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Alteration of the glucocorticoid receptor subcellular localization by non steroidal compounds

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Abstract

The glucocorticoid receptor (GR) engages transient or stable interactions with chaperones (hsp90, hsp70), co-chaperones (p60/hop, hsp40) and several other polypeptides such as immunophilins (Cyp40, FKBP59) and p23 to achieve a high affinity ligand binding state. This complex dissociates in response to hormonal stimuli and holo-GR translocates into the nucleus, where it regulates the activity of glucocorticoid-sensitive genes. GR activity is controlled through its ligand binding domain by steroids displaying either agonistic or antagonistic activity. An alternative approach to modulate GR activity is to target receptor-associated proteins (RAPs), and several non steroidal compounds binding to RAPs affect GR transcriptional activity. We have studied the effect of such drugs on the intracellular localization of a EGFP-GR fusion protein, which has wild type GR pharmacological properties. Agonist and antagonist binding induced nuclear translocation of GR, whereas rifampicin was found to be inactive in our system. Immunosuppressants FK506 and cyclosporin A were able to induce partial nuclear translocation of GR, suggesting that potentiation of glucocorticoid action by these compounds may also proceed through enhanced GR nuclear transfer. Short treatment of cells with the hsp90 inhibitor geldanamycin (GA) did not prevent nuclear translocation of GR. However, longer treatments, in parrallel to the inhibition of GR transcriptional activity, strongly perturbed GR subcellular localization concomitantly to the disruption of the actin network, and caused GR aggregation and down-regulation. The GA-induced transcriptional shutdown was also observed for other nuclear receptors which do not interact stably with hsp90. Thus RAP-binding compounds may exert their effects at least in part through perturbation of the GR cytosol to nucleus partitioning, and identify these proteins as valuable therapeutic targets to control nuclear receptor activity.

1. Introduction

In the absence of its cognate ligand, the glucocorticoid receptor (GR) is transcriptionally inactive (non activated) and associated to several proteins (receptor-associated proteins or RAPs) in the cytoplasm. Regardless of their actual individual role within the non activated complex, which is yet to be clearly defined, RAPs have been identified as being either chaperones (hsp90, hsp70), co-chaperones (hip, hop), immunophilins (FKBP59, Cyp40) and others (p23). Some of these proteins are found to be transiently associated with the receptor complex (hsp70, p23, hip, hop), serving the general role of aporeceptor assembly catalysts (reviewed in [1,2]). RAPs assembly generates a GR-chaperoning complex which maintains GR under a high affinity ligand-binding form. GR is maintained in a poised state that will respond to hormonal stimuli [3]. The cellular pool of aporeceptor complexes is assembled, after protein synthesis, by an ATP-, heat shock protein 70 (hsp70)- and DnaJ like-dependent pathway ([4]; reviewed in [3]). This large, non activated

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form of GR is cytoplasmic but the exact mechanism of cytoplasmic retention is not elucidated yet. However, hsp90 has been shown to associate to actin [5,6] and anchoring of GR to cytoskeleton components through RAPs has been evoked [5,7,8]. Thus glucocorticoid stimulation of target cells leads to GR dissociation from RAP and subsequent migration of the receptor into the nuclear compartment, where it can bind to chromatin-associated hormone response elements [9,10]. This process, commonly referred to as receptor transformation or activation, has been shown to be modulated in-vivo by steroidal or non steroidal compounds that interact directly with the receptor moiety [11,12] or with RAPs [13–18].

The ligand-dependent nuclear translocation of GR has been described for both agonists and antagonists [19] and therefore does not necessarily reflects ligand ability to activate transcriptionally the receptor. However, we [12,20] and others [11] showed that GR dissociation from the chaperon complex is inhibited, in-vivo, by antiglucorticoids and RU486 did not induce detectable binding of GR to genomic GREs [21], a property potentially related to the particular nuclear sublocalization of RU486-bound GR [19]. Thus in some instances, non activated receptor complexes can be detected in the nucleus of target cells. On the other hand, RAP-binding compounds have been shown to influence strongly GR function, although their actual mechanism of action remains debated. The 59 kDa component of GR aporeceptor complex (FKBP52) has been identified as a FK506 and rapamycin binding protein [22], and Ning and Sanchez reported a clear potentiation of dexamethasone effect in the presence of both of these immunosuppressive compounds [23]. Cyclosporin A, another immunosuppressant that binds to cyclophilins (CyPs) but not to FKBP [24], has equally been shown to potentiate dexamethasone effect in a similar cellular system [18]. However, Pratt and coworkers were unable to find evidence for significant effects of these compounds on either the molecular structure of apo-GR or on its transcriptional activity [22]. Hsp90 is an ATP/ADP-binding protein [25,26], and p23 associates to the ATP-bound hsp90, stabilizing the hsp90-GR complex [27,28]. Geldanamycin (GA) and others benzoquinone ansamycin class compounds bind to the ATP-binding site of hsp90 and inhibit p23 association to hsp90 [29]. Treatment of cells with geldanamycin leads to GR or progesterone receptor (PR) loss of ligand binding capacity [29,30] which can be attributed in part to an increased cellular proteolytic degradation via the 20S proteasome pathway [29]. These experiments exemplify pharmacological efforts to modulate GR functions in a ligand-independent fashion. In addition, we reported recently that overexpressing an acidic peptide from hsp90 prevented GR association to hsp90 in vitro and disrupted selectively the glucocorticoid signalling pathway in-vivo [13]. Thus targeting RAP binding to GR may reveal pharmalogical tools that have clinical potential.

The body of data describing effects of RAP-binding compounds has been obtained using either in vitro aporeceptor assembly systems such as rabbit reticulocyte lysate, or immuno precipitation from lysed cells. Visualizing directly GR in intact cells may circumvent some of the limitations encountered when using immunological techniques, and bring valuable information on biological functions of RAP by studying GR subcellular localization. Hence, using a green fluorescent protein (GFP)-tagged GR (EGFP-rGR) may provide insights into the actual contribution of protein–protein interactions within the non activated, heterooligomeric GR. We have examined the influence of several non steroidal compounds on GR subcellular trafficking, using a chimeric EGFP-rGR fusion protein which displays wild-type receptor pharmalogical properties. While confirming that nuclear translocation is a separated step from DNA binding, our results suggest that potentiation of dexamethasone by FK506 and cyclosporin A effects occured through partial facilitation of nuclear accumulation of GR. Dramatic effects of geldanamycin on GR subcellular localization were noted upon long term treatment of target cells but did not abrogated its ability to translocate into the nucleus. These results led us to reconsider some of our previous conclusions as well as those from others concerning the mechanism of action of some GR modulators.

2. Material and methods

2.1. Chemicals and enzymes

Steroids were purchased from Sigma (St Louis, MO, USA) or Steraloids Inc. (Newport, RI, USA). Restriction and DNA modification enzymes were purchased from Promega (Madison, WI, USA). FK506, cyclosporin A and geldanamycin were obtained from Calbiochem-Novabiochem Corp. (San Diego, USA).

2.2. Plasmids

Plasmids encoding the rat GR (pT3.1118 and pRSV rGR) were kindly provided by Dr K.R. Yamamoto (University of California, San Francisco, CA). The EGFP-rGR expression vector was constructed as follows: the rat glucocorticoid receptor cDNA was excised from pT3.1118 [31] as a *Bam*HI-XbaI fragment and inserted into pEGFP-C1 (Clontech, Palo Alto, CA, USA) cut with the same enzymes. This generated a fusion protein coding for amino acid 4 to 795 of rGR. pEGFP-hRARα was constructed by inserting a

*Bam*HI-*Eco*RI fragment encoding hRARα from amino acid 2 to 462 into pEGFP cut with *Bgl*II and *Eco*RI. All sequences were checked by automatic sequencing. The p1187 Luc reporter gene and the CMV-hsp90 expression vector are described elsewhere [13,32] as well as the apoC3-Luciferase and HNF-4 expression [33].

2.3. Cell culture, transient transfections and luciferase assay

HeLa and COS-7 cells were grown in monolayer culture in phenol red-free medium (OptiMEM, Gibco-BRL) supplemented with 10% steroid-free serum calf serum (FCS). FCS was stripped free of contaminating steroids by double dextran-coated charcoal adsorption (0.3% dextran, 3% charcoal). Transfections were carried out using the polyethyleneimine (PEI) coprecipitation method as described [34]. Briefly, 1.5×10^5 cells were transfected with 50 ng of p1187 Luc and 400 ng of pEGFP-rGR. The next day, the medium was renewed twice and cells treated for the indicated times with GR modulators. p1187 Luc was omitted for studies of GR subcellular localization. Luciferase assay were performed using the LucLite system from Packard, according to the manufacturer's guidelines, and RLU measured using a LumiCount plate reader (Packard Instruments, Rungis).

2.4. Immunofluorescence

Anti-tubulin, anti-vimentin, and rhodamine-conjugated phalloidin were purchased from Sigma as well asTRITC-conjugated antimouse IgG. HeLa cells were grown in 6-wells plates containing sterile coverslips and transfected with pEGFP-rGR as described above. Transfected cells were treated for the indicated times with GR inducers or inhibitors at appropriate concentrations. Cells were then washed three times with 1X Phosphate Buffered Saline (PBS) and fixed with 95% methanol in 1X PBS for 30 min at room temperature. Cells were washed twice with 1X PBS and fixed with 3% paraformaldehyde and permeabilized by incubation with 0.1% Triton X-100 for 5 min. After three washes, non specific binding sites were blocked by incubation in 5% non immune serum, and specific antibodies were added at a 1:100 to 1:500 final concentration. Immune complexes were then detected using TRITC-conjugated IgG (1:200 dilution), and actin was detected using rhodamine-conjugated phalloidin at 200 U/mL (ca. 6.6 μM). Stained samples were then mounted on glass slides in Vectashield (Vector Labs, Burlingame, CA, USA).

2.5. Confocal microscopy

Slides were examined with a Leica TCS NT confocal microscope (Leica Microsystemes, Rueil Malmaison, France), equipped with a 15 mW Argon-Krypton laser, configured with an inverted Leica DM IRBE. The 488 nm line was used to excite EGFP, and the 568 nm line was used to detect the TRITC fluorophore. Each image consisted of the projection of 16–36 optical sections performed at intervals of 200 nm in the z axis. {XY} field of 1024×1024 pixels were scanned using oil Pl Apo 40× or 100× (NA = 1,4) objectives. Images were processed using the Leica TCS NT software (Power Scan module), mounted in CorelDraw and printed on a Sony HD-8800 color printer.

3. Results and discussion

3.1. Structure and nuclear translocation of the EGFP-rGR fusion protein

Green fluorescent protein (GFP) fluorescence can be observed in fixed and live cells without any requirement for cofactor and exogenous substrate. The EGFP variant can be excited at 488 nm by argon ion laser used in confocal scanning laser microscope, or easily detected by fluorescence microscopy with filters used to detect fluorescein isothiocyanate (FITC)-conjugated antibodies. In addition, EGFP forms a chromophore in a temperature-independent manner, in opposition to earlier versions of this protein (wtGFP and S65T variants) which require incubation of GFP expressing cells at 30°C. Thus the EGFP-rGR construct encodes for a fusion protein between EGFP and rat GR from amino acid 4 to 795 (Fig. 1), which is strongly and spontaneously fluorescent. The subcellular localization of the EGFP-rGR fusion protein was then examined in HeLa cells and compared to that of a EGFP-hRARα fusion protein (Fig. 1B). Fluorescence of the EGFP-rGR revealed that unliganded GR was located in the cytoplasmic compartment (Fig. 1B-1), whereas intense staining of nuclei was observed upon incubation of target cells with 1 μM Dex for 30 min (Fig. 1B-2), therefore demonstrating a rapid translocation of GR into this cellular compartment. On the contrary, hRARα appeared to be constitutively located in the nucleus irrespective of the presence of ligand (Figs. 1B-3 and B-4).

3.2. Pharmacological characterization of the EGFP-tagged rat GR

Although the ligand binding domain (LBD) of GR and of other nuclear receptors have been shown to function autonomously [35–39], we compared EGFP-rGR transactivating properties to that of wild type (wt) GR, in order to assess any possible effect of the GFP moiety on GR transcriptional activity (Fig. 2). In

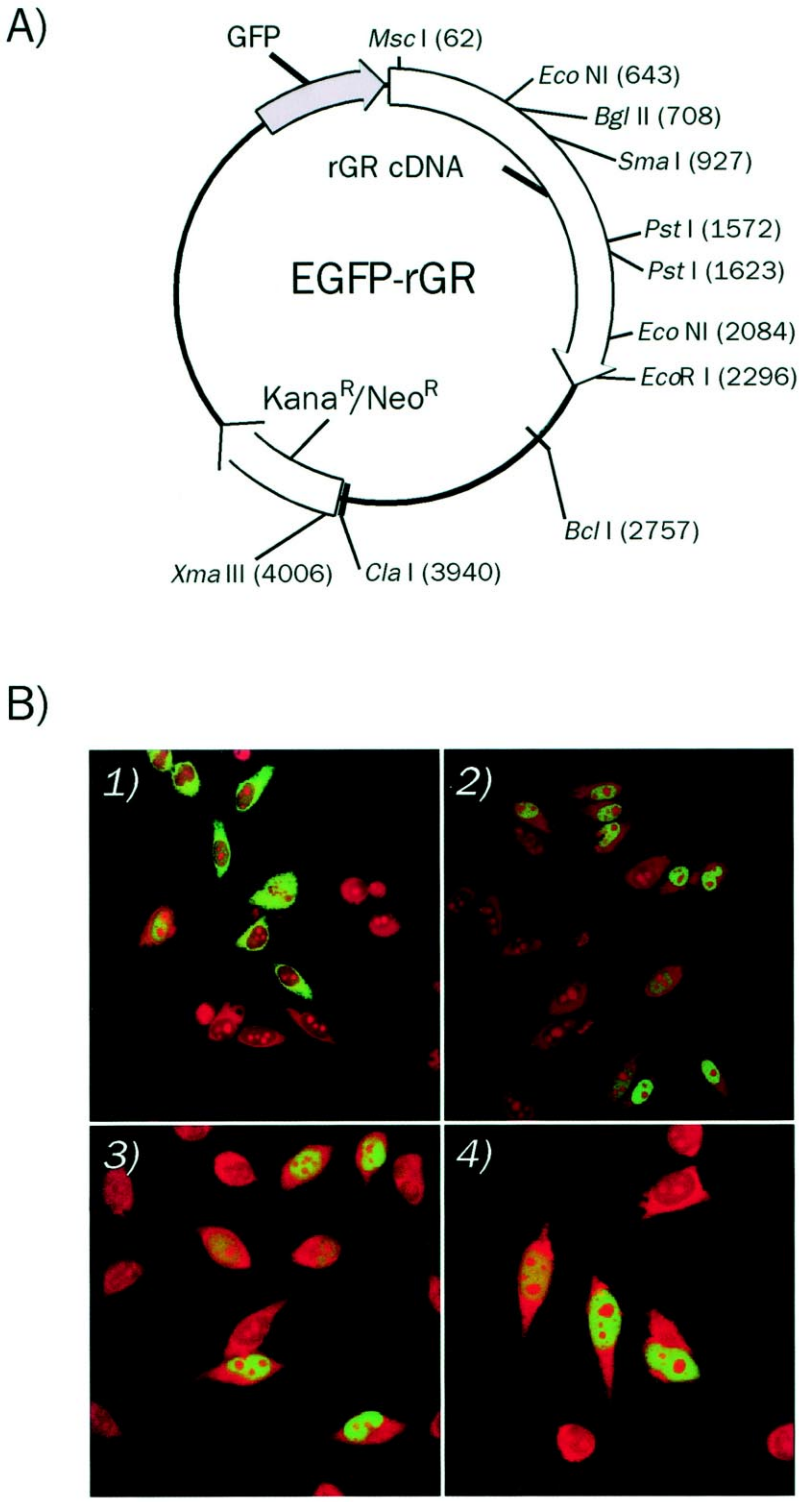


Fig. 1. Structure and expression of the EGFP-rGR expression vector: (A) This eukaryotic expression vector encodes for the EGFP variant fused C-terminally to the rat glucocorticoid receptor cDNA (amino acids 4–795). This chimeric gene is under the control of the cytomegalovirus (CMV) promoter which allows high level of expression in most eukaryotic cell lines. The kanamycin/neomycin resistance gene is useful for establishing stably transfected cell lines. (B) Subcellular localization of the EGFP-rGR and of the EGFP-hRARα fusion proteins. (1) 10^5 Hela cells transfected with 100 ng of the EGFP-rGR vector were fixed, counterstained with Blue Evans and observed by confocal microscopy as described in Section 2. (2) A similar procedure was followed except that cells were treated overnight with 100 nM Dex. (3) HeLa cells were transfected with 100 ng of the EGFP-hRARα vector and processed as above. (4) HeLa cells expressing the EGFP-hRARα fusion protein were treated overnight with 1μM *all-trans* retinoic acid.

the presence of two potent synthetic glucocorticoids, dexamethasone (Dex) and triamcinolone acetonide (TA), a strong induction of the glucocorticoid-inducible promoter from the mouse mammary tumor virus (MMTV) was observed. We noted that TA was a more potent inducer than Dex at 1 μM, suggesting that this steroid might be metabolically inactivated more slowly, or reached higher intracellular levels than Dex at these receptor saturating concentrations. Corticosterone (B) and deoxycorticosterone (DOC) are glucocorticoids found naturally in rodents, and both compounds activated EGFP-rGR and wtGR to a similar extent. RU486 and progesterone, which are antiglucocorticoids, were as expected found to be inactive in our assay when used alone at 10 nM and 1 μM concentrations. Likewise, non steroidal modulators of GR such as FK506, cyclosporin A, geldanamycin and the hsp90 peptidic antiglucorticoid (CMV-hsp90 [13]) did not displayed any intrinsic activity. More surprisingly, the recently identified glucocorticoid agonist rifampicin [40], a macrocyclic antibiotic, was unable to induce detectable transcription from the MMTV promoter as well as from other glucocorticoid-regulated promoters (data not shown).

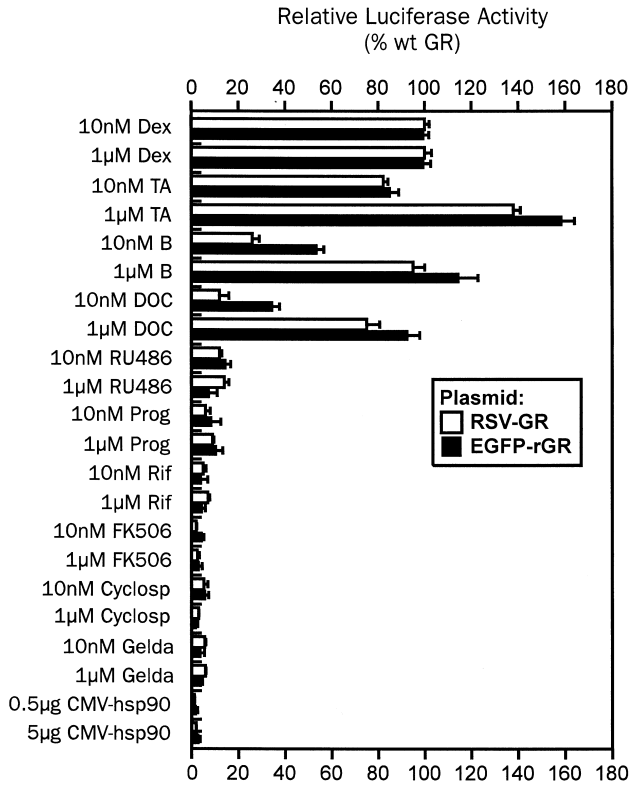


Fig. 2. Ligand-dependent activation of the wt rGR and EGFP-rGR: COS-7 cells were transfected with the reporter gene p1187-Luc and with either pRSV-GR, coding for the wild type rat GR, or pEGFP-rGR, coding for the EGFP-rGR fusion protein. Results are expressed as the percentage of maximal wild-type activity in the presence of 1 μM Dex, and are the mean ± SEM of at least four independent determinations of the luciferase activity. A 25 to 40-fold induction was typically observed with the wild type receptor. White bars: Luciferase activity observed with wt rGR; shaded bars: Luciferase activity observed withEGFP-rGR. Cells were treated overnight with the indicated concentration of GR modulator: Dex, dexamethasone; TA, triamcinolone acetonide; B, corticosterone; DOC, deoxycorticosterone; Prog, progesterone; Rif., rifampicin, Cyclosp, cyclosporine, GA, geldanamycin.

Some of the compounds used above are unable to activate GR and possess either antagonistic or synergistic activities on wtGR-controlled transcription. Their effects were thus tested on the EGFP-rGR chimera challenged with 1 nM Dex (Fig. 3), which induced a 20–30% activation of the MMTV reporter gene when compared to that observed in the presence of saturating (1 μM) Dex concentration (Fig. 2). RU486 is a potent antiglucocorticoid which acts by preventing hsp90 dissociation from GR [11,12]. When added concomittently to the culture medium with 1 nM Dex, it suppressed effectively transcriptional activation of the reporter gene. Rifampicin failed to display either antagonist or synergistic effect on Dex-mediated transcriptional induction. Cyclosporin A, an immunophilin binding exclusively to Cyp40, was able to potentiate Dex effect approximately 3 to 4-fold, as well as FK506 which binds to the p59 (FKBP52) component of the aporeceptor complex. Higher potentiation rates were reported by Renoir and colleagues [18] and Ning et al. [23] but these authors used a different cell line, a difference that may account for the lower potentiation rate observed in our system (4 to 5-fold vs. 40-fold). GA and the hsp90 peptide prevented activation of the reporter gene by Dex. Thus RAP-binding compounds and other non steroidal GR modulators displayed activities similar to those reported using wtGR. The EGFP-rGR fusion protein responded to pharmacological agents strictly as its wild type counterpart, and therefore appears to be a reliable and relevant molecular tool to investigate rGR subcellular localization in response to various glucocorticoid receptor modulators.

3.3. Alteration of the subcellular localization of GR by RAP-binding compounds

We examined then the effect of these compounds on GR ligand-induced nuclear translocation at short incubation times, in order to minimize any possible secondary effects of these compounds on cellular physiology (Fig. 4). RU486 induced a complete translocation of GR into nuclei, demonstrating that nuclear localization and transcriptional activation are clearly distinct steps in the GR activation process. Rifampicin was inefficient at inducing nuclear translocation of GR, therefore confirming its lack of activity in transient transfection assays. GA had no detectable effect on the cellular localization of GR in the presence or in the

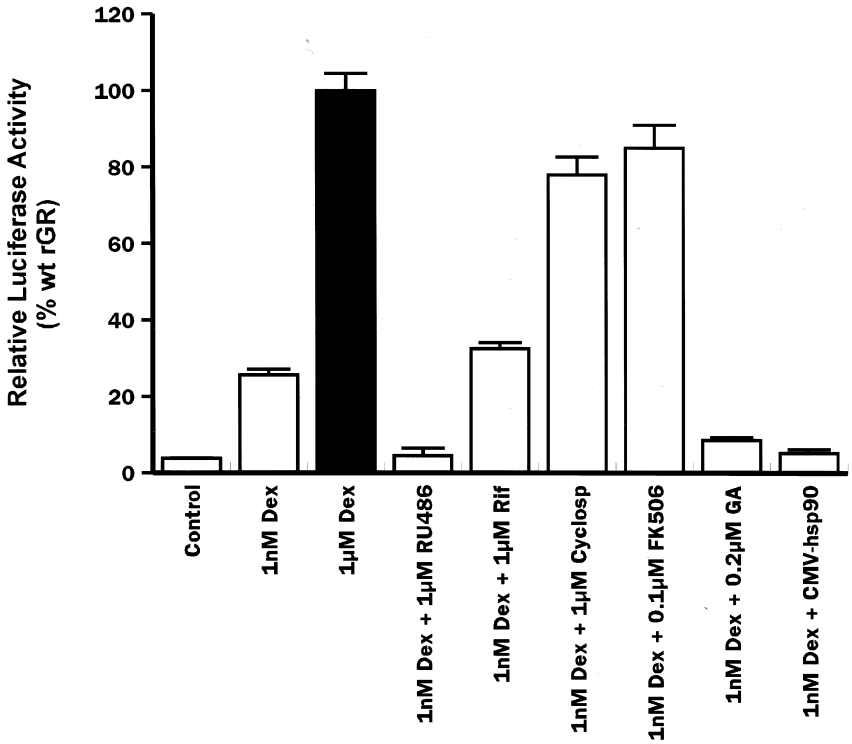


Fig. 3. Modulation of GR activity at sub-saturating concentrations of dexamethasone by steroidal or non steroidal compounds: COS-7 cells were transfected as described above with EGFP-rGR and p1187-Luc, and stimulated with 1nM dexamethasone. Results are expressed as the percentage of maximal wild-type activity in the presence of 1 μM Dexamethasone (black bars), and are the mean ± SEM of at least four independent determinations of the luciferase activity. Dex, dexamethasone; Rif., rifampicin, Cyclosp, cyclosporine, GA, geldanamycin. Cells were treated overnight with the indicated compounds.

absence of Dex, suggesting that inhibition of hsp90 does not significantly impair GR responsiveness to ligand. Similarly, overexpression of the hsp90 peptide did not prevent nuclear accumulation of GR upon Dex treatment. FK506 and cyclosporin A, two RAP-binding compounds which potentiated Dex activity used at receptor sub-saturation concentrations (Fig. 3), induced partial nuclear translocation of GR in the absence of steroid. This effect was quantified by counting several hundred transfected cells in independent cell preparations and defining five classes of signal distribution between cytoplasm (C) and nucleus (N), (Table 1). Control cells exhibited a GR subcellular localization which was clearly cytoplasmic (80%), as well as rifampicin and geldanamycin treated cells. Both RU486 and Dex induced a complete translocation of GR into the nucleus. An intermediate staining pattern was clearly detected with CsA and FK506, with which a partial but significant shift of the GR population into nuclei was observed, with less than 30% of cells exhibiting a predominant cytoplasmic staining. This observation is consistent with the reported inhibition of GR nuclear translocation by anti-FKBP52 antibodies [41] and the CsA-induced translocation of a PR mutant [43]. However, the nuclear translocation was in this case not correlated to an increased transcriptional activity (see Fig. 2), indicating again that no correlation exists between nuclear localization and transcriptional activation, for which agonist binding is an absolute prerequisite.

Table 1
GFP-rGR translocation to nucleus in the presence of RAP-binding compounds: EGFP-rGR was transfected as above and treated with modulators at concentrations indicated in the legend to Fig. 4. After fixation, fluorescence was visualized and GR subcellular localization was subdivided into five groups: C: exclusively cytoplasmic (see Fig. 1B); C > N: predominantly cytoplasmic, C = N: the two compartments are hardly distinguishable; C < N: predominantly nuclear; N: exclusively nuclear. Three independent experiments were carried out in duplicate for each conditions and 200–300 cells were counted per slide. Numbers thus represent the average population for a given subcellular distribution out of 1200–1500 cells

	C	C > N	C = N	C < N	N
Control	47 ± 3.8	33 ± 5.3	20 ± 6	–	–
Dexamethasone	–	–	–	5 ± 2.2	100 ± 2.3
Rifampicin	32 ± 4.5	45 ± 7	18.5 ± 2.3	7 ± 0.8	–
RU 486	–	–	–	4 ± 0.8	100 ± 4.8
Cyclosporin	–	9.8 ± 4	41 ± 0.3	45 ± 3	–
FK 506	7 ± 3	20 ± 7	44 ± 11	26 ± 0.5	–
Geldanamycin	25 ± 1.2	72 ± 4	4.5 ± 2.1	13 ± 4	–

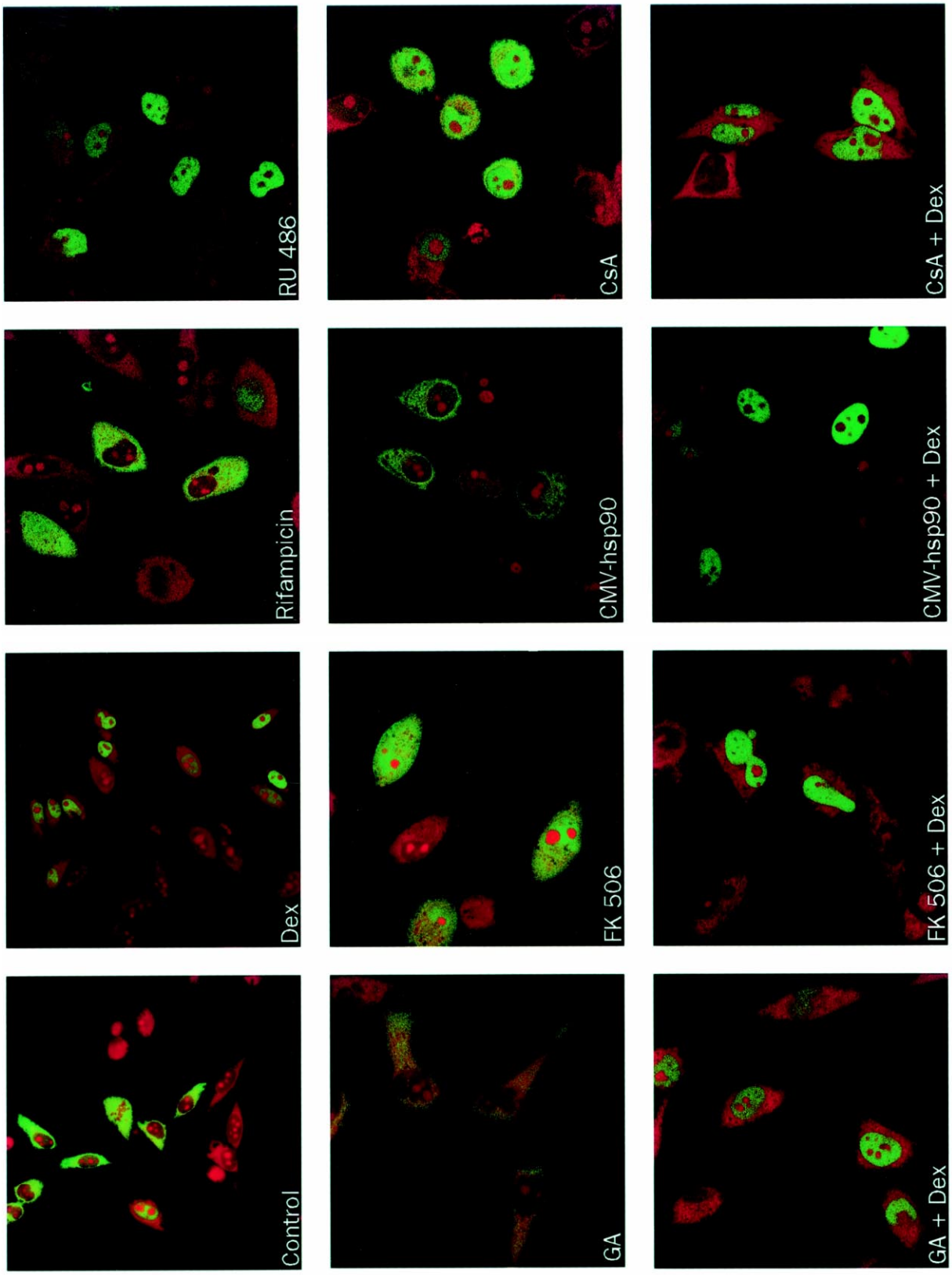
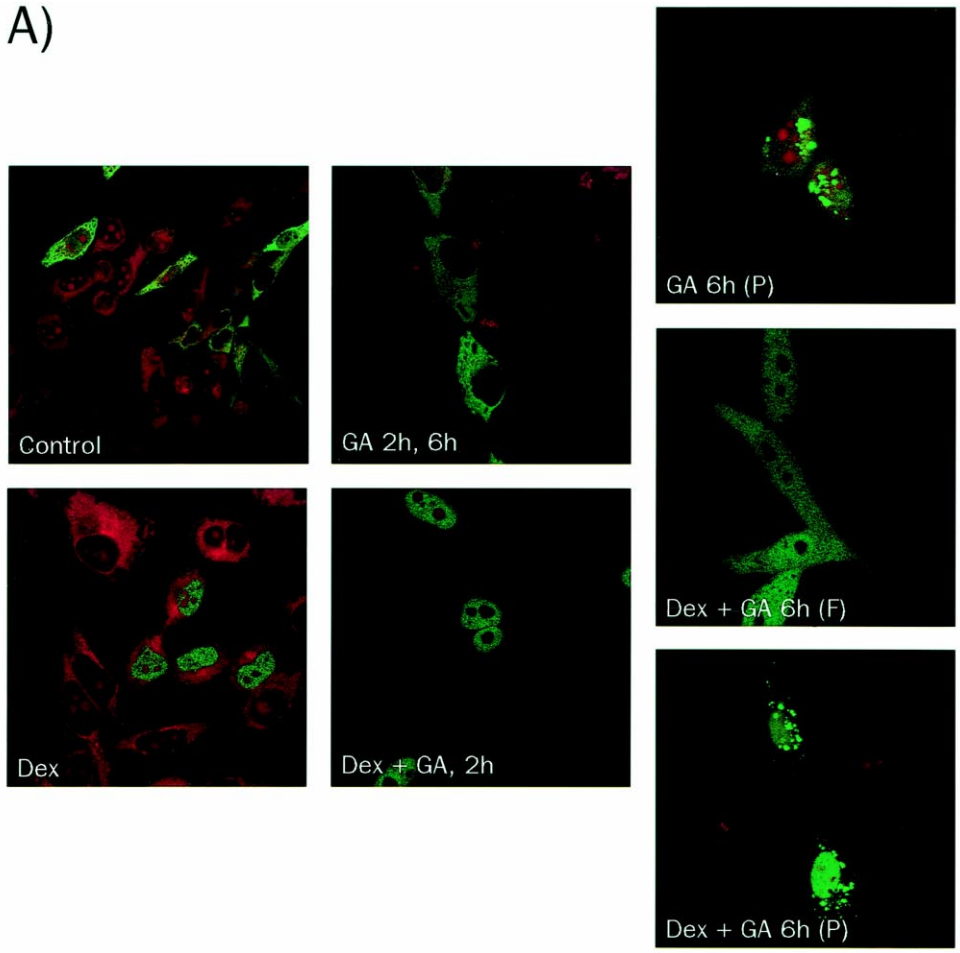


Fig. 4. Influence of GR modulators on GR subcellular localization: HeLa cells were transfected with the EGFP-rGR and submitted to various treatments as indicated for 30 min. Cells were then fixed and EGFP-GR molecules were visualized with confocal laser scanning microscope. Concentrations used in these experiments are similar to those used in Fig. 3. Dex., 1 nM; RU486, 1 μM; Rifampicin, 1 μM, Cyclosporin A 1 μM; FK506, 0.1 μM; Geldanamycin, 0.2 μM; CMV-hsp90, 5 μg expression vector per 35 mm dish.



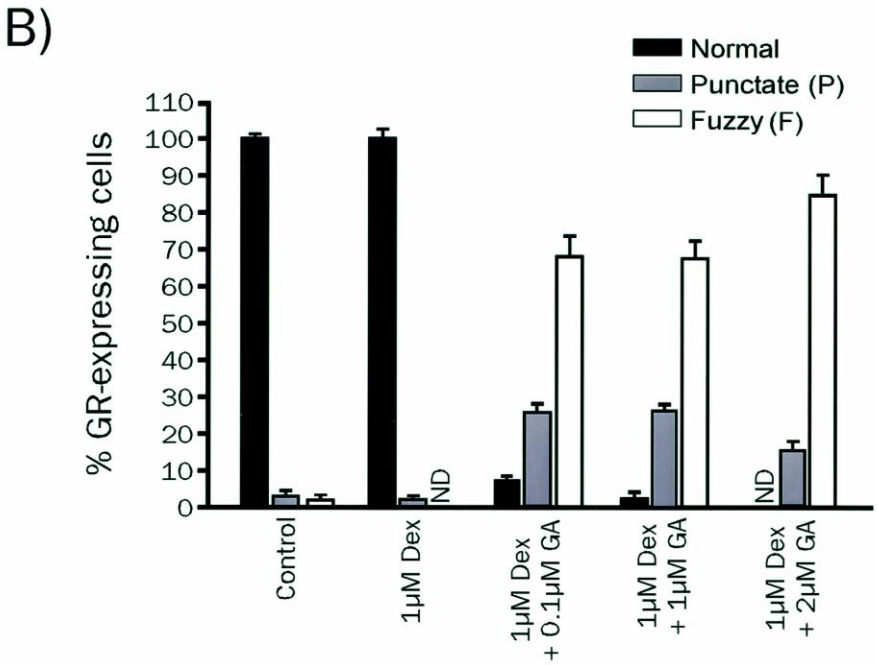


Fig. 5. Geldanamycin blocks GR nuclear translocation and promotes cytoplasmic aggregation: A) Intracellular distribution of GR in response to GA treatment. HeLa cells were transfected with EGFP-rGR and treated with 100 nM Dex and/or 0.2 μM GA for the indicated times (0, 2 or 6 h). Cells were examined using confocal microscopy and representative fields are shown. P: punctate; F: fuzzy. B) Cellular population after a 6 h treatment with increasing GA concentrations. Cells were transfected as above and slides were examined for their contents in cells exhibiting either a normal, punctate or fuzzy staining pattern. Three independent experiments were carried out in duplicate for each condition and 200 to 300 cells were counted per slide. Numbers thus represent for a given subcellular distribution the average population out of 1200 to 1500 cells.

Potentiation of glucocorticoid action by these compounds has been hypothesized to result either from direct binding to GR aporeceptor complex [18,42] or from inhibition of MDR pumps that extrude actively some glucocorticoids and therefore lower the intracellular concentration in steroid [43]. Our data rather support the first hypothesis, since immunosuppressant effects were observed in steroid-free medium and at incubation times of 6 and 24 h. Of course, we cannot at this stage rule out mechanisms involving other signalling pathways.

3.4. Hsp90 inhibition promotes GR aggregation and degradation

Assembly of p23 is necessary to maintain PR under a ligand-binding form in intact cells [30], and a fast and clear decrease of GR ligand binding activity and intracellular concentration was observed in HeLa cells treated with GA [29]. However, our data show that GR is still ligand-responsive after short exposure to GA (30 min, Fig. 4), and that ligand-induced nuclear translocation is also observed at longer exposure time (6 h, Fig. 5Fig. 1 8 h, data not shown), suggesting that a significant fraction of the cellular pool of GR is still able to interact productively with steroid in GA-exposed cells. Quantitation of GR-expressing cells revealed that GA promoted strongly GR downregulation, with an observed decrease of 60% after a 2 h treatment, and no detectable GR after a 24 h treatment (data not shown). It is worth noting that GA also decreased the number of living cells (~40% after 24 h). We conclude from this that the observed loss of glucocorticoid responsiveness of the reporter gene is more likely to reflect an overall decrease in GR expression rather than a loss of ligand binding capacity. We characterized then the subcellular localization of GR in GA-treated cells (Fig. 5A). A 2 h treatment revealed an essentially normal staining pattern in naive or Dex-treated cells. However, a longer exposure (6 h) evidenced two types of GR localization. The first type (referred to as "punctate") was observed irrespective of the presence of Dex and was characterized by the occurrence of GR aggregates in the cytoplasm. These aggregates were not observed with cells expressing EGFP, showing that these aggregates are formed through the GR moiety of the EGFP-GR fusion protein, and were reduced in size, but not eliminated upon treatment with lactacystin, a proteasome inhibitor (data not shown). The second staining pattern (referred to as "fuzzy") suggested that GR was unable to translocate completely into the nucleus in response to Dex treatment, a phenomenon likely to reflect the occurrence of misfolded GR. Thus GA treatment caused either aggregation of GR in the cytoplasm, or interfered with GR ligand-induced translocation. The importance of GR relocations were quantified in the presence of Dex (Fig. 5B). It turned out that the fuzzy pattern was observed at low GA concentrations and remained constant at higher concentrations, representing 70% of GR-expressing cells. The punctate pattern was less abundant, with 25% of GR-expressing cells. Time-course experiments did not allow to establish whether a pattern appeared prior to the other. How-

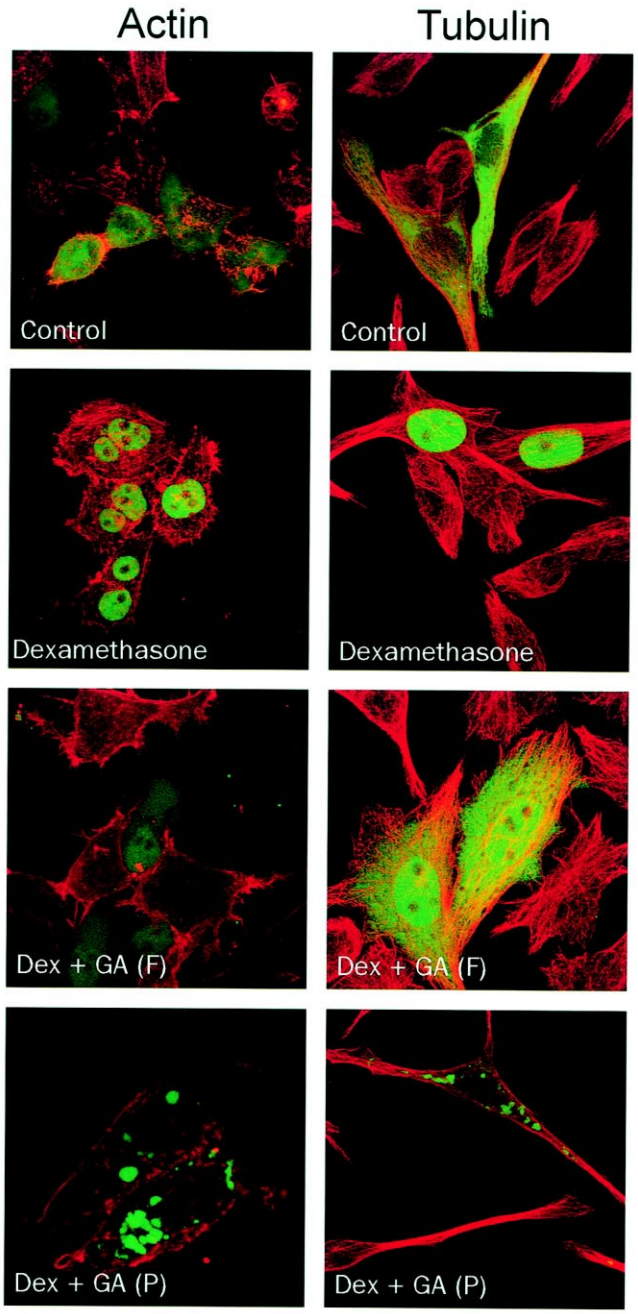


Fig. 6. GA disrupts the actin network: EGFP-rGR expressing cells were treated for 6 h with 0.2 μM GA, with or without 100 nM Dex. Actin and tubulin were detected using TRITC-conjugated phalloidin or anti-tubulin antibodies and networks visualized by confocal microscopy as described in the Section 2.

ever, since the outcome of GA treatment is the down-regulation of GR and considering that aggregation is an irreversible phenomenon, and probably related to impaired chaperoning, we favor the hypothesis that the punctate pattern represents the end point of misfolded GR degradation.

3.5. GA affects selectively actin network organization

hsp90 is known to bind to components of the cytoskeleton such as actin [44] and tubulin [45], and hsp90 inhibition could predictably lead to an alteration of the cytoskeletal network. The integrity of microfilaments (actin), microtubules (tubulin) and intermediate filaments (vimentin) was therefore characterized prior to and after GA treatment (Fig. 6). While vimentin (data not shown) and tubulin (Fig. 6) networks displayed an unaltered organization in the presence or absence of Dex and GA, actin filaments were much less abundant [Fig. 6, Dex + GA (P)] or even absent in GR expressing cells harboring the fuzzy pattern [Fig. 6, Dex + GA(F)]. We noted however that some cells presented a normal phenotype, suggesting that additional physiological parameters may regulate the sensitivity of cells to GA. The potential role of the actin network in conditioning GR responsiveness is not clear at present, since GR has been shown to translocate to the nucleus in cells treated with drugs disrupting the cytoskeleton [46,47]. We note that this disruption was concomitant to receptor aggregation, but the relation between these two phenomenon is still unclear. However, the integrity of the actin network is necessary to observe glucocorticoid responsiveness of the glutamine synthetase gene [48], suggesting that an interaction between actin and GR is a prerequisite for GR-mediated transcriptional activation.

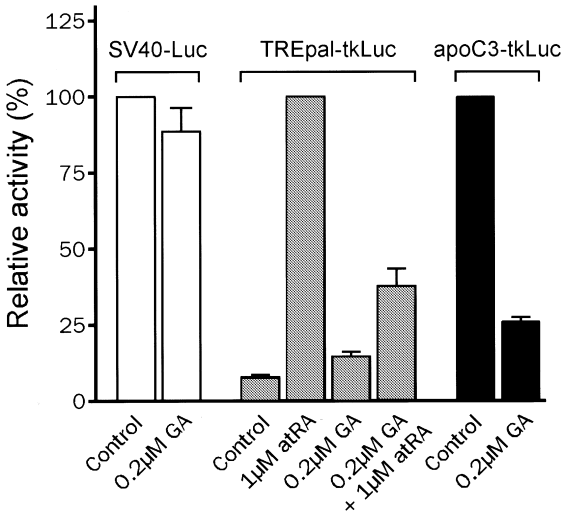


Fig. 7. Transcriptional activity of various promoters in response to GA treatment: HeLa cells were transfected with the indicated reporter gene and with hRARα and hRXRα expression vectors (TREpal tk Luc) or pSG5-hHNF4 (apoC3-tkLuc). Luciferase activities were assayed after a 18 h induction and bars are the average of at least two experiments (± S.D.) carried out in quadruplicate.

3.6. GA inhibits the transcriptional activity of nuclear receptors

Nuclear receptors (in opposition to steroid receptors) such as RAR, T_3R do not associate detectably with the hsp90-containing chaperoning complex [49]. However, indirect evidence suggest that RXR/RAR heterodimers activity is conditioned by intracellular levels of hsp90-mediated chaperoning [50]. We thus asked whether hsp90 inhibition could affect RAR activity, as well as that of HNF-4, an orphan receptor displaying a high constitutive activity. As shown in Fig. 7, the activity of a SV40-driven reporter gene was not affected by GA treatment. On the opposite, the transcriptional activity of RAR/RXR and HNF-4 was abolished by GA. Thus sensitivity to GA inhibition is a feature common to nuclear receptors, suggesting a dependance of these signalling pathways on specific cellular chaperoning activities. We cannot however rule out at this point a possible contribution from other signalling pathways, such as a possible effect of the inhibition of the ras/raf pathway on nuclear receptors activity.

Thus examination of EGFP-rGR expressing cells allowed to study GR shuttling between cytoplasm and nuclei in response to various steroidal or non steroidal GR modulators. In particular, immunosuppressants induced a partial glucocorticoid-independent nuclear translocation of GR, whereas GA triggered GR degradation and promoted its aggregation in the cytoplasm, a phenomenon likely to reflect improper folding of the receptor. GA also affected the activity of RAR, RXR and HNF-4, suggesting that hsp90-mediated chaperoning is required for nuclear receptors activity. While these observations do not fully elucidate the mechanism of action of these drugs, they give valuable piece of information on their effect on GR subcellular localization, and refinements in data analysis will allow both quantitative and qualitative approaches to be undertaken. In addition, our data provide clear conclusions about the subcellular localization of GR, which despite intense investigations, was much debated. The lack of clear-cut data is often attributed to the diversity of biological systems, to the mode and rate of expression of the receptor and mainly to different cell fixation protocols followed by immunochemical detection (reviewed in [51]). Moreover, cross-reactivity of antibodies with the constitutively nuclear GRβ which was not systematicaly addressed in early studies may account for the reported nuclear localization of GRα. Indeed, the use of a specific anti-GRα

antibody demonstrated a cytoplasmic localization of GRα [52]. Thus many of these drawbacks are due to technical problems that can be avoided by using a direct detection method allowing visualization of receptors in living or fixed cells. Obviously, GFP fusion proteins are a powerful tool to achieve such a goal.

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