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Overexpressed glucocorticoid receptor negatively regulates gene expression under conditions that favour accumulation of non-hormone-binding forms of the receptor☆

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Abstract

Previous reports have suggested that the native hormone-responsive glucocorticoid receptor is a heterocomplex with hsp90 and that the receptor constantly cycles between the hormone-responsive and an inactive state, with complex assembly and turnover being driven by hsp70 and hsp90, respectively. Since hsp70 appears to be titrated in cells that transiently overexpress the receptor, assembly intermediates may accumulate when more receptor is produced than can be assembled to hormone-responsive complex. Comparison of receptor protein and hormone-binding levels in extracts from transiently transfected COS-7 cells revealed the presence of non-hormone-binding receptor forms in addition to the native heterocomplex. The receptor was predominantly nuclear in the majority of the transfected cells even in the absence of hormone, with the DNA-binding domain (DBD) being necessary for nuclear localisation. Moreover, the unliganded receptor exhibited constitutive DNA-binding activity and reactivity towards antibodies against the hinge region where NLS1 is known to reside. By comparing fluorography to immunoblotting of two-dimensional SDS-PAGE of cross-linked [^{3}H]dexamethasone-mesylate-labelled receptor, we detected non-hormone-binding receptor species capable of binding DNA in vitro. In addition, using a constitutively active receptor mutant, we found that the overexpressed wild-type receptor was capable of repressing mutant-activated transcription of transiently and stably transfected reporter genes alike in a DBD-dependent manner.

Keywords: Glucocorticoid receptor; Overexpression; Transcriptional repression

1. Introduction

The glucocorticoid receptor (GR) is a segmented ligand-activated transcription factor containing an amino-terminal domain (NTD), a central DNA-binding domain (DBD) and a carboxy-terminal ligand-binding domain (LBD) repressing receptor-mediated activation of transcription in the absence of hormone. Compiled in the DBD are separate activities of DNA binding, DNA-induced homodimerisation and transcriptional activation. Additional transcription activation functions (AFs), the constitutively active AF-1 and the ligand-activated AF-2, are located in the NTD and LBD, respectively [1–3]. In addition to AF-2, the LBD harbours a hormone-dependent dimerisation function and a hormone-dependent nuclear localisation signal (NLS2), with a constitutively active NLS1 being located in the hinge region between LBD and DBD [4,5]. Moreover, the LBD contains binding sites for the 90 and 70 kDa heat shock proteins (hsp90 and hsp70, respectively), both of which are ATPases that function as protein chaperones binding unfolded proteins to protect them against aggregation and assist their folding to the native state ([6,7], reviewed in [8]). Previous reports have suggested that steroid hormone receptors (SHRs) constantly cycle between a hormone-responsive and an inactive state, with the ATPase activities of hsp90 and hsp70 believed to drive turnover and assembly, respectively, of the hormone-responsive state; and that in this state SHRs are heteropentamers that contain 2 mol of hsp90 per mole of receptor but lack hsp70 which, however, is necessary for hormone-responsive complex (HRC) assembly (HRCA) ([9–12], reviewed in [13]). SHR cycling is inhibited in the presence of hormone which is known to promote dissociation of the HRC and stabilisation of receptor homodimers which, in the case of GR bind to the glucocorticoid-responsive elements (GREs) of target genes

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to stimulate transcription in a manner that is dependent on a multitude of co-regulators ([2,4,14,15], reviewed in [16]). In addition to cycling between a hormone-responsive and an inactive state, SHRs constantly shuttle between the cytoplasm and the nucleus, with their nucleocytoplasmic distribution apparently reflecting receptor affinities for retention sites in either cellular compartment as well as rates of nuclear import and export. Notably, hsp90 and hsp70 have been implicated in SHR shuttling as well as cycling (reviewed in [17]).

Given that the endogenous heat shock factor (HSF) is activated in cells that overexpress the GR in the absence of hormone [18] as well as under conditions that favour sequestration of hsp70 by unfolded proteins [19], inhibition of HRCA and accumulation of assembly intermediates in such cells may be postulated. Moreover, since unliganded overexpressed GR binds to GRE in vitro [20], interference of the putative HRCA intermediates with transcription of glucocorticoid target genes may be postulated as well. In support of this reasoning, it has been reported that the GR of WCL2 cells, a stably overexpressed entity located in the nucleus and recovered in the cytosol in association with hsp70 [21], exhibits an intra-nuclear distribution in the absence of hormone that it is indistinguishable from that in its presence [22]. In this light, we looked for interference of the unliganded overexpressed GR with GRE-dependent activation of transcription by N525, a constitutively active mutant of GR [3]. We found that transient overexpression of GR in the absence of hormone results in accumulation of non-hormone-binding receptor forms capable of binding to DNA in vitro; and that, whether by enhancer binding, co-regulator squelching or both, the unliganded overexpressed GR negatively interferes with N525-mediated activation of transcription from GRE-dependent promoters. The data suggest that failure to recruit non-hormone-binding GR to HRCA can retain receptor in the nucleus in a DBD-dependent manner and convert it from an activator to a repressor of target gene transcription.

2. Materials and methods

2.1. Materials, constructs and antibodies

Unless specified otherwise, all materials used in the present study were from Sigma. Plasmid CH110 (β-galactocidase expression vector) was from Pharmacia. Expression vectors coding for hGR (pRShGRα) and hGR deletion mutants Δ9–385, Δ77–262, Δ262–404, Δ428–490 and Δ491–415 [1], were generously provided by Dr. R.M. Evans. The expression vector for the constitutively active rat GR mutant N525 [3] (comprises the 525 amino-terminal amino acids of rat GR) was kindly provided by Dr. K.R. Yamamoto. pHisGR (a 6× His-tagged hGR expression vector) was constructed by introducing a DNA sequence coding the amino acids MHHHHHHSS, immediately upstream of codon Met^1 of pRShGRα. Chloramphenicol acetyltransferase (CAT)-coding reporter vectors GRE-37Tk and 2GRE-37Tk [23] were kindly provided by Dr. G. Schütz. Rabbit antiserum EP against hGR amino acids G499–E597 has been described already [10]. It was used for immunoblotting at a dilution of 1:100. Rabbit antiserum HH against hGR amino acids A304–428 has been described also [10]. It was used for immunoadsorption at a dilution of 1:10. Mouse monoclonal antibody 2F8 was raised against a bacterially expressed chimera of the hGR peptide A304–428 and MS2 polymerase using standard methodology [24]. The chimera was prepared as previously described [10]. 2F8 was used for immunocytochemistry at a concentration of 250 μg/ml. Antiserum 10–26 against the hGR segment G10–V26 was prepared by immunising rabbits with the peptide GREENPSSVLAQERGDVC (corresponds to hGR sequence G10–V26 extended by one cysteine) coupled to keyhole limpet haemocyanin using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester and standard methodology [24]. While antiserum EP and antibody 2F8 were found to cross-react with GR from different species, antiserum 10–26 was found to react only with human GR; it was used for immunocytochemistry as well as immunoadsorption at a dilution of 1:10. The rabbit antiserum against hsp90 and hsp70 [25] was a generous gift from Dr. C.W. Anderson. It was used for immunoblotting at a dilution of 1:500.

2.2. Cell culture, immunocytochemistry, transfection and CAT assay

HeLa 1β5 cells, a clone of HeLa cells stably transfected with the 2GRE-37Tk CAT reporter, have been described [26]. HeLa, HeLa 1β5 and COS-7 cells were cultured in Dulbecco's modified Eagles medium with 0.45% (w/v) D-glucose (high-glucose DMEM) supplemented with 10% steroid-stripped fetal calf serum (ssFCS), 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. Immunocytochemistry of methanol/acetone and paraformaldehyde-fixed COS-7 cells was carried out using monoclonal antibody 2F8, biotinylated second antibody and peroxidase-labelled avidin as reported by Ylikomi et al. [5].

Unless specified otherwise, HeLa, HeLa 1β5 and COS-7 cells growing in 10 cm dishes were transiently co-transfected using the calcium phosphate co-precipitation method with 2 μg of 2GRE-37Tk, (omitted when using HeLa 1β5 cells), 5 μg of CH110 (to monitor transfection efficiencies), 0.5 μg of N525 and 10 μg of hGR or the empty expression vector. Eighteen hours after transfection, the cells were washed twice with phosphate-buffered saline (PBS) and incubated for 24 h with fresh medium containing 10% ssFCS. CAT activity was assessed as already described [26] using aliquots of cell extracts containing equal amounts of total protein (HeLa 1β5 cells) or β-galactosidase activity (all other cells).

2.3. GR labelling, cross-linking, immunoadsorption, binding to DNA and purification

Cytosol was prepared in buffer A (30 mM sodium phosphate, pH 7.8, 5 mM EGTA, 10 vol.% glycerol, 10 mM Na_2MoO_4, 0.2 mM phenylmethylsulphonylfluoride (PMSF), 1 mM leupeptin, 10 mg/ml soybean trypsin inhibitor) containing 2 mM dithiothreitol (DTT), unless specified otherwise. Preparation of the cytosol, covalent and non-covalent labelling of the GR in intact cells and in cytosol using [^{3}H]dexamethasone-21-mesylate (DM) or [^{3}H]triamcinolone acetonide (TA), respectively, cross-linking of the cytosolic GR with dithio-bis(succinimidyl propionate) (DSP) and receptor immunoadsorption using protein A–Sepharose CL-4B have been described [10]. To inactivate [^{3}H]TA binding by the receptor, cytosolic GR in DTT-free buffer A was incubated for 1 h at 4 °C with 0.1 mM sodium arsenite as reported by Stancato et al. [27].

To assess DNA binding of the overexpressed hGR, 100 μl aliquots of cytosolic receptor were incubated with rotation for 1 h at 4 °C with the pellet of a 100 μl suspension of 30% (w/v) DNA–cellulose in buffer B (20 mM Tris–HCl, pH 7.6, 1 mM EGTA, 10 mM Na_2MoO_4, 10 vol.% glycerol, 10 mM β-mercaptoethanol). Following centrifugation at 2500 × *g* for 3 min, the pellet was washed three times with 1 ml of buffer B. Proteins bound to DNA–cellulose were eluted with buffer B containing 30 mM $MgCl_2$. Receptor in the wash and the eluate of DNA–cellulose, with or without immunoadsorption using antiserum HH, was analysed by one- or two-dimensional SDS-PAGE and immunoblotting as described below. To assess GRE binding of the transiently expressed hGR the procedure of Rusconi and Yamamoto [2] was endorsed with minor modifications. Briefly, cytosolic receptor that was immunoadsorbed using antiserum 10–26 and then suspended in buffer B containing 2 mM $MgCl_2$, 50 mM NaCl and 1.25% (w/v) BSA, was incubated with rotation for 2–4 h at 4 °C with 10 ng of [γ-^{32}P]-end-labelled GRE (a 35 nt long *Bam*HI–*Pst*I fragment from GRE-37Tk), in the presence or absence of five-fold excess of unlabelled GRE, then washed with buffer B containing 0.03% (w/v) BSA and 100 mM NaCl and finally counted in a liquid scintillation counter. Alternatively, the procedure of Denis et al. [14] was adopted with minor modifications. Briefly, hGR immunoadsorbates were incubated on a rotator with *Pvu*II-restricted [γ-^{32}P]-end-labelled 2GRE-37Tk and washed as above, DNA bound to the immunoadsorbates was eluted with 17 mM $MgCl_2$ in buffer B, then precipitated with ethanol and finally analysed by agarose gel electrophoresis followed by autoradiography.

To purify 6× His-tagged hGR, cytosol from COS-7 transiently transfected with pHisGR was prepared in buffer C (20 mM HEPES, pH 7.9, 10 mM Na_2MoO_4, 1 vol.% glycerol, 10 mM β-mercaptoethanol, 0.2 mM PMSF, 1 mM leupeptin, 10 mg/ml soybean trypsin inhibitor), and mixed with rotation for 1 h at 4 °C with 10 vol.% settled Ni^{2+}-NTA-agarose (Qiagen). The settled resin was washed three times by centrifugation followed by suspension in 1 ml of ice-cold buffer C and then eluted three times with five volumes of ice-cold 0.25 M imidazole. Eluates were precipitated using TCA and then prepared for SDS-PAGE as described below.

2.4. SDS-PAGE, immunoblotting and fluorography

Proteins were analysed by discontinuous and continuous SDS-PAGE, using samples prepared in SDS sample buffer (3 M Tris–HCl, pH 8.9, 10% (w/v) SDS, 1.44 M β-mercaptoethanol) and in SDS/urea sample buffer (100 mM sodium phosphate, pH 7.0, 1% (w/v) SDS, 6 M urea, 1.44 M β-mercaptoethanol), respectively, as already described [10]. For two-dimensional SDS-PAGE, samples were analysed by continuous SDS-PAGE under non-reducing conditions, then the gel was incubated at 65 °C for 20 min in the presence of 3 vol.% β-mercaptoethanol and 10 mM DDT, and finally embedded on top of a discontinuous SDS-PAGE slab gel and subjected to electrophoresis under reducing conditions. Quantitative immunoblotting of the receptor protein using antiserum EP and [^{125}I]-labelled protein A has been carried out as already described [26]. Calibration curves were constructed with increasing amounts of [^{3}H]TA-labelled GR immunoadsorbed from the cytosol of HeLa cells. Fluorography of [^{3}H]DM-labelled receptor has been described [10].

2.5. Statistics

Unless stated otherwise, data are the mean ± S.E.M. of three or more independent experiments. Data were analysed using the SPSS 10.0 statistical package for Windows. [^{3}H]TA and [^{3}H]DM binding to GR, receptor binding to DNA–cellulose and N525-driven CAT expression in the absence or presence of co-transfected GR were compared using one-way ANOVA with a Tukey post-hoc test for multiple comparisons. Differences were considered statistically significant for values of $P < 0.05$.

3. Results

3.1. Non-hormone-binding receptor forms exist in cells that transiently overexpress GR in the absence of hormone

The level of receptor protein in COS-7 cells transiently transfected with 5 μg hGR expression vector per 10 cm culture dish, as quantified by immunoblotting of whole cell protein and normalised to the efficiency of transfection as determined by receptor immunocytochemistry, averaged ~300,000 molecules per cell in the absence of glucocorticoids (GCs), which is almost eight times the level of GR we have reported for HeLa cells [26]. Transfected and HeLa GR were labelled with [^{3}H]TA and [^{3}H]DM and receptor

protein in immunoadsorbates of equal hormone-binding activity was quantified by immunoblotting. Having observed that levels of receptor protein in hGR immunoadsorbates were consistently higher than those of HeLa GR, we immunoblotted hGR immunoadsorbates together with increasing amounts of immunoadsorbed HeLa receptor, as assessed by hormone binding (Fig. 1A, line plot and inset), to accurately quantify transfected receptor protein levels. Similar results were obtained whether transfected wild-type hGR or mutant Δ77–262 was analysed and whether antiserum HH or antiserum 10–26 was used to immunoadsorb the receptor. Analysis of Δ77–262 was given a preference over wild-type hGR in order to prevent endogenous GR of COS-7 cells from interfering with assessment of the level of expression of the transfected receptor. Fig. 1A shows that the amount of Δ77–262 protein was higher than predicted from the amount of [^{3}H]TA in Δ77–262 immunoadsorbates (open triangle). Treatment of the cytosol with sodium arsenite, which is known to block hormone binding to GR [27], nullified the amount of immunoadsorbed [^{3}H]TA (filled triangle), confirming that the hormone was specifically bound to Δ77–262. Similarly, Fig. 1B shows that the amount of receptor protein in similarly analysed immunoadsorbates of [^{3}H]DM-labelled Δ77–262 was higher than predicted from the amount of covalently bound hormone (open triangle). To prevent non-specifically labelled proteins from interfering with assessment of the level of receptor-bound [^{3}H]DM, this was quantified by densitometry of fluorographs of SDS-PAGE analysis of transfected receptor immunoadsorbates along with increasing amounts of immunoadsorbed [^{3}H]DM-labelled HeLa GR (line plot and inset). Cytosol treatment with sodium arsenite abolished covalent labelling of Δ77–262 (filled triangle), confirming that [^{3}H]DM was specifically bound to Δ77–262. Fig. 1C shows that the amount of transfected hGR capable of binding [^{3}H]TA was significantly lower than that of receptor protein ($P < 0.001$) but appreciably higher ($P < 0.01$) than that which was labelled with [^{3}H]DM, suggesting that it is unlikely that the non-hormone-binding receptor was generated by dissociation of [^{3}H]TA during immunoadsorption. The above data suggest that overexpression of hGR results in accumulation of non-hormone-binding forms of the receptor.

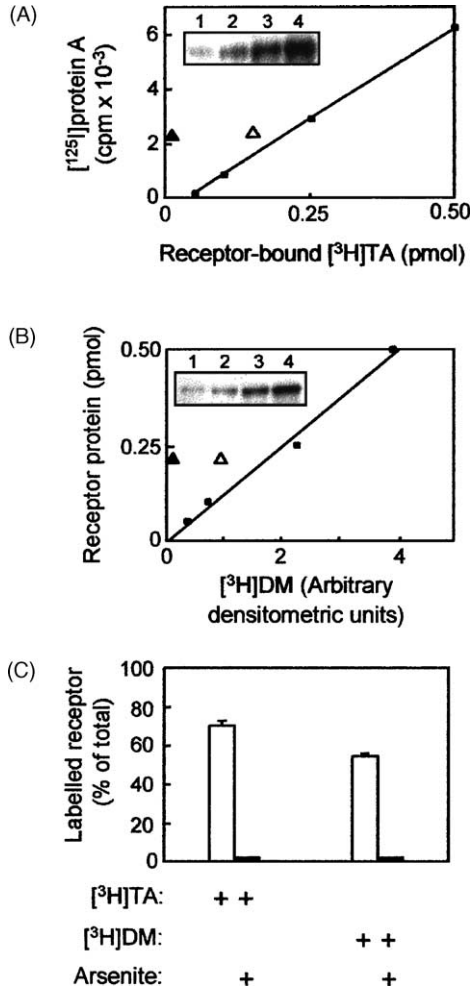

Fig. 1. Non-hormone-binding receptor forms are present in COS-7 cells overexpressing hGR. (A) Cytosol from COS-7 cells overexpressing hGR mutant Δ77–262 was treated (▲) or not treated (△) with sodium arsenite, labelled with [^{3}H]TA and subjected to immunoadsorption using anti-GR serum HH. Aliquots of receptor immunoadsorbates were either counted for [^{3}H]TA or immunoblotted using anti-GR serum EP and [^{125}I]-labelled protein A together with increasing amounts (■) of immunoadsorbed HeLa GR (inset) to quantify Δ77–262 protein (▲, △). (B) Alternatively, untreated (△) and arsenite-treated cytosol (▲) was labelled with [^{3}H]DM and receptor immunoadsorbates were either subjected to fluorography (inset) and densitometry to quantify Δ77–262-bound [^{3}H]DM, or immunoblotted as in panel A together with increasing amounts (■) of [^{3}H]DM-labelled HeLa GR to quantify Δ77–262 protein (▲, △). (C) [^{3}H]TA and [^{3}H]DM binding to Δ77–262 as normalised to the amount of receptor protein. DM, dexamethasone-21-mesylate; hGR, human glucocorticoid receptor; TA, triamcinolone acetonide.

3.2. De-repression of DNA binding and NLS1 functions of unliganded overexpressed GR

The unliganded transfected GR is usually found in the cytoplasm and is recovered in the cytosol as a non-DNA-binding entity [5,28]. As not expected, therefore, we found that in the absence of hormone the overexpressed hGR as well as mutants Δ77–262, Δ262–404 and Δ491–415 were predominantly located in the nucleus in the majority of the transfected cells irrespectively of whether methanol/acetone or paraformaldehyde was used to fix the cells and despite that the cells were growing in high-glucose medium in the absence of hormone and phenol red (Fig. 2 and data not shown). By contrast, DBD deletion mutant Δ428–490 was located in the cytoplasm, suggestive of DNA-dependent nuclear localisation of the overexpressed receptor.

We next tested whether the unliganded overexpressed receptor possess DNA-binding activity [8]. Fig. 3A shows that while the amount of unliganded HeLa GR that was retained by DNA–cellulose was much less than that which was retained following heat-induced transformation in the presence

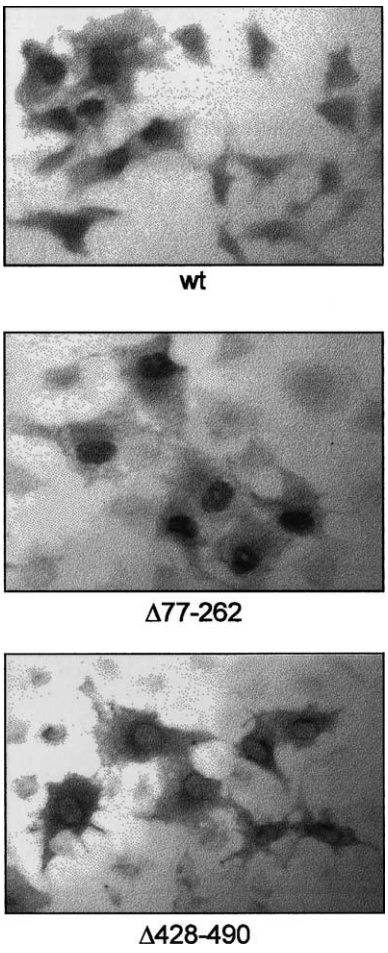

Fig. 2. DBD-dependent nuclear localisation of the unliganded overexpressed hGR. Paraformaldehyde-fixed COS-7 cells overexpressing wild-type (wt) hGR, mutant Δ77–262 (lacks AF-1) or mutant Δ428–490 (lacks the DBD) were probed using monoclonal anti-GR antibody 2F8, biotinylated second antibody and peroxidase-labelled avidin. AF-1, activation function 1; DBD, DNA-binding domain.

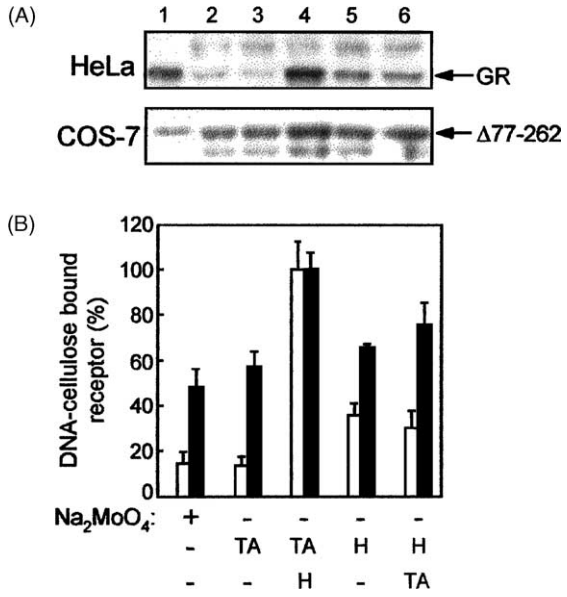

Fig. 3. De-repression of the DNA-binding function of the unliganded overexpressed hGR. (A) Cytosol from COS-7 cells overexpressing hGR mutant Δ77–262 and HeLa cells was prepared in the presence (lane 2) or the absence (lanes 3–6) of sodium molybdate. Equal amounts of cytosol were subjected to no treatment (lane 2), incubation with TA (lanes 3 and 4) followed by heating at 25 °C for 30 min (lane 4), or heating at 25 °C for 30 min (lanes 5 and 6) followed by incubation with TA (lane 6), and DNA–cellulose-bound receptor was immunoblotted using anti-GR serum EP and [^{125}I]-labelled protein A. Lane 1: Δ77–262 from COS-7 cells and endogenous GR from HeLa cells as purified by immunoadsorption using anti-GR serum HH. (B) Protein levels of DNA–cellulose-bound GR from HeLa cells (□) and Δ77–262 from COS-7 cells (■), as assessed by quantitative immunoblotting, were expressed relative to those binding to DNA–cellulose following thermal activation of the receptor in the presence of hormone (panel A, lane 4). H, heat; Na_2MoO_4, sodium molybdate.

of TA, retention of overexpressed hGR was high even before transformation. Quantitative immunoblotting analysis showed that, while the amount of unliganded HeLa GR retained by DNA–cellulose was 14.7 ± 5.4% (mean ± S.E.M.) of what was retained following heat-induced transformation in the presence of TA, the respective amount of unliganded overexpressed hGR was 48.0 ± 8.0% of the transformed (Fig. 3B). Transformation resulted in equivalent fractions of transfected and HeLa receptor binding to DNA–cellulose (37.4 ± 2.6 and 37.5 ± 4.5% of the total receptor, respectively). Similarly, DNA binding of TA-labelled overexpressed hGR at 4 °C was significantly higher ($P < 0.05$) than observed for HeLa GR, and this was the case also following heat treatment in the absence of hormone (Fig. 3B). The percentage of unliganded transfected hGR binding to DNA–cellulose over that of unliganded HeLa GR (33.3%) closely matches the percentage of the receptor that is not capable of binding [^{3}H]TA (29.8%) (Fig. 1C), implying that the two fractions are the same.

To further study the overexpressed hGR we performed immunoadsorptions using antiserum EP that is known not to react with the HRC of HeLa GR (Mitsiou et al., in preparation). We found that while the amount of unliganded HeLa GR that was immunoadsorbed with a 1:50 dilution of antiserum EP from cytosol prepared in the presence of sodium molybdate (stabilises the HRC) was 8% of that prepared in the presence of 0.5 M NaCl (dissociates the HRC), the respective percentage of overexpressed hGR was as high as 50%; and that the amount of specifically bound hormone in the EP immunoadsorbates of hGR was negligible. Since antiserum EP was raised against hGR peptide G499–E597 and the minimal hsp90-binding segment of the receptor is reportedly located between residues 550–598 [8], these findings may be taken to suggest that NLS1 (R479–K498) is not occluded by hsp90 in the non-hormone-binding forms of hGR. Given that a productive association of GR with hsp90 is necessary for occlusion of NLS1, repression of DNA binding and maintenance of the steroid-binding activity of the receptor [6–8], the inference from the data of Figs. 1–3 is that the non-hormone-binding receptor species likely possessing a non-occluded NLS1 and those possessing constitutive DNA-binding activity are the same entity(ies).

3.3. The major non-hormone-binding species of unliganded overexpressed GR is a DNA-binding monomer of the receptor

We examined whether the non-hormone-binding hGR species lack hsp90 or interact with the chaperone non-productively. We found that, in addition to hsp90, hsp70 was present in immunoadsorbates of the unliganded overexpressed hGR (not shown). To demonstrate that the chaperone was bound to the receptor rather than to the immunoadsorbent [9], His-tagged hGR that was overexpressed in the absence of hormone was purified from cytosol using Ni^{2+}-affinity chromatography and then subjected to immunoblotting. Fig. 4A shows that the His-tagged receptor (lane 2) was associated with hsp70 as well as hsp90 (lane 4) and that affinity-purified material from COS-7 cells overexpressing unliganded non-tagged hGR contained neither the receptor (lane 1) nor the chaperones (lane 3). Given that an association of hsp70 with the native hormone-responsive GR is reportedly possible [7], we cross-linked overexpressed hGR labelled with [^{3}H]DM in the cell (Fig. 4B, lane 1) or in the cytosol (lane 2) with DSP and analysed receptor immunoadsorbates using non-reducing SDS-PAGE and fluorography [10]. We detected no complexes other than those of apparent molecular masses of 350 and 290 kDa, previously reported to correspond to the native HRC and the product of dissociation of the immunophilin component of this complex, respectively [10]. Since these complexes are known to lack hsp70 [9,10], we examined whether the chaperone was associated with the non-hormone-binding form(s) of the receptor by combining the non-reducing SDS-PAGE of Fig. 4B with reducing SDS-PAGE in the second dimension followed by immunoblotting. Fig. 4C (upper panel) shows that, in addition to the 290 and 350 kDa complexes, non-labelled hGR forms, namely the monomer and higher order complexes with apparent molecular mass in the range of 450–700 kDa, were present in the transfected cells; and that the higher order complexes contained hsp70 as well as hsp90 (lower panel). Similar results were obtained irrespectively of whether the cross-linked receptor was labelled with [^{3}H]DM in the cytosol or in the cell. Since DSP treatment of cytosol from WCL2 cells that stably overexpress mouse GR consistently failed to reveal the monomer (not shown), its presence in our transiently transfected COS-7 cells is likely not the result of inefficient cross-linking of the transfected hGR.

The DSP-cross-linked overexpressed hGR was also analysed by two-dimensional SDS-PAGE and immunoblotting following fractionation using DNA–cellulose. Fig. 4D shows that in contrast to the 290 and 350 kDa complexes that were found in the flow-through fraction (upper panel), the receptor monomer was found in the protein fraction retained by DNA–cellulose (lower panel), with the higher order complexes presumably distributed in either fraction in amounts that cannot be detected by immunoblotting. This is possible considering that reaction with DSP compromised

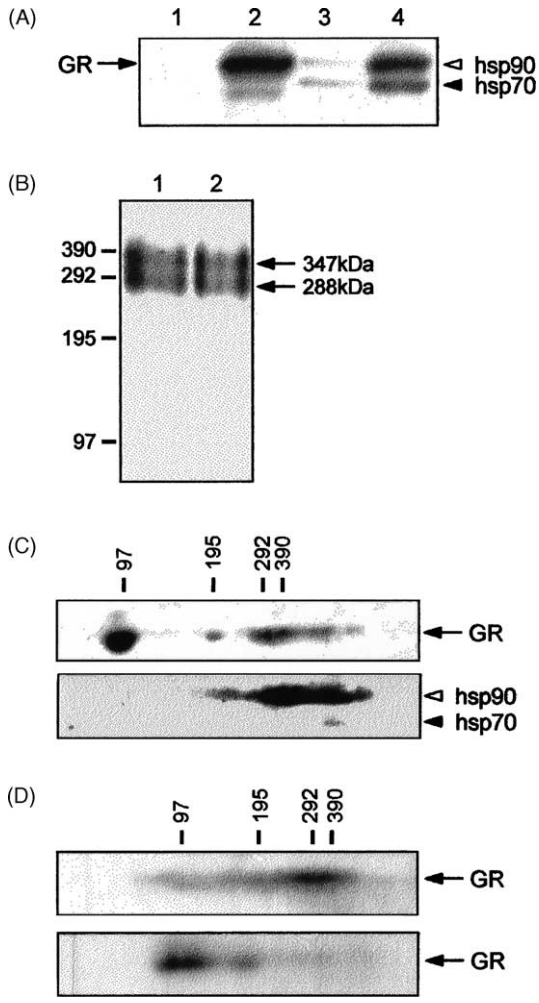

Fig. 4. Monomeric and heterooligomeric non-hormone-binding forms of the overexpressed hGR. (A) Cytosol from COS-7 cells overexpressing hGR (lanes 1 and 3) or His-tagged hGR (lanes 2 and 4) was purified using Ni^{2+}-affinity chromatography and analysed by SDS-PAGE and immunoblotting using anti-GR serum EP (lanes 1 and 2) or the antiserum to hsp90 and hsp70 (lanes 3 and 4). (B) Overexpressed hGR covalently labelled in the cell (lane 1) or the cytosol (lane 2) with [^{3}H]DM was cross-linked in the cytosol with DSP, immunoadsorbed with anti-GR serum HH and analysed by non-reducing SDS-PAGE and fluorography. Dashes indicate the positions of EGS-cross-linked phosphorylase *b* species used as marker proteins. (C) Alternatively, immunoadsorbates of DSP-cross-linked hGR (as described in panel B) were analysed by two-dimensional SDS-PAGE under non-reducing and reducing conditions in the first and second dimensions, respectively, and then immunoblotted using anti-GR serum EP and an antiserum to both hsp90 and hsp70. (D) Cytosol from COS-7 cells overexpressing hGR was treated with DSP and incubated with DNA–cellulose. Unbound (upper panel) and DNA-bound protein fractions (lower panel) were analysed by two-dimensional SDS-PAGE and immunoblotting using anti-GR serum EP. EGS, ethylene glycol(succinimidyl succinate); DSP, dithio-bis(succinimidyl propionate).

GR binding of our antisera. Quantitative immunoblotting analysis of non-cross-linked receptor immunoadsorbates obtained with a 1:50 dilution of antiserum EP from the flow-through fraction and the eluate of DNA–cellulose revealed apparent receptor associations with 4 molecules of

Table 1
An excess of hsp90 and hsp70 is associated with the non-hormone-binding fraction of hGR which is transiently overexpressed in COS-7 cells

Cell extract	Untreated	DNA–cellulose-treated	
		Unbound	DNA-bound
hsp90[a]	3.3	3.9	0
hsp70[a]	1.9	0.5	2.6

[a] Stoichiometries of hsp90 and hsp70 (mol/mol of receptor) associated with untreated and DNA–cellulose-treated receptor mutant Δ77–262. Following treatment, DNA–cellulose-bound receptor was eluted using 30 mM $MgCl_2$ in the presence of 20 mM sodium molybdate. Receptor in DNA-bound and unbound fractions as well as in cell extracts not treated with DNA–cellulose was immunoadsorbed using a 1:50 dilution of antiserum EP. Extract from mock-transfected cells was used as control. Quantitative immunoblotting of the receptor, hsp90 and hsp70 in the immunoadsorbates was carried out using appropriate calibration curves obtained with increasing amounts of immunoadsorbed HeLa receptor or purified hsp90 and hsp70, respectively, as described in Section 2.

hsp90 and 2.6 molecules of hsp70, respectively (Table 1). Taken together, the data of Fig. 4C and Table 1 suggest that the 450–700 kDa complexes comprise non-DNA-binding, non-hormone-binding species associated with an excess of hsp90 and DNA-binding, non-hormone-binding species associated with an excess of hsp70.

We next asked whether the unliganded overexpressed hGR possess constitutive GRE-binding activity. Receptor immunoadsorbates from extracts of cells transfected with Δ77–262, Δ9–385 (lacks the 10–26 epitope) or with the empty expression vector were incubated with [γ-^{32}P]-labelled GRE. Fig. 5A shows that GRE binding of Δ77–262 is reduced in the presence of a five-fold excess of unlabelled GRE to the level of non-specific binding observed with Δ9–385 or the empty expression vector. To find out whether overexpressed hGR can distinguish between GRE and non-specific DNA, similarly obtained receptor immunoadsorbates were challenged with γ-[^{32}P]-labelled fragments of 2363, 1687 and 420 bp from *Pvu*II-restricted plasmid GRE-37Tk (Fig. 5B, lane 1), of which only the latter contains a GRE. Fig. 5B shows that only the 420 bp fragment was retained by immunoadsorbed Δ77–262 (compare lanes 1 and 2) and that none of the fragments was specifically retained by mock immunoadsorbates (compare lanes 1 and 3). The data of Fig. 5 are taken to suggest that the non-hormone-binding fraction of overexpressed hGR, whether in monomer form or in association with hsp70, possesses GRE-binding activity.

3.4. The unliganded overexpressed hGR inhibits GRE-dependent activation of transcription

We next examined whether unliganded overexpressed hGR affects the ability of constitutively active GR mutant N525 to drive CAT gene transcription from the 2GRE-37Tk reporter. The reporter and the N525 expression vector alone or together with hGR, Δ428–490 or the empty expression

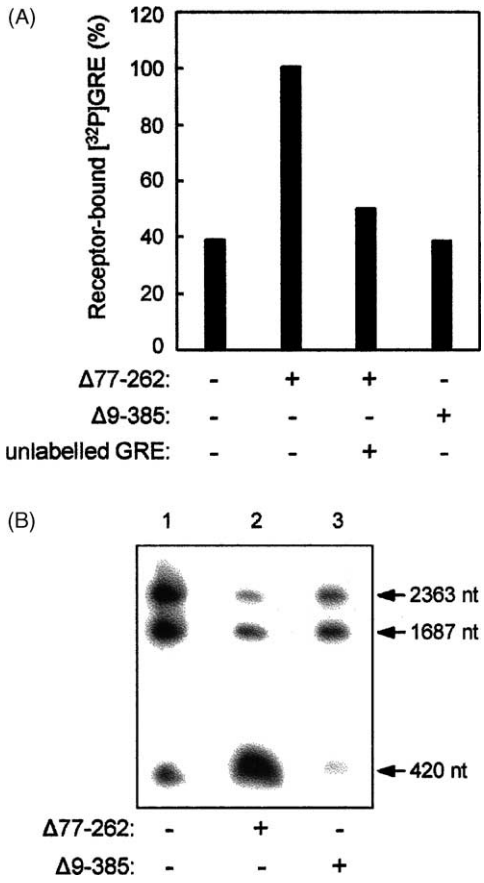

Fig. 5. The unliganded overexpressed GR exhibits GRE-binding activity. (A) Cytosol from COS-7 cells transfected with hGR mutant Δ77–262, mutant Δ9–385 or the empty expression vector was subjected to immunoadsorption using anti-GR serum 10–26. Immunoadsorbates were incubated with [^{32}P]-labelled GRE in the presence or absence of an excess of unlabelled oligonucleotide. GRE binding to the immunoadsorbates was measured using a β-counter. Data represents the mean of duplicate samples of a representative experiment. (B) Alternatively, Δ77–262 (lane 2) and Δ9–385 (lane 3) immunoadsorbates were incubated with [γ-^{32}P]-labelled, *Pvu*II-restricted GRE-37Tk and DNA bound to the receptor was eluted and analysed by agarose gel electrophoresis and fluorography. The shorter of the three GRE-37Tk fragments contains a GRE (lane 1). GRE, glucocorticoid-responsive element.

vector, were co-transfected in HeLa cells and these were assayed for CAT activity. We observed significant inhibition (63%; $P < 0.05$) of N525-induced CAT expression in the presence of the wild-type hGR but not Δ428–490 (Fig. 6). In similar experiments, Δ77–262 and Δ262–404 inhibited N525-induced CAT expression by 56 and 34%, respectively (data not shown), suggesting that the inhibitory effect was exerted predominantly by the C-terminal half of the NTD. Co-expression of N525 and hGR in HeLa 1β5 cells (HeLa cells stably transfected with the 2GRE-37Tk reporter) inhibited N525-induced CAT expression by 50%, suggesting that a native-like nucleosome structure at the GRE is compatible with the inhibition (Fig. 6). Whether unliganded overexpressed hGR inhibits GRE-dependent CAT expression solely by competing with N525 for binding to GRE or

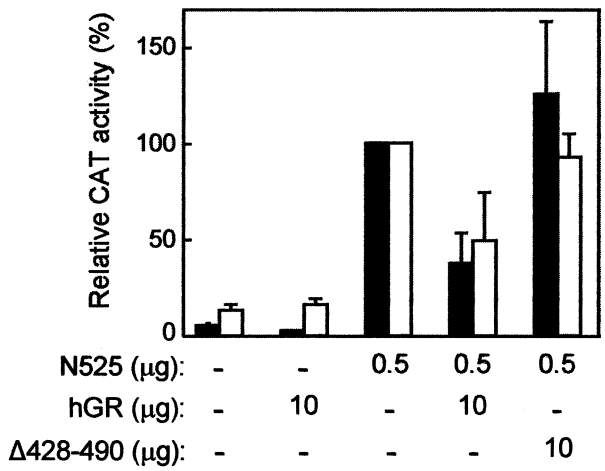

Fig. 6. The unliganded overexpressed hGR inhibits GRE-dependent activation of transcription. HeLa cells (■) were transfected with the 2GRE-37Tk CAT reporter (along with a plasmid expressing β-galactosidase) and plasmids expressing the wild-type hGR, receptor mutant Δ428–490, mutant N525, or none, as indicated. Alternatively, HeLa 1β5 cells (□) were transfected only with the indicated expression plasmids. CAT activities were normalised to β-galactosidase activity (HeLa cells) or total protein (HeLa 1β5 cells) and expressed relative to the normalised CAT activity of cells expressing N525 alone.

for limiting transcription factors as well, is not clear from these data.

4. Discussion

Based on a report that HSF is activated in cells that transiently overexpress SHRs in the absence of hormone [18], we hypothesised that the hsp70/hsp40 chaperone machine may be titrated in such cells and that HRCA intermediates may accumulate when more GR is produced than can be assembled to HRC. Direct comparison of the levels of receptor protein and receptor-bound hormone in hGR immunoadsorbates from COS-7 cells transiently overexpressing the receptor in the absence of GCs, revealed the presence of non-hormone-binding receptor species (Fig. 1). In contrast to unliganded hGR transiently expressed in COS-7 cells to levels of 50–100,000 molecules per cell, which has been reported to be predominantly cytoplasmic and free of hsp70 [28], hGR overexpressed to levels of ∼300,000 molecules per cell was predominantly located in the nucleus in the absence of hormone and was recovered in the cytosol in association with the chaperone. We found that DBD was necessary for nuclear localisation of the unliganded overexpressed hGR (Fig. 2), indicative of a constitutive DNA-binding activity being responsible for its nuclear retention. In support of this notion, we observed that the DNA-binding activity of the unliganded overexpressed hGR, as normalised to that of the heat-transformed liganded receptor, was ∼3.3 times higher than the similarly normalised activity of the endogenous GR of HeLa cells (Fig. 3). Moreover, using antiserum EP to the hinge region of GR where by NLS1 is located [4,5,8], we obtained data indicating that NLS1 is likely not occluded in the non-hormone-binding fraction of the unliganded overexpressed hGR, consistent with retention within the cell nucleus of non-hormone-binding species possessing de-repressed NLS1 and DNA-binding functions. NLS1 comprises a pair (R479, K480) and a stretch (R491–K498) of basic amino acids [5,28]. Since the unliganded overexpressed Δ491–515 was also located in the nucleus, the pair is probably sufficient for nuclear import of the de-repressed receptor species. Given the involvement of hsp90 and hsp70 in receptor shuttling as well as cycling [8,13,17], their titration by receptor overexpression is likely to affect the nucleocytoplasmic distribution of the HRC as well, allowing for nuclear localisation of steadily (the monomer) and transiently de-repressed (the HRC) receptor species alike.

The fraction of the unliganded overexpressed hGR that failed to bind [^{3}H]TA was comparable to that which was not labelled with [^{3}H]DM (Fig. 1C), implying that these fractions comprise similar receptor entity(ies). The major non-labelled species that was detected by two-dimensional SDS-PAGE analysis of cross-linked [^{3}H]DM-labelled hGR was a non-hormone-binding monomer (Fig. 4C). The cysteine residue of GR which is specifically labelled with [^{3}H]DM is located in a region of the molecule that is critical for hsp90 as well as hormone binding to the receptor [29]. In addition, both the [^{3}H]TA and the [^{3}H]DM-binding activities of the receptor are known to be adversely affected following dissociation of the chaperone from the HRC. In fact, only low-affinity binding of [^{3}H]TA and non-specific labelling of several cysteines with [^{3}H]DM has been observed with GR LBD produced in *E. coli*, suggestive of a loosely structured steroid-binding pocket being formed when hsp90 is absent or not productively associated with the receptor [30]. This observation contrasts the loss of both [^{3}H]TA and [^{3}H]DM binding caused by the thiol-specific reagents methyl methanethiolsulphonate and sodium arsenite by way of intra-molecular disulphide bond formation between the DM-reactive cysteine and a neighbouring thiol [29]. In the above light one may speculate that the monomer is unable to bind [^{3}H]TA with high affinity because it is conformationally constrained by an intra-molecular disulphide bond. It has been reported that disulphide–thiol exchange mediated by reduced thioredoxin restores the hormone-binding activity of in vitro inactivated GR [31]. Whether thioredoxin can restore [^{3}H]DM labelling of the monomer is not known. Should this were the case, however, it could mean that rendering thioredoxin limiting by receptor overexpression generates a conformationally constrained hGR monomer in which repression of the hormone-binding function is linked to de-repression of its NLS1 and DNA-binding functions; and that the enzyme is probably required to relieve the constrain before the monomer can be recruited by hsp70/hsp40 to HRCA.

Using DNA–cellulose we fractionated unliganded overexpressed hGR to find that, in addition to the

non-DNA-binding, hormone-binding 290 and 350 kDa complexes and the DNA-binding, non-hormone-binding receptor monomer (Fig. 4D), presumptive complexes of the monomer with an excess of hsp90 or hsp70 were also recovered by immunoadsorption of the fractionated receptor with antiserum EP (Table 1). These complexes likely originate from the 500–700 kDa species of Fig. 4C. hsp90 in these species may have a role in preventing the non-hormone-binding monomer from aggregating when hsp70 is limiting for HRCA. Significantly, Yonehara et al. [32] have reported that hyperthermia generates oligomeric forms of hsp90 capable of maintaining denatured dihydropholate reductase in a refoldable state in vitro; and that oligomerisation of the chaperone is promoted by unfolded proteins. On the other hand, Kosano et al. [11] have shown that hsp70/hsp40 chaperone machine is required to maintain the heat-inactivated progesterone receptor in a refoldable state in vitro. In this light it is tempting to speculate that with hsp70/hsp40 likely being limiting in cells that highly overexpress hGR, the fraction of the monomer which is recruited by hsp70/hsp40 to HRCA is transferred to hsp90 to be maintained in a refoldable state by the latter chaperone. Significantly, it has been shown that titration of hsp90 by non-native proteins induces HSF activation in vitro [33] much like titration of hsp70 by heat denatured proteins is believed to induce the heat shock response of the cell [19]. Alternatively, hsp90 in the 500–700 kDa species may have a role in promoting release of the non-hormone-binding monomer from DNA. Significantly, an excess of hsp90, in addition to hsp70, was found associated with GR from yeast overexpressing the receptor in a YDJ1/hsp40 mutant background, which allows for receptor activation of transcription in the absence of hormone [34]. Moreover, hsp90 has been reported to reverse oestrogen receptor binding to ERE to form large complexes with the released receptor [35]. That hsp90 alone or in combination with hsp70/hsp40 can drive turnover of GR complexes with DNA is currently a matter of conjecture. Interestingly, recent studies revealed that p23 and hsp90 can promote disassembly of transcriptional GR complexes in vivo [36]. The transient transfection system described here may help to study the chaperoning of receptor interaction with DNA and chromatin in the proper cellular milieu.

Using an immunoprecipitation assay previously reported by Rusconi and Yamamoto [2], we found that the unliganded overexpressed hGR exhibits GRE-binding activity (Fig. 5A). In addition, using a variation of this assay initially described by Hollenberg et al. [1], we found a preference of the non-hormone-binding receptor for DNA fragments housing a GRE over fragments of non-specific DNA in the reaction mixture (Fig. 5B). Moreover, using a transient co-transfection assay, we found that the unliganded overexpressed hGR repressed N525-mediated GRE-dependent rather than basal transcription; and that, while deletion mutants Δ77–262 and to a lesser extent Δ262–404 were effective in repressing N525-mediated transcription, the DBD deletion mutant Δ428–490 was not, implicating the C-terminal half of the NTD in a DBD-dependent repression of transcription from GRE-dependent promoters. Previous reports have convincingly argued that the unliganded LBD of SHRs represses all their functions including squelching of limiting cofactors necessary for transcriptional activation in the presence of hormone [1–3,37,38]. By selecting DBD mutations of GR that promoted cofactor squelching in yeast overexpressing the receptor, Lefstin et al. [37] have shown that the effect was primarily mediated by the C-terminal half of the NTD; and that squelching was exacerbated by DBD mutations that strengthened receptor binding to DNA in the presence of hormone. Interestingly, the same mutants but not the wild-type receptor exhibited modest squelching also in the absence of hormone. The present data suggest that, whether by enhancer binding, cofactor squelching or both, overexpression of wild-type GR in mammalian cells allows for repression of GRE-dependent activation of transcription. The present findings may have relevance to the inhibition of transcription observed with the beta isoform of GR, a non-hormone-binding nuclear entity endowed with constitutive DNA-binding activity and surmised to repress transcription from GRE-dependent promoters [39,40].

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