wide range of dilutions. Increasing concentrations of the peptide-BSA conjugate standard (5–25 ng or 70–360 fmol of the peptide-BSA conjugate/lane as assessed by the BCA Assay kit (Pierce) and corrected by the approximate molecular weights of the conjugates (~69 kD) were used in each of the Western blots performed, in addition to the protein extracts to be analyzed. The band densities of standards and samples developed on the film were scanned and saved on computer disks for densitometry, using the NIH image system 1.57 program. Following densitometry measurements, a standard curve

was constructed of known quantities of the peptide-BSA conjugates versus densitometry measurements, and the densitometry measurements of the unknown samples were converted to a numerical value calculated from the standard curve (Instat Graph Pad, San Diego, CA, U.S.A.).

Immunocytochemistry

HeLa cells grown to near confluence in multichamber slides were treated with 10^{-7} M dexamethasone or placebo for 15, 30, 60, or 120 min. Subsequently, the cells were washed three times with PBS. Fixation and permeabilization were performed in tandem by incubation with 4% paraformaldehyde in PBS for 15 min, followed by treatment with 0.2% Triton X-100 in PBS for 15 min. After three additional washes with PBS, the cells were incubated with 50% normal goat serum for 5 min. Then, the "first" antibodies, 5 μg/ml of the purified anti-GRα or -GRβ, respectively, or 1 μg/ml of the commercially available antibody, were added. Incubation was allowed to take place for 60 min. After another three washes with PBS, the "second" antibody, Cy3-conjugated AffiniPure Goat Anti Rabbit IgG (Jackson Immuno Research, West Grove, PA, U.S.A.), was added, and incubation was allowed to proceed for 60 min. After another three washes, we applied three drops of mounting buffer (150 mM Tris, pH 8.8, containing 10 mg Dabco, 0.1% azide, and 25% glycerol) on the bottom of each chamber, removed the chamber walls from the bottom plastic slide and covered the latter with a coverslip. Cy3 is maximally excited by light at 553–555 nm and emits at 568–574 nm. All pictures were taken at 400× magnification using immersion oil.

The specificity of the immunoreaction was controlled by substituting the primary antiserum with nonimmunized rabbit serum or PBS. No staining was observed in these experiments. Dilution of each of the three antisera from 10 to 0.1 μg/ml decreased markedly the amount of immunopositivity, as expected. The specificity of the anti-hGRα versus anti-hGRβ antisera against the corresponding proteins was tested in COS-7 cells expressing either protein. There was concordance between the recombinant proteins and the appropriate antisera.

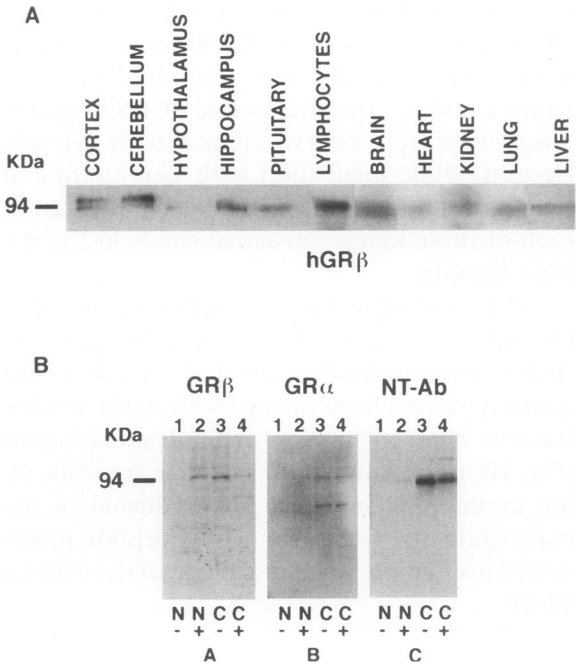

FIG. 2.

(A) Expression of hGRβ in human tissues. hGRβ protein is expressed in multiple human tissues. Protein extracts (25 μg/lane) were run on a 8% SDS polyacrylamide gel. After transfer to nitrocellulose membranes, samples were probed with anti-hGRβ specific antibody. The positions of the molecular-mass marker expressed in kD are indicated by arrows on the left. **(B) Cytosolic and nuclear localization of the hGR isoforms in the presence and absence of dexamethasone.** hGRα (left) and hGRβ (right) immunoblotting analyses of nuclear (N, Lanes 1 and 2) and cytosolic (C, Lanes 3 and 4) fractions of HeLa cells. Proteins (8 μg/lane) were run on a 8% SDS polyacrylamide gel, in the absence (Lanes 1 and 3) or presence (Lanes 2 and 4) of 10^{-7} M dexamethasone. The positions of the molecular-mass markers expressed in kD are indicated by arrows on the left.

RESULTS

The Nonligand-Binding hGRβ Isoform Protein Is Present and Widely Expressed in Human Tissues and the Cytosol and Nucleus of HeLa Cells

Using our purified hGRα and hGRβ isoform-specific antibodies in Western blots of extracts from multiple human tissues (Fig. 2A) and HeLa cells (Fig. 2B), we detected a 94-kD immunoreactive band corresponding to each of the isoforms. In both instances, we also detected a second smaller molecular weight band, which has been reported previously with commercial antibodies and which, most likely, represents an N-terminal

proteolytic product of the receptor, earlier called "meroreceptor" (18). In the absence of dexamethasone (Fig. 2B, left and middle panels, Lanes 1 and 3), most hGRα and hGRβ immunoreactivity of HeLa cells was found in the cytosolic fraction, while incubation with dexamethasone (Lanes 2 and 4) resulted in a visible increase of each of these immunoreactive bands in the nuclear fraction.

Our positive controls, employing the commercial anti–N-terminal antibody, also gave us a similar immunoreactive band at 94 kD, whose quantity shifted from the cytosolic to the nuclear fraction after incubation with dexamethasone (Fig. 2B, right panel). Our negative controls, using preimmune antisera, buffer devoid of primary antibody, or immunogenic peptide preabsorbed antisera, gave no immunoreactive band at 94 kD.

The hGRβ Isoform is Present in Quantities Sufficient to Produce Antagonism of hGRα

To measure the amount of hGRα and hGRβ protein present in our panel of central nervous system tissues and HeLa cells, we utilized quantitative Western analyses using a standard curve prepared by linking the GRα or GRβ immunizing peptides to bovine serum albumin. A standard curve of band densities was generated, corresponding to known amounts of the peptide-conjugate loaded per lane, and used in each of the Western blots to derive the amount of immunoreactivity corresponding to the hGRα and hGRβ proteins contained in each sample.

Figure 3A shows the standard curve corresponding to 5–25 ng or 70–360 fmol of the hGRβ peptide-conjugate/lane (band at $\cong$69 kD). The densitometry values obtained from the immunoreactivity bands of the hGRβ peptide-conjugate are shown in Fig. 3B. A computer-generated standard curve obtained from densitometry measurements of the peptide-conjugate (y-axis) and the corresponding fmoles of hGRβ peptide-conjugate (x-axis) is presented in Fig. 3C. Using this assay, we quantified hGRα and hGRβ in a panel of human tissues and in whole HeLa cells, as well as in cytosol and nuclear extracts of HeLa cells. The hGRα-to-hGRβ ratio in human tissues varied between 0.2 and 1.0, while this ratio was 0.2 in HeLa cells (see below).

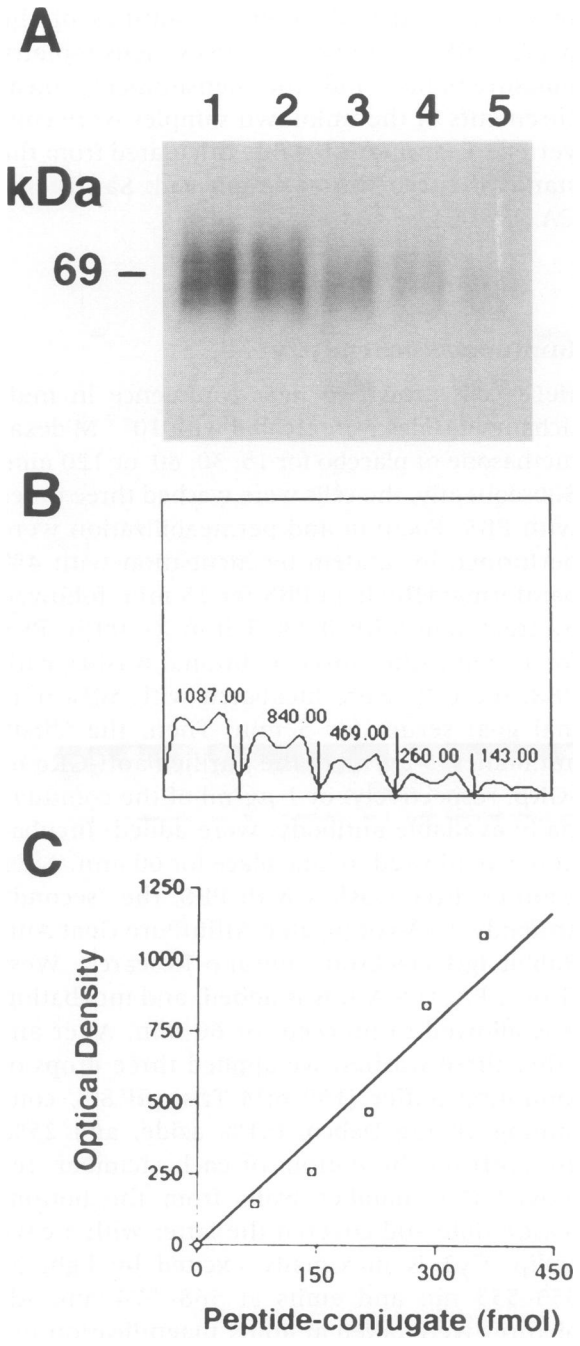

FIG. 3. Quantitative Western blot assay

The immunizing peptide, modified by the addition of an N-terminal cysteine and conjugated to maleimide-activated albumin, was used as the immunoreactive peptide standard for the assay. (A) Representative standard curve corresponding to 70–360 fmol (Lanes 1–5) of the hGRβ peptide-conjugate/lane (band at 69 kD) used to measure hGRβ protein. (B) Densitometry values obtained from the immunoreactive bands of the hGRβ peptide-conjugate. (C) Standard curve generated from the densitometry measurements (y-axis) and corresponding fmol of the hGRβ peptide.

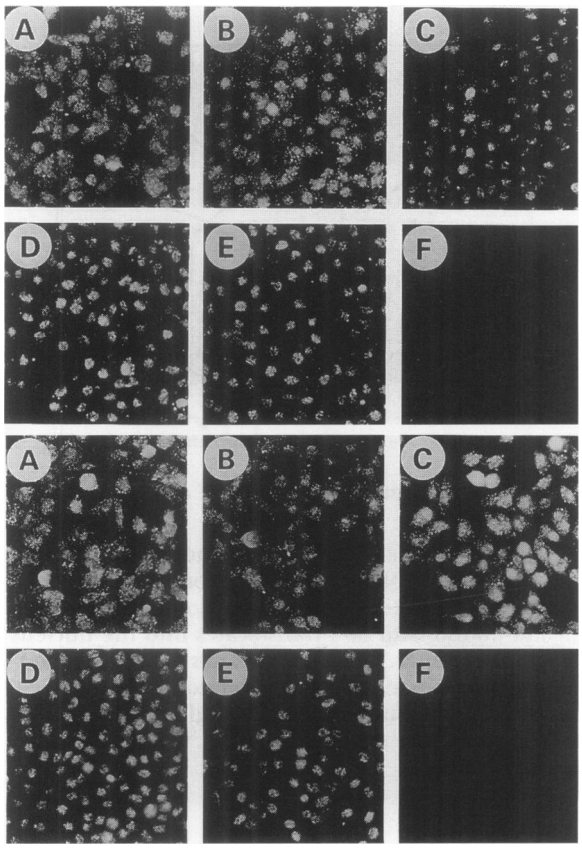

FIG. 4. Representative micrographs of the hGRα (top) and hGRβ (bottom) immunofluorescence analyses in HeLa cells

The subcellular distribution of GRα and GRβ are shown prior to (A) and after 15 (B), 30 (C), 60 (D), and 120 (E) min of incubation with 10^{-7} M dexamethasone. The cells were fixed with 4% buffered paraformaldehyde, permeabilized with 0.2% Triton X-100, and processed for immunocytochemical detection, as described in Materials and Methods. In the bottom panels (F), no primary antibody was employed. Magnification: 400×.

Immunocytochemical and Western Blot Evidence That Both hGRα and hGRβ Are Cytosolic and Translocate into the Nucleus in the Presence of Glucocorticoid

Using our hGRα and hGRβ isoform-specific purified antibodies we detected specific immunofluorescence in both the cytoplasm and nucleus of HeLa cells (Fig. 4). In the absence of dexamethasone, about two-thirds of hGRα immunoreactivity was found in the cytoplasm, and the remaining one-third in the nucleus (Fig. 4, top). Under the same conditions, about two-thirds of the hGRβ immunoreactivity was in the cytoplasm, with the remaining immunofluorescence in the nucleus (Fig. 4, bottom). Translocation of both hGRα and hGRβ immunoreactivity into the nucleus took place after incubation with dexamethasone, which was virtually maximal for both isoforms by 30 min. Our positive controls, using the commercial anti–N-terminal antibody also detected immunoreactivity in both the cytoplasm and nucleus of HeLa cells, with increasing nuclear content after incubation of the cells with dexamethasone (data not shown). Our negative controls, using preimmune sera or buffer devoid of primary antibody, demonstrated no specific immunofluorescence.

Table 1 shows the amount of immunoreactive hGRα and hGRβ in Western blots from extracts of whole HeLa cells and in the cytosolic and nuclear fractions of these cells before and after 60 min of incubation with 10^{-7} M of dexamethasone. Although a shift of both hGRα and hGRβ took place from the cytosol to the nucleus after addition of the hormonal ligand, the hGRα-to-hGRβ ratio in HeLa cells remained ≅0.2, independently of the fraction or treatment. This

TABLE 1. Quantities of immunoreactive hGRα and hGRβ in HeLa cells and their fractions[a]

	hGRα (fmol/μg protein)	hGRβ (fmol/μg protein)	Ratio α/β[b]
Whole cell	240.00	1032.00	0.23
Cytosol − dexamethasone	153.00	711.00	0.22
Cytosol + dexamethasone	120.00	561.00	0.21
Nucleus − dexamethasone	36.00	258.00	0.14
Nucleus + dexamethasone	54.00	288.00	0.19

[a]Data from one representative of two experiments.

[b]There was no significant difference in the α/β ratios in HeLa cells or their fractions in either experiment.

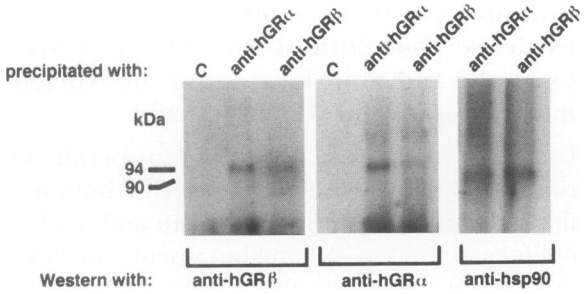

FIG. 5. Western blots analysis of hGRα, hGRβ, and hsp90 performed using total protein extracted from HeLa cells after pre-adsorption with nonimmune rabbit IgG and anti-hGRα and -hGRβ antibodies, and precipitation with protein A-Sepharose

Aliquots (100 μl) were incubated with nonimmune rabbit IgG and anti-hGRα-, and -hGRβ-specific antibodies and precipitated with protein A-Sepharose, as described in Materials and Methods and shown at the top. After washing, the immunoadsorbed/precipitated receptors were solubilized in SDS-sample buffer, each sample was divided into aliquots and resolved by SDS-PAGE. After transfer to nitrocellulose membranes, samples were immunoblotted with anti-hGRα (left panel), -hGRβ (middle panel) and -hsp90 (right panel). Anti-hGRβ antibody precipitated hGRα and hsp90, while anti-hGRα antibody precipitated hGRβ and hsp90. The position of identified immunoreactive bands are indicated against molecular weights standards.

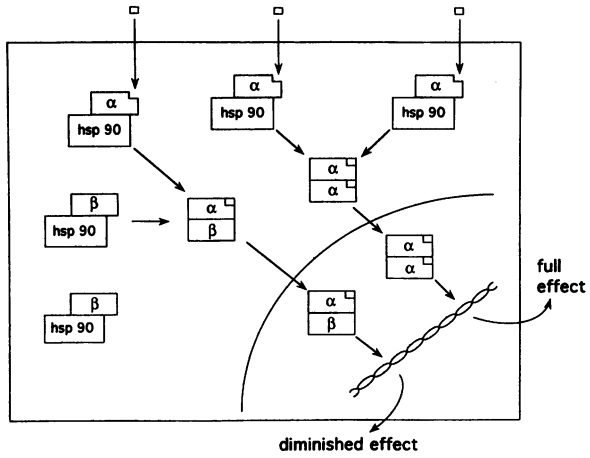

FIG. 6. Putative mechanism of action of the hGRβ isoform

hGRβ, like unliganded hGRα, is anchored to hsp90 as part of a cytoplasmic heterooligomer. In the presence of glucocorticoid, hGRβ heterodimerizes with ligand-bound GRα and translocates into the nucleus as a heterodimer. There, it interacts with GREs and acts as a dominant negative inhibitor of the classic receptor, decreasing the effects of glucocorticoids on modulating gene transcription.

suggests that both isoforms translocated into the nucleus from the cytoplasm in parallel and at roughly equal proportions.

Evidence That the hGRβ Isoform Is Part of a Cytosolic Hetero oligomer Which Contains hsp90 and That hGRβ Probably Heterodimerizes with hGRα

To examine the hypothesis that cytosolic hGRβ is bound to hsp90 and to test the ability of the hGRα and hGRβ to form heterodimers, we performed appropriate immunoadsorption/precipitation studies using anti-hGRα and anti-hGRβ antibodies followed by SDS electrophoresis and Western blot studies. Figure 5 shows the Western blots of these immunoprecipitates indicated at the top, immunoblotted with anti-hGRα (Panel A), -hGRβ (Panel B), and -hsp90 (Panel C) polyclonal antibodies indicated at the bottom. The anti-hGRα antibody adsorbed both hGRα and hGRβ, as well as hsp90; similarly, the anti-hGRβ antibody precipitated both hGRα and hGRβ, as well as hsp90. These results show clear interaction between each hGR isoform and hsp90 and potential interaction with each other.

A Simplified Model of How Heterodimerization of GRα and GRβ Might Modulate the Glucocorticoid Sensitivity of Target Tissues

Figure 6 shows the classic model of GRα "transformation" induced by hormone binding, including ligand binding, liberation of the cytoplasmic hormone-receptor complex from a heat shock protein complex, formation of putative hGRα-hGRα homodimers, translocation of this complex into the cell nucleus, interaction with nuclear elements, and modulation of transcription of glucocorticoid-responsive genes. We found evidence that the hGRβ isoform is also present in the cytoplasm in complex with hsp90. In the presence of glucocorticoid, we hypothesize that hGRβ heterodimerizes with ligand-bound GRα and translocates into the nucleus as a heterodimer, which interacts with GREs and acts as a dominant negative inhibitor of the classic receptor, decreasing the effects of glucocorticoids on their target tissues.

DISCUSSION

In Western blots of human tissues and HeLa cell extracts, a specific protein band of approximately 94 kD was seen, with the respective antibodies reacting with each of the hGR isoforms. To our knowledge, this is the first time that the natural existence of hGRβ *protein* has been demonstrated in human cells and tissues. By our quantitative Western blot assay, the levels of the β-isoform were approximately 1- to 5-fold higher than those of the α-isoform, proportions that would allow this antagonist protein to play a physiologic role in some tissues, by modulating the sensitivity of these tissues to glucocorticoids (7,8). The hGRα-to-hGRβ protein ratio was inverse to the previously computed ratio of the corresponding isoform specific mRNAs amplified by quantitative RT-PCR (8). This discrepancy between the protein and mRNA data might be artifactual or may be explained by differential mRNA and protein stabilities and/or by differential transcription/ translation rates between the two mRNA species.

Several other dominant negative physiologic or pathologic isoforms of nuclear receptors have been previously described, including an aberrant hGR causing severe generalized glucocorticoid resistance (19) and the naturally occurring thyroid hormone receptor α_2 and progesterone receptor A (20–25). However, the physiologic/ pathophysiologic tissue concentrations and roles of the latter naturally expressed isoforms have not been clearly defined as yet.

In the absence of ligand, the majority of specific hGRα and hGRβ immunoreactivity was present in the cytoplasm of the HeLa cells in a distinct subcellular distribution. This was demonstrated in the Western blots of subcellular fractions of our HeLa cells as well as in our immunocytochemistry stainings. While previous studies demonstrated that HeLa cells contained high-affinity, saturable glucocorticoid-binding sites (26), chronic culturing of HeLa cells might have resulted in changes of the GRs. Therefore, we performed binding studies, which confirmed the earlier findings. These data suggest that hGRβ, like the unliganded hGRα, is retained in the cytoplasm as a component of a cytoplasmic heterooligomer (26–28). One might expect this, since like the hGRα the hGRβ isoform despite its C-terminal "truncation" contains the sequences that are essential to form complexes with cytoplasmic chaperone molecules. This was supported by the immunoadsorption/precipitation of hsp90 with the anti-hGRβ antisera.

Apparently, the hormonal ligand liberates hGRα from the hsp heterooligomer, thereby uncovering nuclear localization signals (NLS), such as NLS1, which is located in the hinge region of the receptor, next to the C-terminal side of the DNA-binding domain, and NLS2, which is within the ligand-binding domain (10,29). The NLSs permit interaction of the hormone-receptor complex with nuclear pore proteins and passage into the nucleus through the pore. Thus, in the presence of dexamethasone there was the expected increase of the nuclear-to-cytosolic hGRα ratio, demonstrated both by Western blots and by immunocytochemistry. Interestingly, however, under the same conditions and using the same methods there was a similar and parallel increase of nuclear-to-cytosolic hGRβ ratio. Since hGRβ does not bind ligand, we hypothesize that this isoform is probably released from the hsps by heterodimerizing with hGRα receptors already liberated with the assistance of the ligand. Likewise, ligand-activated hGRα receptors might interact with uncomplexed hGRβ-isoforms in a steady state of reaction with the hsps. Indeed, using cross-immunoadsorption/precipitation studies, we demonstrated that the hGRβ isoform was bound to hsp90 and/or to hGRα.

We summarize the proposed model in Fig. 6. This model presupposes the presence of a series of dynamic equilibria between hGRα and its ligand, hsp90, itself etc., as previously proposed for the progesterone receptor (30), along with a parallel dynamic equilibrium between hGRβ and hsp90. In contrast to our data, in their recent study Oakley et al. suggested that hGRβ was non–hsp90-binding and, hence, nuclear (8). This conclusion was based on the findings of "hGRβ immunoreactivity" in the nucleus of COS-1 cells transfected with hGRβ cDNA. This difference might be explained by the use of a different cell type and an artificial transfection system or by the fact that this group employed an antibody directed against the N-terminal portion of the receptor, which can recognize C-terminally truncated polypeptides. Such truncated polypeptides could have easily translocated into the nucleus since they would not normally bind to hsp90. Also, it is possible that the binding of hGRβ to hsp90 is not as tight as that of hGRα, favoring non–ligand-dependent translocation of this isoform into the nucleus. Further studies will allow clarification of this issue.

We suggest that changes in the alternative

splicing rate of the human GR gene leading to abnormally low expression of hGRα and high expression of hGRβ might participate in the development of generalized or tissue-specific glucocorticoid resistance, while, conversely, abnormally high expression of hGRα and low expression of hGRβ might lead to generalized or tissue-specific glucocorticoid hypersensitivity states (31,32). The former may present with psychiatric, endocrine, or autoimmune manifestations, the latter with metabolic and/or cardiovascular manifestations (33). We have obtained strong preliminary evidence that the hGRα-to-hGRβ ratio is markedly decreased (~50-fold) in cultured lymphoblasts from patients with glucocorticoid resistant asthma type II, a severe form of early life asthma, which is very poorly responsive to glucocorticoids (34,35), suggesting that hGRβ may indeed be directly involved in human pathophysiology. The molecular defect(s) leading to this change remains to be examined.

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