Evidence That the β-Isoform of the Human Glucocorticoid Receptor Does Not Act as a Physiologically Significant Repressor*

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Katrin Hecht‡, Jan Carlstedt-Duke‡, Pontus Stierna§, Jan-Åke Gustafsson‡, Mikael Brönnegård¶, and Ann-Charlotte Wikström‡‡

From the Departments of ‡Medical Nutrition, §Otorhinolaryngology and ¶Pediatrics, Karolinska Institute, Huddinge University Hospital, Novum, S-141 86 Huddinge, Sweden

Alternative splicing of the human glucocorticoid receptor (hGR) primary transcript generates two receptor isoforms, hGRα and hGRβ, with different carboxyl termini diverging at amino acid 727. By reverse transcriptase-polymerase chain reactions it was previously demonstrated that the hGRβ message had a widespread tissue distribution. To demonstrate the presence of hGRβ as protein we produced specific rabbit antisera to hGRβ, as well as a hGRβ-specific mouse monoclonal IgM antibody, by peptide immunizations. By SDS-polyacrylamide gel electrophoresis and Western immunoblotting we showed that hGRβ is endogenously expressed at the protein level in HeLa cells and human lymphatic leukemia cells. Using an antibody directed against an epitope shared by both isoforms we showed a relatively lower expression of the hGRβ form. We also showed that hGRβ bound to hs90 by immunoprecipitation of in vitro translated hGRβ in reticulocyte lysate with hs90-specific antibodies, a coprecipitation occurring also in the presence of dexamethasone. We could not demonstrate that hGRβ inhibited the effects of dexamethasone-activated hGRα on a glucocorticoid-responsive reporter gene. In conclusion, low hGRβ expression levels and hGRβ-hs90 interaction maintained in the presence of ligand and lack of inhibition of hormone-activated hGRα effects challenge the concept of the hGRβ isoform as a proposed dominant negative inhibitor of hGRα activity.

The cloning of the full-length human glucocorticoid receptor (hGR) in 1985 predicted the existence of two receptor isoforms differing at their carboxyl termini (1). Characterization of the genomic structure of the hGR gene suggested two alternative exons for the COOH-terminal part of GR and that alternative splicing of exons 9a and 9b was the mechanism responsible for generating the two receptor isoforms (2). However, Oakley et al. (3) have recently shown that the hGR gene is processed as one single exon 9 containing both exon 9a and 9b. Both hGR isoforms share the same amino acid sequence through amino acid 727 but diverge beyond this position with hGRα having an additional 50 amino acids and hGRβ with an additional non-homologous 15 amino acids.

Human GR in similarity to the other members of the steroid receptor superfamily consists of a poorly conserved amino-terminal region containing a major transactivation domain responsible for gene activation, a highly conserved cysteine-rich central DNA-binding domain, and a relatively well conserved carboxyl-terminal domain important for hormone binding (4). In addition to steroid binding, the ligand-binding domain also has a number of other functions including dimerization, heat shock protein 90 (hs90) binding and transactivation (5). Most previous studies have not distinguished between hGRα and hGRβ and have mainly taken into consideration hGRs which is widely expressed and functions as a ligand-dependent transcription factor. Without making any distinction between hGR isoforms, earlier studies could be summarized to have shown that hGR resides both in the cytoplasm and in the cell nucleus and forms a heteroligomeric complex containing one steroid binding hGR and several non-steroid binding components (6), including a dimer of hs90 and monomers of hs70 and hs56. Hs90 has been suggested to maintain hGR in a conformation that is unsuitable for DNA (GRE) binding but favorable for ligand binding. Once hormone binds to the receptor, a conformational change of the receptor results in the dissociation of hs90 and some of the other associated proteins. Upon ligand binding GR is preferentially located in the nucleus, where it binds as a homodimer to GREs usually localized in the promoter regions of glucocorticoid-responsive genes (7). It has also been demonstrated that hGR, in addition to either enhancing or repressing transcription of a specific gene by binding to its promoter or to further upstream regions, may modulate gene expression by interacting with other transcription factors such as AP-1 (8) and NF-κB (9).

Only a few recent studies have addressed the function of the β isoform of hGR (3, 10, 11) and thus many aspects of the possible physiological role of hGRβ remain to be further elucidated. Oakley et al. (3) have shown that hGRβ does not bind dexamethasone or RU486 (3). hGRβ has been assigned a role as a dominant negative inhibitor of hGRα activity (3, 10). In the absence of hGRα, however, hGRβ seems to be transcriptionally inactive (3), although it can bind to a GRE as demonstrated in a gel mobility shift assay. The presence of hGRα and β at the mRNA level has also been demonstrated (3, 10) and a relatively lower amount of hGRβ message (0.2–0.3% of total GR mRNA) has been suggested (3). A recent study by de Castro et al. (11) has implicated that hGRβ binds hs90, but has not demonstrated this in a direct fashion. In the same study this group also demonstrated hGRβ protein in various tissues. However,
the relative levels of hGRb as compared with hGRa remain to be unequivocally established. Reports of the intracellular localization of hGRb are somewhat conflicting and it also remains to be established under which conditions, normal and/or pathological, glucocorticoid-dependent repression or activation of gene transcription may be modulated by the interaction of the two hGR isoforms. The exact mechanism of such an effect also warrants further study.

To demonstrate the presence of hGRb as an expressed protein and to enable further studies of its function, we have performed peptide immunizations of rabbits and mice and produced specific rabbit antisera to hGRa and hGRb as well as an hGRb-specific monoclonal IgM antibody. We confirm the expression of specific hGRb transcripts in several human tissues and extend these findings to the demonstration of the expression of the hGRb protein in two cell lines. We also show a faster relative mobility of hGRb in SDS-PAGE/Western blotting as compared with hGRa. Furthermore, our data indicate a lower relative amount of hGRb protein expression as compared with hGRa. Finally, we demonstrate that hGRb can bind directly to sp,900, that this binding is not affected by ligand in the same way as hGRa-hsp90 binding and, based upon cotransfection studies in COS-7 cells, we challenge the current belief that hGRb is a general, negative modulator of hGRa function.

**EXPERIMENTAL PROCEDURES**

**Screening of a Human cDNA Library by PCR—**A cDNA library panel (CLONTECH) of 5 different human 5′-Stretch Plus™ cDNA libraries was screened by PCR for hGRb specific expression. The sense primer corresponded to the α and β common exon 8 hGR sequence bp 2219–2238, *i.e.* 5′-AGCTAGGAAAAAGCATTGTC-3′. The specific hGRb primer corresponded to the exon 9β sequence bp 2317–2335 generating the antisense primer 5′-CTGGTTTTAACCACATAAC-3′. PCR reactions were performed in the presence of 3 μl of the primer set, 3 mM MgCl2, 0.5 μg of DNA, 12.5 nM nucleotide mixture, and 0.5 units of Taq polymerase (Promega). After a 2-min denaturing step at 95 °C, 28 PCR cycles were run with an annealing temperature of 45 °C for 1 min and an extension temperature of 72 °C for 2 min. A PCR product of approximately 117 bp was generated in all tested tissues, *i.e.* heart, pancreas, lung, liver, brain, placenta, kidney, and skeletal muscle (data not shown).

**Plasmid Construction and Plasmids Used for in Vitro Transcription/Translation—**Plasmid pGem7Sp6hGRa (RiboProte System, Promega) contained the full-length human GR α isoform under control of the SP6 promoter (generously provided by Dr. Sam Okret, Karolinska Institute). For construction of plasmid pGEM7Sp6hGRb, a ClaI-XbaI fragment was amplified from pRSShGRb (kindly provided by Dr. R. Evans, Salk Institute, La Jolla, CA) by PCR. The NH2-terminus primer, GRI, 5′-CCATCGATAAATTGGAGA-3′, contained an internal ClaI site corresponding to bp 1525 within the GR gene (1) and the COOH-terminal antisense primer, GR8, 5′-GCTCTAGACGGTCGGGATACACATACA-3′, corresponding to bp 2606–2627 contained an added 5′ XbaI site (1). The GRI-GR8 fragment was amplified by PCR using Vent-Polymerase (New England Biolabs) and the following program: 25 cycles of 45 s at 94 °C, 60 s at 44 °C, 90 s at 72 °C, and a final elongation step of 10 min at 72 °C. The amplified segment was subcloned as a ClaI-XbaI fragment into Bluescript (Stratagene), resulting in plasmid pS8-1 and subsequently sequenced. pGem7Sp6hGRb was finally constructed by replacing the COOH-terminal ClaI-XbaI fragment in pGem7Sp6hGRa (RiboProte System, Promega) with fragment pS8-1. Plasmid pRShGRbΔSaIII was constructed by cutting pRSShGRb with SaII, at a unique restriction site within the r1 sequence in the GR-coding region. The protruding ends were blunt ended by mung bean nucleases treatment (covarying 20 U/ml) and a calf intestine phosphatase from R 131. The truncated protein produced from this construct corresponded to the NH2 terminus of the hGR and was 168 amino acids long, with the last 37 amino acids differing from the wild type protein. This protein did not activate the GRE-dependent reporter gene used in our transfection experiments. The reporter plasmid pSALP contained the gene for secreted placental alkaline phosphatase under the control of the GR-inducible MMTV promoter. Plasmid pAp contained the same reporter gene under control of a non-inducible promoter. Both plasmids were kindly provided by KaroBio, Huddinge, Sweden. Plasmids used in transfection experiments were prepared from *Escherichia coli* XL-1 Blue on Sepharose columns (QIagen, KEBO, Sweden).

**Cotransfection and Transfection of COS-7 Cells—**Cotransfection cultures of COS-7 cells were grown in Dulbecco’s modified Eagle’s medium containing 4.5 mg/ml glucose; Life Technologies, Inc.) supplemented with heat-inactivated 10% fetal bovine serum, 2 mM glutamine, penicillin (5 units/ml), and streptomycin (50 μg/ml). Transfection was performed using DOTAP as a transfection agent according to the manufacturer’s recommendation (Boehringer Mannheim). Cells were grown in 35-mm dish plasmid pMT-GRa, pRSShGRb, and pRSShGRbΔSaIII, respectively, for 6 h. Cells were transfected by adding fresh medium containing dexamethasone (Sigma), 24 h after transfection. 48 h after dexamethasone induction, alkaline phosphatase activity was assayed in cellular supernatants. Concentrations of plasmids and dexamethasone are as indicated in the figures.

**Immunization of Alkaline Phosphatase—**Transient transfections were assayed for alkaline phosphatase activity. For this purpose, 1 ml of medium of induced cells was cleared by centrifugation, the supernatant was transferred to a fresh tube and incubated at 65 °C for 30 min and thereafter reincubated. 250 μl of the supernatant was added to 50 μl of alkaline phosphatase assay solution (5 μl/ml Sigma 104 phosphatase substrate in 1.12 mM NaCl, 4 mM MgCl2, 0.4 mM Tris-HCl, pH 10) in microtiter plates incubated at room temperature. Enzymatic activity was monitored by following the rate of change of absorption of 2-nitrophenylphosphate at 405 nm.

**Immunization—**Peptides were selected based on the predicted antigenicity or based on unique sequences of the two hGR receptor isoforms. Peptide E17P corresponds to amino acids 510–526 in both hGRa and hGRb. Peptide K15P corresponds to the 15 COOH-terminal amino acids of hGRb and N15 to the 15 COOH-terminal amino acids of hGRa. The peptides were ordered from NeoSystem Laboratoire (Strasbourg, France). Peptides were conjugated to keyhole limpet hemocyanin by the one-step glutaraldehyde method. Rabbits were immunized with 50 μg of peptide-keyhole limpet hemocyanin initially in Freund’s complete adjuvant and thereafter in incomplete adjuvant or phosphate-buffered saline. Immunization was repeated monthly and after several boosters, the animals were bled and the antisera tested in enzyme-linked immunosorbent assay against the relevant peptide. Positive sera were further tested in enzyme-linked immunosorbent assay against hGR purified by immunofinity chromatography (cf. below) from HeLa cells or a baculovirus-expressed hGR (kindly provided from KaroBio, Huddinge, Sweden). Monoclonal antibodies were prepared according to standard procedures.

**Immunofinity Purification of hGR—**A monoclonal antibody previously generated against the rat GR with a known cross-reactivity to hGR (mAb5, also called 293 (14–16) was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia,Upsala, Sweden) according to a procedure described by the manufacturer. CytoS from approximately 5 ml of HeLa cell pellets, stored frozen at −70 °C, was prepared by homogenization in low salt buffer as described previously (16). The cytosol was slowly passed over the column, washed with 10 column volumes of EPC (1 mM EDTA, 20 mM sodium phosphate, pH 7.0, 10% (w/v) glycerol, 10 mM dithiothreitol) containing 50 mM NaCl and further with 10 column volumes of EPG with 1 mM NaCl. Human GR was then eluted with 0.1 mM sodium citrate buffer, pH 3.0. A similar procedure was used for hGR purification from plasmapheresis-enriched lymphocytes from patients with chronic lymphatic leukemia (a kind gift from Dr. Adam Smolovitz, Dept. of Hematology, Karolinska Hospital, Stockholm, Sweden) or from SF1 cells expressing hGR obtained from KaroBio (Huddinge, Sweden).

**In Vitro Transcription/Translation—**The radioiodinated glucocorticoid receptors GRa and GRb, respectively, were generated by *in vitro* transcription/translation of plasmids pHGr and pGEM7SP6hGRb, in the presence of [35S]methionine (Amersham) in rabbit reticulocyte lysate using a coupled *in vitro* transcription/translation kit (Promega) according to the manufacturer’s instruction.

**Immunoprecipitation—**Immunoprecipitation of [35S]methionine labeled glucocorticoid receptor-bound to hsp90 by monoclonal IgM antibodies against hsp90 (antibody 3G3, purchased from Affinity Bioreagents) and subsequent SDS-polyacrylamide electrophoresis was carried out as described previously (17), with an additional step to block nonspecific binding using 3% fat-free milk. The SDS gels were dried under vacuum and subjected to autoradiography. The same protocol
was used with polyclonal anti-hGR antibodies, with the modification that samples were blotted to nitrocellulose before autoradiography.

Western Immunoblotting— SDS-PAGE and Western blotting to nitrocellulose of immunofinity-purified hGR were performed according to standard procedures. To allow discrimination between hGRa and hGRb, strips were cut from single lanes of purified hGR subjected to SDS-PAGE and Western blotting, marked to ensure proper alignment, and probed with peptide-specific antibody and the relevant secondary antibodies coupled to alkaline phosphatase (DAKO-Patts, Denmark). Apart from primary antibodies described in this paper we also used antisera derived from patients with chronic lymphatic leukemia (CLL) was enriched for hGR using a monoclonal antibody directed against the NH2-terminal domain of the hGR isoforms and previously shown to be cross-reactive with the hGR NH2 terminus (15). Enriched cytosols were subjected to SDS-PAGE and Western blotting. Strips cut from one lane were probed with various antibodies. Panel A represents HeLa cell cytosol: lane 1, control without primary antibody; lane 2, mAb5; lane 3, mAb7; lane 4, rabbit anti-N15I (hGRb); lane 5, rabbit anti-E17P (hGRb); lane 6, preimmune serum from rabbit anti-N15I. Panel B represents CLL cell cytosol: lane 1 and 2, controls without primary antibody; lane 3, mAb7; lane 4, a mouse monoclonal antibody to N15I (hGRb); lane 5, rabbit anti-E17P (hGRb); lane 6, rabbit anti-N15I (hGRb). Antibodies against hGRb react preferentially with a band with a slightly lower molecular mass than antibodies directed against both hGRa and hGRb. hGRa migrates at molecular size ~ 94,000.

RESULTS

We confirmed that hGRb is expressed at the mRNA level in human tissues by PCR screening of a commercial human cDNA library panel using an exon 9b-specific primer together with a common internal GR primer corresponding to an exon 8-specific sequence. With all tissues investigated a PCR product corresponding to the expected size, 117 bp, was generated indicating the widespread presence of hGRb-mRNA transcripts (data not shown), similar to what has been demonstrated previously (3, 10). Primer specificity was tested by incubating the β-primers with plasmids containing the gene for hGRa and hGRb, respectively. No PCR product was obtained with the combination of β-primers/a-plasmid (data not shown).

To study the expression of the hGRb protein we produced and tested a number of anti-hGRb antibodies. Upon extensive immunization the three selected peptides, K15K, N15I, and E17P, corresponding to hGRa, hGRb, and a peptide common to both isoforms, elicited immune responses in rabbits. We also produced a monoclonal antibody to hGRb. To ensure that the antisera were specific for each isoform we tested for cross-reactivity. Proteins corresponding to each of the isoforms, hGRa and hGRb, were specifically expressed in reticulocyte lysate. Fig. 1, shows that antibodies raised against peptides only recognized the cognate receptor isoform, i.e. antibodies raised against the hGRb specific peptide K15K precipitated hGRb but not hGRa and antibodies raised against N15I precipitated hGRa but not hGRb.

The expression of hGRb was studied in HeLa and CLL cells. Cytosol from HeLa cells grown in culture or from lymphocytes obtained from plasmapheresis of patients with chronic lymphatic leukemia (CLL) was enriched for hGR using a monoclonal antibody directed against the NH2-terminal part of rat GR, mAb5, that cross-reacts with hGR and presumably should recognize both GR isoforms equally. Fig. 2 shows strips cut from a nitrocellulose filter after SDS-PAGE and Western immunoblotting. Strips from one large lane of cytosol were cut into several narrower strips and probed with antibodies and the relevant secondary antisera coupled to alkaline phosphatase. In Fig. 2A, hGR-enriched HeLa cell cytosol, when probed with a hGRb specific antibody raised against peptide N15I, was shown to contain a major immunoreactive band of slightly lower relative molecular weight than that of the predominant band, which was seen when the blot was probed with the antisera raised against peptide E17P, which was common to both hGR isoforms. A similar result for CLL cells is shown in Fig. 2B. These results clearly showed that hGRb was expressed at the protein level in two different cell types of human origin. The expression levels of hGRb, however, seemed to be significantly lower as compared with hGRa expression levels indicated by the fact that the intensity of the β-band was much lower when an antibody recognizing an epitope situated approximately 200 amino acids more NH2 terminally in the ligand-binding domain and common for both receptor isoforms was used. This was also suggested by experiments where mAb7, recognizing an epitope within the NH2-terminal domain of hGR, separate from the mAb5 epitope (15) at increasing concentrations and recognized an additional band of lower molecular weight, corresponding in size to the hGRb isoform, as tested on CLL cytosol enriched for both GR isoforms by mAb5 immunoaffinity chromatography as described above (Fig. 3).

Thus, the results in Figs. 2 and 3, although not directly quantitative, indicate a lower relative amount of hGRb protein in both HeLa and CLL cells.

To test whether hGRb is present as a heterocomplex with hsp90 and, if so, whether hsp90 could be released by addition of dexamethasone, in vitro translation of hGRa or hGRb in reticulocyte lysate in either the absence or presence of 100 μM dexamethasone was carried out. Aliquots of the lysate were divided and immunoprecipitated using either Sepharose-coupled monoclonal hsp90 antibodies (cf. below) or, as a control, unspecific IgM antibodies (Fig. 4, lanes 2, 5, 9, and 12). As indicated in Fig. 4, 35S-labeled protein was detected in lysate
FIG. 3. Relatively lower expression of hGRβ in HeLa cells. Cytosol derived from HeLa cells was enriched for hGR by immunoaf-finity chromatography using anti-ratGR antibody mAb5 directed against an epitope within the NH₂-terminal domain of GR, previously shown to be cross-reactive with the hGR NH₂-terminal (15). Enriched cytosol was subjected to SDS-PAGE and Western blotting. Strips cut from one lane were probed with increasing dilutions of mAb7. Lane 1, 10 μg/ml; lane 2, 2 μg/ml; lane 3, 0.4 μg/ml; lane 4, 80 ng/ml; lane 5, 16 ng/ml; and lane 6, 3 ng/ml. Note the rapid disappearance of the lower band, which in size corresponds to hGRβ, with decreasing antibody concentrations.

FIG. 4. Effect of dexamethasone on hsp90 binding of hGRs and hGRβ. [35S]Methionine-labeled hGRα and hGRβ were expressed in vitro, in rabbit reticulocyte lysate as described under "Experimental Procedures." Aliquots of the lysates in the absence of dexamethasone(hGRβ in lanes 1–3 and hGRα in lanes 4–6) were immunoprecipitated with either anti-hsp90 monoclonal antibody 3G3 (lanes 1 and 4) or with an irrelevant monoclonal IgM TEPC 183 antibody (lanes 2 and 5). The precipitates were separated by SDS-PAGE and coimmunoprecipitated hGRs and β, respectively, were visualized by autoradiography (cf. "Experimental Procedures"). Lanes 3 and 6 corresponds to the total input amount of labeled hGRα or hGRβ lysate, subjected to immuno-precipitation. Expression in the presence of 100 μM dexamethasone of hGRβ (lanes 8–10) and hGRα (lanes 11–13), in vitro was also performed. Lanes 10 and 11 represent the total input amount of labeled hGRβ and hGRα lysate. Aliquots of the lysates in the presence of 100 μM dexamethasone were immunoprecipitated with either anti-hsp90 monoclonal antibody 3G3 (lanes 8 and 11) or with an irrelevant monoclonal IgM TEPC 183 antibody (lanes 9 and 12). Lane 7 contained a non-radioactive molecular mass standard. The arrow indicates the position of hGRα at 85 kDa.

containing phGRα (lanes 6 and 13) and pGem7SP6hGRβ (lanes 3 and 10), respectively. hGRβ, which is 35 amino acids shorter than hGRα, runs slightly ahead, demonstrating the difference in size between the two hGR isoforms (85 and 81 kDa). Furthermore, both hGR isoforms were detected in immunoprecipi-tates using hsp90 antibodies, indicating that hGRs (Fig. 4, lane 4) as well as hGRβ (Fig. 4, lane 1) is bound to hsp90. To test whether hsp90 was released in the presence of hormone, hGRs or hGRβ were synthesized in reticulocyte lysate in the presence of 100 μM dexamethasone and immunoprecipitated with hsp90 antibodies in the presence of dexamethasone. As shown in Fig. 4 (lane 11), hGRα was not immunoprecipitated with hsp90 antibodies in the presence of dexamethasone. In contrast, hGRβ (Fig. 4, lane 8) was immunoprecipitated with hsp90 antibodies in the presence of dexamethasone. hGRβ coprecipitated with hsp90 to the same extent in both the absence (Fig. 4, lane 1) and presence (Fig. 4, lane 8) of dexamethasone.

FIG. 5. Induction of the reporter gene pSALP in COS-7 cells by hGRα and dexamethasone. COS-7 cells were transfected with a plasmid containing the dexamethasone-inducible MMTV promoter linked to an alkaline phosphatase reporter gene (pSALP) and simultaneously cotransfected with either pMT-GRα (filled circles) or pRShGRβ (open squares). The cells were treated with increasing concentrations of dexamethasone (0.05–100 nM) as indicated in the figure and secreted alkaline phosphatase activity was measured in the cell media as described under "Experimental Procedures."

To test whether hGRβ had any effect on the hormone-in-duced hGRα-mediated stimulation of gene expression, COS-7 cells were transfected with pSALP as a reporter gene and constant amounts of hGRα or hGRβ plasmid. In hGRα trans-fected cells, dexamethasone induced alkaline phosphatase activity in a dose-dependent fashion, whereas no induction was observed in hGRβ transfected cells (Fig. 5). In a second set of experiments, cells containing pSALP were transfected with increasing amounts of hGRβ and a constant amount of hGRα. As indicated in Fig. 6, panel A, alkaline phosphatase activity decreased accordingly. However, we obtained the same effect when cells were transfected with a constant amount of a GR independent reporter gene (pAP) and increasing amounts of hGRβ (Fig. 6, panel B), indicating that the hGRβ effect on the GR-inducible pSALP activity might be due to unspecific squelching and not to a specific inhibitory effect of hGRβ. A caveat in this set of experiments is that the total plasmid concentration was changing. Therefore to keep the total concentration of transfected plasmid DNA constant, cells were transfected with increasing amounts of hGRβ in the presence of hGRΔ. As shown in Fig. 7 (panel B), the problem with unspecific squelching in the GR independent reporter gene system was eliminated. However, we did not obtain a significant inhibitory effect of hGRβ on dexamethasone-induced hGRα-mediated stimulation of the pSALP reporter gene activity (Fig. 7, panel A).

DISCUSSION

Based on characterization of multiple receptor cDNA clones and receptor protein analysis by immunoblotting, where only hGRα was demonstrated, it was initially concluded that the predominant physiological form of hGR is hGRα (1). Results have recently been published demonstrating expression of hGRβ transcripts in a variety of human tissues and a potential role for hGRβ as a dominant negative inhibitor of hGRα activity (3, 10). In contrast to the well characterized hGRα isoform, very little is known about the hGRβ splice variant. In this report, we examined the expression of the hGRβ transcript and protein, association of hGRβ with hsp90 and physiological function of hGRβ. By PCR we confirmed that hGRα and hGRβ mRNA transcripts were co-expressed in several human tissues.

We have previously produced anti-rat GR antibodies that
cross-react with hGR and recognize epitopes in the amino-terminal domain, thus recognizing both the hGRα and hGRβ (14). In this report, we have produced isoform-specific anti-hGRα and hGRβ polyclonal antibodies in rabbits, which are noncross-reactive in immunoprecipitation experiments and which specifically recognize the hGRα and hGRβ proteins in Western blotting. In this study we also produced a monoclonal antibody against hGRβ, raised against the 15 unique COOH-terminal amino acids of hGRβ here called peptide N151. This antibody also recognizes a specific immunoreactive band in CLL cell cytosol (Fig. 2B) as well as in HeLa cell cytosol (not shown), demonstrating that hGRβ was expressed at the protein level in human cells. As compared with hGRα, hGRβ expression levels seemed to be significantly lower, as indicated by results using two different antibodies recognizing epitopes common for both isoforms, one in the NH2-terminal part of hGR and the other in the ligand-binding domain, approximately 200 amino acids NH2 terminally of the diverging point of hGRα and -β. A low hGRβ expression is in better agreement with a recent report by Oakley et al. (3), where hGRβ mRNA levels are estimated to be only 0.2–0.3% of total mRNA. These data and ours indicate that hGRβ may not necessarily be of significant importance under normal physiological conditions. However, a recent study by de Castro et al. (11) suggests a high level of hGRβ protein expression, in most cases exceeding hGRα expression, based on quantitation in Western blotting experiments, using peptides coupled to albumin to create standard curves. It is unclear to what extent coupling efficiency for the different peptides is controlled in this experiment and whether a quantitative comparison between the two isoforms in this fashion really is valid. Furthermore, we noted that the antisera raised by de Castro et al. (11) did not differentiate between the sizes of the two hGR isoforms. We believe that the use of an antibody that recognizes a common epitope in the two hGR isoforms, as described in this paper, is better suited for quantitative comparison of the isoforms as no difference in affinity to this epitope between isoforms is to be expected in Western blotting. This enables a direct comparison of relative levels of the proteins. Whether there exists a varying expression of the different hGR isoforms in normal tissues as well as in pathological tissues remains to be further studied.

In addition to steroid binding, the ligand-binding domain also harbors other functions including dimerization, hsp90 binding, and transactivation (6). It is well established that the hGRα receptor isoform translocates from the cytoplasm to the nucleus in a hormone-dependent manner and that, in the absence of hormone, the association of hsp90 with hGRα appears to inactivate the nuclear localization signal (18, 19). The hormone-dependent dissociation of hsp90 from hGRα is probably important in the nuclear translocation of hGR. In this report we showed that hGRβ is also associated with hsp90, but in contrast to hGRα still maintains the hsp90 association in the presence of ligand. Receptor derivatives of rat GR terminating at amino acids 766 (hGR 748) and 671 (hGR 653) were found to coprecipitate together with hsp90, whereas further truncation at the COOH-terminal end interfered with this interaction (20). Thus the site of hsp90 interaction appears to lie within a common region of the two hGR isoforms located at least 75 residues N-terminal of the diverging point of hGRα and hGRβ. Relating to the involvement of hsp90 in determining the intracellular localization of GR, a recent report by Oakley et al. (3) demonstrates that hGRβ resides primarily in the nucleus of transfected cells independent of hormone treatment. However, this is in contrast to another study showing essentially the same distribution pattern for hGRα as for hGRβ (11), i.e., the intracellular localization of hGRβ and its relation to hsp90 interaction call for further studies.

In our experimental system, and in contrast to previous reports (3, 10), we were not able to demonstrate that hGRβ inhibits the effect of hormone-activated hGRα on a glucocorticoid-responsive reporter gene in COS-7 cells. In cotransfection experiments, using the reporter gene pSalP containing an
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MMTV promoter positively regulated by glucocorticoids and hGRα, alkaline phosphatase activity was clearly induced by dexamethasone in a dose-dependent manner, whereas no induction was obtained in cells transfected with pSALP and hGRβ. When COS-7 cells were transfected with a constant amount of hGRα-plasmid and increasing concentrations of hGRβ-plasmid, hGRα-mediated activation of the MMTV promoter was inhibited. However, the increasing expression of hGRβ inhibited a glucocorticoid-independent constitutive reporter gene to the same extent, indicating that this effect was due to nonspecific squelching. When hGRα and hGRβ were expressed in the same cell and transfected DNA was kept constant by adding the truncated pRSV-GRβ∆SalI plasmid, we did not obtain a significant hGRβ inhibition of glucocorticoid-induced hGRα-mediated activation of the MMTV promoter. Thus, we conclude that the suggested hGRβ-mediated repression of hGRα is not a universal phenomenon and also that the interaction between hGRα and hGRβ may be more complex than previously suggested and warrants further studies. In the case of repression occurring in systems other than ours, it also remains to be determined to what extent hGRα/hGRβ-heterodimers or hGRβ/hGRβ-homodimers participate in the occupation of GRE sequences. In addition, interactions with other steroid hormone receptors and other proteins and transcription factors, such as AP-1 (8) and NFκB (9), may further contribute to the complexity of hGRα and hGRβ regulation of gene expression.

Studies of progesterone receptor isoforms in different animal models have identified variations in the levels of progesterone receptor-A and -B as a consequence of endocrine manipulations as well as during development (21). Despite the issues raised above regarding hGRα-hGRβ interaction, the possible resulting effects on specific gene expression and the conflicting data with regard to the absolute and relative levels of hGRα and hGRβ expressed in various human tissues, it may still be possible that during specific circumstances an altered ratio of these GR receptor isoforms may result in an alteration of hormonal responses.

In conclusion, there are a number of important issues yet to be addressed with regard to the physiological significance of hGRβ as a modulatory receptor isoform. Studies by Oakley et al. (3) and de Castro et al. (11) demonstrate that the hGRβ protein indeed is expressed in several tissues at the mRNA level. Based on experiments using antibodies detecting both the hGRα and hGRβ isoform, however, GRα was suggested as the major form expressed in our system. We also found that hGRα was associated with hsp90, and our study indicated that ligand does not result in a significant release of hGRβ from hsp90. These results and the fact that hGRβ did not have a dominant negative action in a glucocorticoid-driven reporter gene system, but rather a nonspecific squelching effect, warrant further studies of the role of GR isoforms in human, and indicate that hGRβ, under normal physiological conditions probably does not have a significant function at observed expression levels.

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