Cytosolic glucocorticoid receptor interaction with nuclear factor- κ B proteins in rat liver cells

Christina WIDÉN, Jan-Åke GUSTAFSSON and Ann-Charlotte WIKSTRÖM¹

Department of Medical Nutrition, Karolinska Institute, NOVUM, S-141 86 Huddinge, Sweden

The glucocorticoid receptor (GR) acts as an anti-inflammatory factor. To a large extent, this activity is exerted by the interference of pro-inflammatory nuclear factor κ B (NF- κ B) activity. In their respective inactive forms, both GR and NF- κ B reside in the cytoplasm and translocate to the nucleus on relevant stimulation. Previously, p65, a component of the NF- κ B complex, and GR have been shown to interact physically *in vitro*, and the interaction is assumed to take place in the nucleus of cells [McKay and Cidlowski (1999) *Endocrine Rev.* 20, 435–459]. We have studied the interaction between GR and NF- κ B using *in vivo*-like conditions. Using immunoaffinity chromatography or immunoprecipitation, combined with Western blotting, we observed that, with endogenous protein levels in cytosolic extracts of rat liver and of H4-II-E-C3 hepatoma cells and in contrast with the current belief, p65, p50 and inhibitory κ B α complex interact with GR, even in the absence of glucocorticoid or an inflammatory signal. The interaction between non-liganded/non-

activated GR and p65/p50 has also been verified by both p65 and p50 co-immunoprecipitations. Intracellular localization studies, using Western blotting, revealed that glucocorticoids can decrease tumour necrosis factor α (TNF α)-induced nuclear entry of p65, whereas glucocorticoid-induced GR translocation was much less affected by TNF α . We were also able to demonstrate a nuclear interaction of GR and p65 and p50 using *in vivo*-like protein concentrations. Furthermore, nuclear GR interaction with heat-shock protein 90 was enhanced distinctly by TNF α treatment. In conclusion, our studies suggest a strong interconnectivity between the NF- κ B and GR-signalling pathways where also, somewhat unexpectedly, a physical interaction in the cytosol constitutes an integral part of GR–NF- κ B cross-talk.

Key words: glucocorticoid receptor, heat-shock protein 90, catalytic subunit of protein kinase A, p50, p65.

INTRODUCTION

The glucocorticoid receptor (GR) and the nuclear factor κ B (NF- κ B) are transcription factors with a well-known functional interplay, counteracting each other in the regulation of immune and inflammatory responses [1–3]. Glucocorticoid hormones, via GR, suppress inflammation by inhibiting the transcription of several cytokines, chemokines and cytokine receptors, and via transrepression of a number of inflammatory mediators. Activated NF- κ B, on the other hand, enhances the expression of many of the cytokines and chemokines that are repressed by GR.

When non-activated, both GR and NF- κ B reside in the cytoplasm of most cells. Non-activated GR forms a multi-protein complex in which, among other proteins, a dimer of heat-shock protein (hsp) 90, as well as monomeric hsp70, heat-shock organizing protein, hsp40 and p23 are included (see [4] for a review). The proteins in this multi-protein complex play a role in the function of GR. For example, the dimer of hsp90 keeps the GR in an inactive and optimal conformation for ligand binding [5]. On ligand binding, GR is activated, i.e. GR dissociates from the dimer of hsp90 and the nuclear localization signals of GR are unmasked, and the receptor is capable of translocating into the nucleus and regulates gene transcription [6,7].

Within the cytoplasm, non-activated NF- κ B is associated with an inhibitory protein, inhibitory κ B (I κ B) [8]. NF- κ B is activated by a wide variety of stimuli, including the pro-inflammatory cytokine tumour necrosis factor α (TNF α), oxidative stress, bacterial and viral proteins. NF- κ B activation

leads to phosphorylation and proteolytic degradation of I κ B, thereby unmasking the nuclear localization signal of NF- κ B, which can translocate into the nucleus where it regulates gene expression. NF- κ B is a dimer and the classical and most studied dimeric NF- κ B transcription factor is the p65/p50 heterodimer, of which p65 is the transcriptionally active subunit (see [3,9] for reviews on NF- κ B).

In various experimental systems *in vitro*, GR has been found to associate with the p65 subunit of NF- κ B and the interaction is known to lead to mutual transcriptional antagonism. The interaction and subsequent cross-talk is considered to occur in the nucleus of cells on stimulation of GR and NF- κ B [3], and is known to involve the Rel homology domain of p65 and the DNA-binding domain (DBD) of GR [1,2,10–12]. However, Doucas et al. [13] showed that the mutual cross-repression of NF- κ B and GR requires a cytoplasmic step, and is dependent on the catalytic subunit of protein kinase A (PKAc) signalling. The methods used to investigate functional GR–p65 interaction have relied on the interaction of overexpressed proteins in cell lines and/or experiments *in vitro*. Hence, we decided to investigate whether GR interacts with p65 in the same way under conditions, where GR and its associated partners are present at concentrations comparable with the situation *in vivo*.

Using immunoaffinity chromatographic purification of GR from liver cytosol of adrenalectomized rats [14], we found that p65, p50 and the inhibiting protein, I κ B α , co-purified with GR from rat liver cytosol both when GR was in a non-liganded/non-activated (i.e. in the presence of molybdate) and in a

Abbreviations used: DBD, DNA-binding domain; DTT, dithiothreitol; GR, glucocorticoid receptor; hsp, heat-shock protein; I κ B, inhibitory κ B; mAb, monoclonal antibody; NF- κ B, nuclear factor κ B; NMIGG, normal mouse IgG; PKAc, catalytic subunit of protein kinase A; TA, triamcinolone acetonide; TNF α , tumour necrosis factor α .

¹ To whom correspondence should be addressed (e-mail lotta.wikstrom@mednut.ki.se).

liganded/activated state (i.e. ligand was added to the cytosol before heat activation). The GR and NF- κ B association was further confirmed as GR co-immunoprecipitated with both p65 and p50 in rat liver cytosol. To investigate further the interaction between GR and p65 *in vivo*, we studied how triamcinolone acetonide (TA), a synthetic glucocorticoid and/or the inflammatory mediator, TNF α , affected the GR–p65 interaction in a rat hepatoma cell line. We employed both short-term and overnight treatment of the cells with TA and/or TNF α . We found both cytosolic and nuclear interactions between GR and the NF- κ B family proteins. We also found that GR on the one hand, and p65 and p50 on the other, although they can interact in both cytoplasm and nucleus, could translocate from cytoplasm to nucleus independent of one another.

In summary, we found that GR is associated with p65, p50, I κ B α , hsp70 and hsp90 in the cytosol of rat liver cells. GR could also associate with p65, p50, hsp70 and hsp90 in the nucleus. The cytosolic GR interaction with NF- κ B proteins indicated greater interdependence and cross-control of these two signalling pathways than that was anticipated previously. The repression of stimulated p65 translocation by TA, in contrast with the more robust GR translocation whenever the ligand was present, indicated mechanistic differences as to how these two signalling pathways control one another. Furthermore, an increase in nuclear GR–hsp90 interaction, subsequent to an inflammatory stimulus, pointed to a novel mechanism for an inflammatory stimulus, such as TNF α , to counteract an anti-inflammatory response.

EXPERIMENTAL

Cytosol preparation and immunoaffinity chromatographic purification of rat GR

Rat liver cytosol was prepared from four 6–8-week-old, adrenalectomized female rats (M&B, Ry, Denmark). The livers were perfused with EPG buffer [1 mM EDTA, 20 mM phosphate buffer (pH 7.4), 10 % (v/v) glycerol, 50 mM NaCl and 2 mM dithiothreitol (DTT)], and homogenized in EPG buffer containing 0.5 μ g/ml leupeptin, 1 μ g/ml aprotinin (Roche Diagnostics Scandinavia AB, Stockholm, Sweden), 0.7 μ g/ml pepstatin and 200 μ g/ml PMSF (Sigma, Stockholm, Sweden) and ultracentrifuged at 210 000 *g* for 30 min at 4 °C. To obtain GR in different functional states, the cytosol was prepared as follows: 20 mM Na₂MoO₄ was added to the EPG buffer in all steps for non-liganded/non-activated GR, and 20 nM TA was first added to the cytosol at 4 °C for 1 h, and then the cytosol was diluted 1:2 in EPG buffer and heat-activated at 25 °C for 30 min, for liganded/activated GR. The cytosol was filtered and then, at 4 °C, run through (1) a column containing 1 ml of Sepharose 4B, (2) a column with 1 ml of normal mouse IgG (NMIgG) antibodies coupled with CNBr-activated Sepharose 4B (referred to as the NMIgG column) and (3) a column with 1 ml of GR-specific mouse monoclonal antibody (mAb) 250 (originally called mAb 7) [15] coupled with CNBr-activated Sepharose 4B (referred to as the 250 column). Chromatography columns were purchased from Bio-Rad Laboratories (Stockholm, Sweden), and Sepharose 4B as well as CNBr-activated Sepharose 4B was from Pharmacia (Uppsala, Sweden). After washing with 15 ml of EPG buffer/50 mM NaCl, 15 ml of EPG buffer/150 mM NaCl (salt wash), and 15 ml of EPG buffer/50 mM NaCl respectively, 4 column volumes of the corresponding peptide D-12-E synthesized by Neosystem (Strasbourg, France) [16], at a concentration of 1.4 mg/ml in EPG buffer, was used for elution of GR and GR-associated proteins at 4 °C. As the elution efficiency was dependent on the functional state of GR, peptide elution was not

sufficient to elute liganded/activated GR, which required 2.5 M NaSCN in EPG buffer for complete elution. The eluted proteins were precipitated with 0.02 % sodium deoxycholate for 15 min and 20 % (v/v) trichloroacetic acid at 4 °C overnight. After centrifugation at 3000 *g* for 30 min and washing with ice-cold acetone, the proteins were resuspended in sample dilution buffer.

SDS/PAGE, Western-blot and immunostaining analyses

The proteins were run on SDS/polyacrylamide gel [7 or 9 % (v/v) gel] under reducing conditions and blotted on to PVDF membrane (Bio-Rad Laboratories). The membrane was stained with Coomassie Brilliant Blue R 250[®] (Fluka, Stockholm, Sweden), cut to obtain 1 lane/strip and destained in methanol. After blocking with 5 % (w/v) defatted dried milk, the strips were incubated with the following primary antibodies: anti-GR mAb 250 (also called mAb 7) [15], and anti-p65, anti-I κ B α and anti-c-Rel (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti-p50 (StressGen Biotechnologies Corp., Victoria, BC, Canada) respectively, in PBS and 1 % defatted dry milk for 2 h at room temperature (20 °C). After washing in washing buffer (PBS and 0.5 % Tween 20), the PVDF strips were incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Uppsala, Sweden and Bio-Rad Laboratories) diluted in PBS and 1 % defatted dried milk for 1 h. The strips were again washed in washing buffer and incubated with SuperSignal[®] (Pierce, Rockford, IL, U.S.A.) for 5 min. The signals were detected according to the manufacturer's instructions. Occasionally, SuperSignal[®] WestFemtoMaximumSensitivitySubstrate (Pierce) was used to detect proteins instead of SuperSignal[®] Substrate.

Co-immunoprecipitation of GR using antibodies directed against p65 and p50

Liver cytosols were prepared as described above, and 10 mM Tris/HCl (pH 7.8), 0.1 % Nonidet P40, 0.15 M NaCl and 1 mM EDTA were added to the cytosol, which was then preabsorbed with agarose-coupled normal rabbit IgG (Santa Cruz Biotechnology). Total amount of protein (1000 μ g) of the preabsorbed cytosol was diluted to a total volume of 300 μ l in 10 mM Tris/HCl (pH 7.8), 0.1 % Nonidet P40, 0.15 M NaCl, 1 mM EDTA, 0.5 mM PMSF and 0.5 μ g/ml each of aprotinin, leupeptin, pepstatin and 2 μ g of antibodies directed against p65, p50-1 or p50-2 (sc-109, sc-7178 or sc-114 respectively; Santa Cruz Biotechnology) were added. After 1 h, 20 μ l of resuspended volume of Protein A/G–agarose (Santa Cruz Biotechnology) was added and after another 3 h, the pellets were washed three times in PBS. Loading buffer (20 μ l) was added to each sample and the samples were boiled before SDS/PAGE and Western-blot analysis. As negative control, 2 μ g of agarose-coupled normal rabbit antibodies were used. All steps were performed at 4 °C.

Salt-gradient analysis of GR and GR co-purifying proteins

Liver cytosols from adrenalectomized rats were prepared to contain either non-liganded/non-activated or liganded/activated GR as described above, and the cytosols were run, in parallel, through three subsequent columns: Sepharose, NMIgG and 250 columns respectively. A stepwise NaCl gradient elution of the 250 columns was performed using EPG buffer with the following increasing salt concentrations: 30 + 20 ml of 50 mM, 20 ml of 300 mM, 20 ml of 600 mM, 20 ml of 1.2 M and 20 ml of 2.4 M. Thereafter, a 20 ml of 50 mM wash followed by a 5 ml peptide

elution and a 10 ml NaSCN elution was performed. The salt-eluted fractions (other than the first 30 ml of the first washing with 50 mM), as well as the peptide and NaSCN-eluted fractions, were precipitated as described above and analysed by SDS/PAGE and Western blotting.

GR co-immunoprecipitation studies in rat hepatoma cells

Rat hepatoma cells H4-II-E-C3 (A.T.C.C., Manassas, VA, U.S.A.) were grown to 75 % confluency in RPMI medium, supplemented with 7.5 % (v/v) foetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin at 37 °C in 5 % CO₂. Then, the cells were exposed to: only the medium as a control, 20 nM TA, 200 units/ml TNF α (Boehringer, Indianapolis, IN, U.S.A.), and 20 nM TA and 200 units/ml TNF α either for 15 min overnight (18 h) or for 30 min. The cells were pelleted and homogenized in homogenizing buffer [50 mM potassium phosphate buffer (pH 7.6), 10 % glycerol, 10 mM Na₂MoO₄, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF and 0.5 μ g/ml of each aprotinin, leupeptin, pepstatin, antipain, N- α -tosyl-L-lysylchloromethane and 0.25 μ g/ml N-tosyl-L-phenylalanylchloromethane). After centrifugation at 800 g for 20 min, the supernatant was decanted and further centrifuged at 100 000 g for 40 min to obtain the cytosol. To obtain the nuclear extract, the pellet was washed twice, first in washing buffer [10 mM Tris/HCl (pH 7.4)/0.33 M sucrose/3 mM MgCl₂/10 mM Na₂MoO₄] containing 1 % Triton X-100, followed by two more washes in washing buffer without Triton X-100. The pellet was resuspended in extraction buffer [50 mM potassium phosphate buffer (pH 7.6)/10 % glycerol/0.4 M KCl/1 mM EDTA/1 mM DTT] and extracted on ice for 1 h with periodical mixing. After centrifugation at 100 000 g for 30 min and dialysis of extraction buffer for 1–2 h, the nuclear extract was obtained. In the studies shown in Figure 6, dilution instead of dialysis was used to obtain a buffer containing 40 mM KCl. The protein concentration of the extracts was determined. The cytosol and the nuclear fractions were preabsorbed with NMIG coupled with CNBr-activated Sepharose 4B. The preabsorbed fractions were immunoprecipitated using mAb 250, linked to CNBr-activated Sepharose 4B for 2 h. Both NMIG- and 250-coupled Sepharoses were washed with 0.5 ml of EPG buffer/50 mM NaCl, 0.5 ml of EPG buffer/150 mM NaCl (salt wash), and 0.5 ml of EPG buffer/50 mM NaCl before sample dilution buffer was added. The samples were boiled before analyses by SDS/PAGE and Western blot as described above. The anti-PKAc antibody was purchased from Transduction Laboratories (Lexington, KY, U.S.A.). All steps were performed at 4 °C.

Time kinetic studies in rat hepatoma cells

Rat hepatoma cells were grown as described above and exposed to: only medium as a control, 20 nM TA, 200 units/ml TNF α , and 20 nM TA and 200 units/ml TNF α for 0, 30, 60 and 120 min, or for 8 and 24 h. Cytosol and nuclear extracts from the cells were obtained as described above, and the protein concentration of the extracts was determined. Cytosol (45 μ g) and the nuclear extract (20 μ g) were loaded on to each well, and separated by SDS/PAGE and analysed by Western blotting.

RESULTS

p65 and I κ B α co-purify with GR from rat liver cytosol

To study cytosolic GR-interacting proteins, we have earlier [14] described and established a GR immunoaffinity chromatographic

purification method, where GR is purified from cytosol prepared from livers of adrenalectomized rats. Briefly, cytosol is prepared and pre-purified, first on a Sepharose column followed by a column containing NMIG immobilized on a Sepharose matrix. The pre-purified cytosol is subsequently applied to a column containing mAb 250 [15]. A peptide corresponding to the identified epitope for mAb 250 [16] is used for elution of GR, and alternatively NaSCN is used as an eluent [14]. The peptide-eluted GR and its co-purified proteins were separated using SDS/PAGE and analysed by Western blotting. The advantage of using such a method to investigate GR and GR-interacting proteins is that the method is based on *in vivo* like conditions, i.e. native proteins at native concentrations.

There are several reports in the literature which show that GR and NF- κ B interact physically [1,10,11]. These studies rely on different *in vitro* methods using, for example, immunoprecipitations of nuclear extract from HeLa cells overexpressing GR and p65 [10], immunoprecipitations of whole-cell extract from COS cells co-transfected with GR and p65 [11] or immunoprecipitations of translated p65 and GR *in vitro* [1].

We decided to investigate whether p65 or c-Rel (another member of the mammalian Rel family [3]), and/or their dimerizing partner p50 could associate with GR in the liver cytosol of adrenalectomized rats. Since I κ B α anchors non-activated p65 in the cytoplasm, we also investigated whether I κ B was associated with GR as well. After immunoaffinity chromatographic purification of GR from rat liver cytosol, Western-blot analysis showed that p65, p50 and the inhibitor protein I κ B co-purified with GR in a non-liganded/non-activated state (Figure 1A), i.e. when molybdate was added to the cytosol. p65, p50 and I κ B also interacted with GR, purified from the liver cytosol of adrenalectomized rats, in a liganded/activated state (Figure 1B), i.e. when TA was added to the cytosol before heat activation. Although c-Rel is present in the rat liver [17], GR did not bind to c-Rel in our experiment. This lack of c-Rel–GR interaction may emphasize the specificity of the p65 interaction. As detailed studies of the interacting domains of p65 and GR have been performed, we assume that p65 and GR interactions are direct. The nature of the GR interactions with p50 and I κ B, however, are possibly indirect, via p65, but direct interactions cannot be excluded.

GR co-immunoprecipitates with p65 and p50

By using a rabbit polyclonal antibody directed against p65 and two different rabbit polyclonal antibodies directed against two different regions of p50, we performed a set of co-immunoprecipitation studies to confirm further that GR associates with the NF- κ B complex in rat liver cytosol. The p65 and p50 co-immunoprecipitates were analysed by SDS/PAGE followed by Western blotting. Indeed, GR was found to co-immunoprecipitate with all three of the polyclonal antibodies directed against NF- κ B complex proteins (Figure 1C). No GR was found to immunoprecipitate with normal rabbit IgG (results not shown). We also investigated the supernatant to examine how much GR did not co-immunoprecipitate with the p65, p50-1 and p50-2 antibodies. We found that most of the GR within the liver cytosol is not actually bound to NF- κ B.

GR interaction with p65 and I κ B α in rat liver cytosol withstands high-salt washing

To examine the strength of the GR interaction with p65 and I κ B α , a stepwise salt elution with increasing concentrations of NaCl from 50 mM to 2.4 M was performed. Liver cytosol

Figure 1 p65, p50 and IκBα associate with both non-liganded/non-activated and liganded/activated GR in rat liver cytosol

(A, B) Cytosol of four livers from adrenalectomized rats was prepared and divided into two equal parts. To one part, sodium molybdate was added to obtain GR in a non-liganded/non-activated state (A), and to the other part ligand was added and heat activation was performed to obtain GR in a liganded/activated state (B). The cytosols were purified using an immunoaffinity chromatographic method [14]. Western-blot analysis was performed to detect GR and p65, p50, c-Rel and IκBα. Molecular-mass markers are shown in kDa. (C) Liver cytosol containing non-liganded/non-activated GR was prepared from adrenalectomized rats as described in the Experimental section and immunoprecipitations (ip) were performed using 2 μg of polyclonal antibody directed against p65, p50-1 or p50-2. The p65 and p50 immunoprecipitates were separated and analysed using Western-blot analysis. Immunostaining showed that GR co-immunoprecipitated with p65, p50-1 and p50-2. The supernatant (s) shows the fraction of GR that has not co-immunoprecipitated with the p65, p50-1 and p50-2 antibodies respectively. The positive control (+) is 5% (50 μg of total protein) of input (1000 μg of total protein).

from adrenalectomized rats was prepared to contain either non-liganded/non-activated GR (Figure 2A) or liganded/activated GR (Figure 2B) and the cytosols were run, in parallel, through three subsequent columns: Sepharose, NMIgG and 250-columns respectively. A stepwise salt gradient elution of the 250-columns was performed using EPG buffer with increasing salt concentrations: 50 mM, 300 mM, 600 mM, 1.2 M and 2.4 M, followed by a salt washing out using 50 mM NaCl. Thereafter, elution with the D-12-E peptide followed by an NaSCN elution was performed. The salt-eluted fractions as well as the peptide and NaSCN-eluted fractions were precipitated and analysed by SDS/PAGE and Western blotting. We found that even after exposure to 2.4 M NaCl, p65 and IκBα were still associated with GR and co-eluted with GR when using the peptide D-12-E for elution as well as with the following GR elution using NaSCN, both when GR was non-liganded/non-activated and when GR was in a liganded/activated state. We also found that part of the cytosolic p65 and IκBα did not seem to be associated with GR (Figures 2A and 2B, lane 2). These results indicate that the interactions among GR, p65 and IκBα in rat liver cytosol are

strong, but that a significant fraction of p65 and IκBα is not GR-associated.

Early effects of TA and TNFα treatment on localization and GR–NF-κB protein interaction

Intrigued by the cytosolic association between GR and NF-κB proteins, we studied the functional consequences of this interaction in a cell-culture system. This was also performed under conditions where the cells were challenged with the respective activator for GR and NF-κB by the addition of the GR ligand TA and/or the NF-κB activator TNFα respectively. The experiments were performed with the rat hepatoma cell line H4-II-E-C3, expressing native levels of GR and NF-κB proteins. The cells were grown to 70% confluency and thereafter exposed to either only the medium as a control, TA, TNFα or a combination of TA and TNFα for 15 min. Cytosolic and nuclear extracts were prepared and immunoprecipitations were performed using the anti-GR mAb 250. Western-blot analysis of the GR precipitates, as presented in Figure 3(A), showed that GR could be immunoprecipitated mainly from the cytosol of cultivated cells (Figure 3A, lanes 1a–1d), and a small amount was found in the nucleus, even in the absence of ligand (Figure 3A, lane 2a). A fraction of GR translocated to the nuclear compartment after 15 min treatment with TA (Figure 3A, lane 2b). The combined addition of TA and TNFα for 15 min led to a less pronounced GR nuclear translocation as compared with the treatment to TA alone. This may reflect that activated p65, at least at this early time point, could decrease GR entry into the nucleus. p65 co-immunoprecipitated with the cytosolic fraction of GR with or without TA (Figure 3A, lanes 1a and 1b) in a similar fashion as in rat liver cytosol (Figure 1). Interestingly, p65 was also found to co-immunoprecipitate with GR from the cytosol on treatment with TNFα (Figure 3A, lanes 1c and 1d). Normally, p65 resides in the cytoplasm of cells and, on treatment with TNFα, p65 translocates to the nucleus. NF-κB translocation is reported to occur with a half-life of 7–8 min in HeLa cells and 12–13 min in chondrocytes [18]. However, in the HTC cells we used, we were not able to detect any p65 in the nucleus 15 min after TNFα treatment, either as free p65 (results not shown) or co-immunoprecipitating with GR (Figure 3A, lanes 2c and 2d). IκBα co-immunoprecipitated with GR from the cytosol independently of TA and TNFα (Figure 3A, lanes 1a–1d). These results indicate that the GR that translocated to the nucleus after treatment with TA and TNFα in combination did not bring along p65 into the nucleus, and thus may represent either a GR population that is not interacting with p65, or a GR population that detaches from p65 on glucocorticoid treatment.

Doucas et al. [13] have shown previously that PKAc and GR are associated, using pull-down experiments as well as immunoprecipitation of GR, overexpressed in HEK-293 cells [13]. They also suggested that the interaction between NF-κB and GR is located in the cytoplasm and dependent on PKAc. Therefore we also investigated if PKAc could co-immunoprecipitate with GR using our system. Indeed, we could detect PKAc in the GR co-immunoprecipitate from the cytosol of the rat hepatoma cells (Figure 3A, lane 1a). However, the PKAc signal was not enriched compared with the input fraction (Figure 3C), thus indicating a weak association with GR.

As a negative control experiment to ascertain that the co-immunoprecipitations are specific, NMIgG antibodies coupled with Sepharose, used for preabsorption, were boiled in sample dilution buffer, run on SDS/polyacrylamide gel and analysed by Western blotting (Figure 3B). Neither GR, p65, IκBα nor PKAc was detected, which indicates that the GR

Figure 2 p65 and I κ B α interaction with GR is maintained at high-salt conditions

A salt elution profile was performed to investigate the strength of the interaction among GR, p65 and I κ B α . Liver cytosol from adrenalectomized rats was prepared to contain either non-liganded/non-activated (A) or liganded/activated (B) GR. A stepwise salt gradient elution of GR was performed using EPG buffer with increasing salt concentrations: 50 mM, 300 mM, 600 mM, 1.2 M, 2.4 M and 50 mM as indicated in the Figure. Thereafter, a peptide elution (D-12-E) followed by an NaSCN elution was performed as described in the Results section. The salt-eluted fractions, apart from the first 30 ml of the first washing with 50 mM, as well as the peptide and NaSCN-eluted fractions, were precipitated and analysed by Western-blot analysis after SDS/PAGE separation using antibodies raised against GR, p65 and I κ B α . Lane 1 represents the cytosol before applying it to the columns and lane 2 contains the flow-through. Both lanes 1 and 2 contain 50 μ g of total protein respectively.

co-immunoprecipitation was specific. Figure 3(C) shows 1 % of the total protein input.

Effects of TA and TNF α treatment on localization of GR/NF- κ B and on GR–NF- κ B protein interaction after overnight treatment

We studied long-term effects of the treatment with TA and/or TNF α on GR and NF- κ B localization and interaction. Rat hepatoma cells were grown to 70 % confluency and thereafter exposed either only to the medium, as a control, TA, TNF α or a combination of TA and TNF α overnight (18 h). Cytosolic and nuclear extracts were prepared and immunoprecipitations were performed. Western-blot analysis, as presented in Figure 4, showed that GR could be immunoprecipitated mainly from the cytosol, but also to a certain extent from the nucleus even in the absence of TA (Figure 4A, lanes 1a and 2a). When adding TA, GR translocated into the nucleus and could be immunoprecipitated mainly from the nucleus, but also from the cytosolic fraction (Figure 4A, lanes 1b and 2b), indicating that, after overnight exposure to TA, a fraction of GR either remains in or re-enters the cytosolic compartment. p65 was capable of associating with GR in the cytosol independently of TA and/or TNF α (Figure 4A, lanes 1a–1d). I κ B α also co-immunoprecipitated with GR from cytosol when TA and/or TNF α was added (Figure 4A, lanes 1a–1d). Interestingly, nuclear p65, activated by TNF α , co-immunoprecipitated with GR in the nuclear fraction (Figure 4A, lane 2c). Furthermore, adding a combination of TA and TNF α overnight, resulted in GR translocation to the nucleus, but no further enhancement of p65 co-immunoprecipitation in the nuclear fraction (Figure 4A, lane 2d). In this experiment, we detected PKAc in the GR co-immunoprecipitate from the cytosol of the rat hepatoma cells irrespective of TA and/or TNF α . However, compared with the input control (Figure 4C, lanes 1a–1d), the signals in Figure 4(A), lanes a–d, are quite weak and not as enriched as, e.g. the I κ B α signals, which were not even detectable in the input control. This result also indicates that GR and PKAc could interact but that their interaction is probably weak. As a negative control experiment, the NMIgG antibodies coupled with Sepharose used for preabsorption were boiled in sample dilution buffer and run on SDS/polyacrylamide gel and analysed by Western blotting as well (Figure 3B). Figure 3(C) shows 1 % of the total protein input. The missing input control

signal of p65 from the cytosol of cells treated with TA and TNF α is due to a technical problem (Figure 4C, lane 1d).

Time kinetic studies of the distribution of GR and p65 in the cytosol and the nuclear compartment after addition of TA or TNF α , or a combination of TA and TNF α

To evaluate further our results shown in Figures 3 and 4, we studied the time kinetics of GR and p65 entry to the nucleus after challenge. Therefore we examined the cellular distribution of GR and p65 at various time points; rat hepatoma cells were grown to 70 % confluency and thereafter exposed to either only the medium as a control, TA, TNF α , or TA and TNF α in combination for 0, 30 or 60 min or for 2, 8 or 24 h. Cytosolic and nuclear extracts were prepared and analysed by Western blotting, as presented in Figure 5. We found that without treatment (–) both GR and p65 reside mainly in the cytoplasmic compartment (Figure 5, –, lanes 1–6, for both GR and p65). Addition of TA to the medium for 30 min was sufficient for GR to translocate from the cytosol to the nucleus (Figure 5, GR and TA) and after 2 h exposure to TA, most of GR resided in the nuclear compartment. Treatment with TA did not lead to nuclear translocation of p65 at any time point. On treatment with TNF α , a maximal presence of nuclear p65 was observed after 30 min, whereas GR remained in the cytosol. These results also indicate that most of the p65 resides in the cytosol and that only a fraction of p65 enters the nuclear compartment on treatment with TNF α . Treatment with TA and TNF α in combination showed that GR entered the nucleus in the same way as treatment with TA alone indicating that GR enters the nucleus independently of p65. However, p65 did enter the nucleus on treatment with TA and TNF α in combination, but not to the same extent as on treatment with TNF α alone. TA treatment seemed to decrease p65 entry to the nuclear compartment. These results indicate that cross-repression is mechanistically different for GR and p65.

Cytosolic and nuclear GR interaction with NF- κ B and hsp family proteins after TA and TNF α treatment for 30 min

Since the results in Figure 5 showed that both GR and p65 entered the nucleus on treatment with TA and TNF α for 30 min, we chose this time point for further investigation of

Figure 3 GR co-immunoprecipitation studies with the cytosol and the nuclear extract of rat hepatoma cells after TA, TNF α or TA and TNF α treatment for 15 min

Rat hepatoma cells were grown to 70 % confluency and thereafter exposed to only medium (a) or medium containing 20 nM TA (b), 200 units/ml TNF α (c), or 20 nM TA and 200 units/ml TNF α (d) for 15 min. Cytosolic (1) and nuclear (2) extracts were prepared as described in the Experimental section, and the total protein concentration of the fractions was estimated to ensure loading of equal protein amounts: cytosolic fractions (1) lanes a–d, 4.4 mg each and the nuclear fractions (2) lanes a–d, 0.63 mg each. Three independent GR co-immunoprecipitations were performed using 30 μ l (cytosolic fraction) and 20 μ l (nuclear fraction) of Sepharose coupled with mAb 250 (A). The GR co-immunoprecipitates were separated and analysed using Western blotting with antibodies as indicated in the Figure and described in the Results section (A). The Figure shows a representative blot from one of the experiments. As a negative control, 30 μ l (cytosolic fraction) and 20 μ l (nuclear fraction) of Sepharose coupled with antibodies directed against NM1gG was used (B). (C) Shows 1 % of total protein input.

Figure 4 GR co-immunoprecipitation studies with cytosol and nuclear extract of rat hepatoma cells after TA, TNF α , or TA and TNF α overnight treatment

Rat hepatoma cells were grown to 70 % confluency and thereafter exposed to only medium (a) or medium containing 20 nM TA (b), 200 units/ml TNF α (c) or 20 nM TA and 200 units/ml TNF α (d) overnight for 18 h. Cytosolic (1) and nuclear (2) extracts were prepared as described in the Experimental section and the total protein concentration in the fractions was: (1) cytosolic fractions: lane a, 6.1 mg; lane b, 4.0 mg; lane c, 4.2 mg; lane d, 6.4 mg; (2) nuclear fractions: lane a, 1.2 mg; lane b, 1.8 mg; lane c, 0.83 mg; lane d, 1.6 mg. GR co-immunoprecipitation was performed using 75 μ l (cytosolic fractions) and 25 μ l (nuclear fractions) of Sepharose coupled with mAb 250 (A). The GR co-immunoprecipitates were separated and analysed using Western blotting with antibodies as indicated in the Figure and described in the Results section (A). The Figure shows a representative blot from one of three independent experiments. As a negative control, 75 μ l (cytosolic fraction) and 25 μ l (nuclear fraction) of Sepharose coupled with antibodies directed against NM1gG were used (B). (C) Shows 1 % of total protein input.

Figure 5 Time kinetic studies of the distribution of GR and p65 in the cytosol and the nuclear compartment in rat hepatoma cells after addition of TA, TNF α , or a combination of TA and TNF α

Rat hepatoma cells were grown to 70% confluency and thereafter exposed to only the medium (—); or medium containing 20 nM TA (TA); 200 units/ml TNF α (TNF); or 20 nM TA and 200 units/ml TNF α in combination (TA and TNF) for 0 min (1), 30 min (2), 60 min (3) or 120 min (4) and for 8 h (5) or 24 h (6). Cytosolic (Cyt) and nuclear (NE) extracts were prepared as described in the Experimental section and analysed by Western blotting using antibodies directed against GR and p65.

Figure 6 GR co-immunoprecipitation studies with cytosol and nuclear extract of rat hepatoma cells after TA, TNF α , or TA and TNF α treatment for 30 min

Rat hepatoma cells were grown to 70% confluency and thereafter exposed to only the medium (a) or medium containing 20 nM TA (b), 200 units/ml TNF α (c), or 20 nM TA and 200 units/ml TNF α (d) for 30 min. Cytosolic and nuclear extracts were prepared as described in the Experimental section and the total protein concentration of the fractions was estimated: cytosolic fractions 2 mg (1) and nuclear fractions 350 μ g (2). GR co-immunoprecipitation of the cytosolic (1) and nuclear fraction (2) was performed using 30 and 20 μ l respectively of Sepharose coupled with mAb 250. The GR co-immunoprecipitates were separated and analysed using Western blotting using antibodies as indicated in the Figure and described in the Results section. The Figure shows a representative blot from one of three independent experiments.

GR and NF- κ B interactions. We investigated the GR co-immunoprecipitation pattern for a panel of proteins, including p65, p50, I κ B α , hsp90 and hsp70. Again, we used rat hepatoma cells, exposed to TA, TNF α or TA and TNF α in combination for 30 min. Cytosolic and nuclear extracts were prepared and immunoprecipitations were performed. Western-blot analysis of the GR precipitates, as presented in Figure 6, shows that, in the absence of treatment, most GR resides in the cytosol but a small

fraction of GR was found in the nuclear extract. When the cells were treated with TA, GR entered the nucleus but a considerable amount of GR remained in the cytosolic fraction. Treating the cells with TA and TNF α in combination for 30 min did not seem to affect the cellular localization of GR compared with TA treatment alone. p65 co-immunoprecipitated with GR from the cytosol independently of any treatment, which is in line with the results described above. After treatment with TNF α for 30 min, p65 co-immunoprecipitated with GR from the nuclear extract. In this experiment, in line with the results presented in Figure 4, it seemed that p65 co-immunoprecipitated with GR in the nuclear extract on combined treatment with TA and TNF α , but not to the same extent as after treatment with TNF α alone (Figure 6, lanes 2c and 2d).

Using a very sensitive substrate, SuperSignal[®] WestFemto-MaximumSensitivitySubstrate, we were able to detect p50 in the GR co-immunoprecipitate. Our results show that p50 was associated with GR in the untreated cytosol and after treatment with TA. On treatment with TNF α or combined TA and TNF α for 30 min, the GR–p50 association in the cytoplasm ceased, but instead p50 appeared as a GR co-purifying protein in the nucleus. I κ B α co-purified with GR from the cytosol independent of any treatment (results not shown).

In this experiment, we also investigated the GR co-immunoprecipitation pattern of hsp90 and hsp70. hsp90 co-immunoprecipitated with GR in the cytosolic fraction independent of TA and/or TNF α , but we also detected a weak association between GR and hsp90 in the nucleus from untreated cells and cells treated with TNF α (Figure 6, lanes 2a and 2c). hsp70 was found to co-immunoprecipitate with GR, independently of treatment, in the cytosol and in the nucleus, although the co-immunoprecipitation signals from the nucleus were much weaker after treatment with TA. The findings of hsp90 and hsp70 as GR-co-immunoprecipitating proteins in the nucleus in the control panel, i.e. with no treatment (Figure 6, lane 2a), may represent non-liganded/non-activated GR, as non-liganded/non-activated GR is capable of shuttling between the cytoplasm and the nucleus [19]. After treatment with TNF α for only 30 min, hsp90, in repeated experiments, was found to co-immunoprecipitate with

GR from the nucleus (Figure 6, lane 2c) and, compared with the control, addition of TNF α for 30 min seemed to enhance the GR–hsp90 interaction. After treatment with TA or TA and TNF, hsp90 dissociated completely from GR in the nucleus (Figure 6, lanes 2b and 2d). The input control showed the same amount of hsp90 in the nucleus regardless of treatment (results not shown).

DISCUSSION

It has been determined previously that GR and p65 interact physically *in vitro*, and that this interaction involves the Rel homology domain of p65 [20] and the DBD of GR [12]. Proposed mechanisms for NF- κ B and GR cross-talk include direct physical interaction in the cell nuclei, resulting in mutual loss of DNA-binding ability, competition for a limiting common transcriptional co-activator or induced synthesis of I κ B α by GR (see [3] and references therein). Recently, a study suggested that transrepression of GR and p65 may depend on a cytoplasmic event that requires PKAc and a PKA phosphorylation site in p65 [13]. Furthermore, a study by Nissen and Yamamoto [21] shows that GR interferes with phosphorylation of Ser-2 of the RNA polymerase II C-terminal domain required for transcription–initiation via the interleukin-8 and intracellular adhesion molecule-1 promoters, and in this fashion GR could repress NF- κ B activated by TNF α . The present study, as well as earlier studies by Lidén et al. [22] indicates that GR does not interfere with the NF- κ B DNA binding, but rather with events involving transcriptional activation. The physical interaction between GR and p65 has been observed in several experiments *in vitro* [1,10,11,20], but until now, GR and p65 interaction has not been confirmed using *in vivo* like conditions, and the intracellular localization of their interaction has not been clear.

We have developed a GR purification method previously, where GR and its associated partners are purified from rat liver cytosol [14]. Using this GR purification method, we have shown that GR interacts with a large number of proteins in the cytosol of livers from adrenalectomized rats [14] (E. Hedman, C. Widén, A. Asadi, T. Kieselbach, W. Schröder, J.-Å. Gustafsson and A.-C. Wikström, unpublished work). In the present study, we have shown that GR interacts physically with p65, p50 and I κ B α in the liver cytosol of adrenalectomized rats. It should be noted that we have observed GR–p65 interaction in the cytoplasm also under non-stimulated conditions and not only on activation of GR and NF- κ B [3] as assumed previously. The GR–NF- κ B interaction was confirmed by using antibodies directed against p65 and p50, and we found that GR co-immunoprecipitated with these antibodies. We also investigated the supernatant and found that, *in vivo*, only a small fraction of GR co-immunoprecipitated with p65 and p50, since most GR remained in the supernatant.

Both the GR–p65 and GR–I κ B α interactions withstood 2.4 M NaCl, thus indicating that the interactions among GR, p65 and I κ B α are strong. We also concluded that not every p65 and I κ B α molecule in the cytosol was associated with GR, since we detected both p65 and I κ B α in the flow-through fraction (Figures 2A and 2B, lanes 2) of the immunoaffinity column and in the supernatant after co-immunoprecipitation (results not shown), although all GR bound to the column.

To investigate further the GR and NF- κ B protein interaction, we studied the impact of their respective activators, TA and TNF α , in a rat hepatoma cell line. Our results show that we were able to immunoprecipitate GR from rat hepatoma cellular cytosol and nuclear extract after treatment with TA for 15 min. Furthermore, we detected both p65 and I κ B α as GR co-immunoprecipitating proteins from the cellular cytosolic extract independently of any

added TA and TNF α . The addition of both TA and TNF α for 15 min did not result in as much GR nuclear translocation as did in treatment with TA alone. We speculate that activated p65 hinders or at least delays GR from entering the nucleus. After treatment with TNF α for 15 min, p65 did not co-immunoprecipitate with GR from the nucleus. The reason could be that GR and p65 do not associate physically in the nucleus *in vivo*, but the probable explanation is that p65 in rat hepatoma cells needed more than 15 min for activation and nuclear translocation. This is in contrast with what has been reported previously for HeLa cells and human chondrocytes [18].

PKAc has been found to be a possible mediator of cross-repression by NF- κ B and GR [13], and we investigated whether PKAc co-purified with GR in the cytosol of rat hepatoma cells. Indeed, we found that PKAc and GR co-immunoprecipitated but the signal was weak and not enriched, as compared with the input signal. Furthermore, overnight treatment with TA and/or TNF α did not affect this interaction. This indicates that GR and PKAc can interact but that the interaction in rat hepatoma cells is weak at physiological protein concentrations.

As shown in Figures 3, 4 and 6, we were able to immunoprecipitate a small fraction of GR from the nucleus, even in the absence of TA (Figure 3A, lane 2a; Figure 4A, lane 2a; Figure 6, lane 2a). There are several possible explanations for this: (1) a small fraction of non-liganded/non-activated GR (i.e. associated with hsps) may reside in the nuclear compartment [23], (2) a small fraction of non-liganded/non-activated GR was activated occasionally and entered the nucleus or (3) non-liganded/non-activated GR recirculates between the cytoplasm and the nucleus [19,24]. It should be added that immunoprecipitation represents an effective enrichment of the available GR and that there is no demonstrable GR in the input nuclear extract fraction (Figures 3C and 4C), indicating that the nuclear GR fraction, as expected, is small in the absence of ligand. Also, the combined overnight treatment with TA and TNF α seemed to increase the GR amounts both in the cytosol and in the nucleus (Figure 4A, lanes 1d and 2d), as compared with TA treatment alone (Figure 4A, lanes 1b and 2b). Usually, the amount of GR is down-regulated after overnight exposure to TA; maybe this effect is inhibited by TNF α .

When investigating the co-immunoprecipitation pattern of hsp90 and hsp70, we found that both hsp90 and hsp70 co-immunoprecipitated with GR in the nucleus in the absence of TA, which is in line with the notion that non-liganded/non-activated GR can shuttle between the cytoplasm and the nucleus [19,24]. More interestingly, the hsp90 and hsp70 co-immunoprecipitation with GR in the nucleus seemed to be enhanced after treatment with TNF α for 30 min (Figure 6, lane 2c). hsp90 is reported to translocate into the nucleus after single or repeated heat-shock stimuli [25] (see [26] for a review), but the observation of potentiated interactions between hsp90 and GR in the nucleus after TNF α stimulus is novel and may indicate additional mechanisms for pro-inflammatory TNF α action.

GR associated with p65, p50 and I κ B α in the cytosol of rat hepatoma cells independent of TA and/or TNF α treatment. On TNF α treatment for 30 min, p65 and p50 entered the nucleus and also appeared as GR co-immunoprecipitating proteins in the nucleus of rat hepatoma cells. Activated p65 and p50 were thus capable of interacting with non-liganded/non-activated GR in the cell nucleus. After combined treatment with TA and TNF α , p65 and p50 were still associated with GR in the nucleus, but to a lesser extent as compared with treatment after TNF α alone. We speculate that this is due to a weaker association of p65 and p50 to liganded/activated GR when compared with non-liganded/non-activated GR or to a lower amount of nuclear p65 and p50 available

GR - NF- κ B interaction

Figure 7 NF- κ B is found within various GR receptosomes in liver cells

If GR and NF- κ B reside in the same cellular compartment in a liver cell, they can associate with each other both when non-activated or activated. We have identified p65, p50 and their inhibitor I κ B α associated with both non-liganded/non-activated GR (A) and with liganded/activated GR (B) in the cytosol of rat liver cells. On activation of GR and NF- κ B, using TA and TNF α , we have identified p65 and p50 associated with liganded/activated GR in the nucleus of liver cells (C). On stimulation with TNF α alone, we have identified p65 and p50 associated with non-liganded/non-activated GR in the nucleus of liver cells (D).

in the nucleus on treatment with TA and TNF α in combination when compared with treatment of TNF α alone. Our findings that GR can interact with p65 and p50 in the cytosol independent of TA and TNF α , and that non-liganded/non-activated GR as well as liganded/activated GR can interact with activated p65 and p50 in the nucleus are somewhat in contrast with previous work by e.g. De Bosscher et al. [27], Lidén et al. [22], and McKay and Cidlowski [2], where only ligand-induced GR–NF- κ B interaction has been observed and where the GR–NF- κ B interaction is assumed to take place only in the nucleus of cells. It is also possible that the GR–NF- κ B association we have observed in the cytoplasm of liver cells may be cell-type-specific.

Interaction between GR and NF- κ B involves the Rel homology domain of p65 and the DBD of GR and the C-terminal zinc finger of the DBD [12]. This zinc finger is important for dimerization and transactivation [12]. The Rel homology domain of p65 is important for DNA binding, dimerization and nuclear localization [3]. Based on this knowledge as well as our results showing that the GR–p65 interaction is independent of ligand activation and the results from Nissen and Yamamoto [21], who have also demonstrated that interaction of GR and p65 is independent of ligand *in vitro*, we assume that GR dimerization is not a prerequisite for the interaction with p65 subunit of NF- κ B.

Taken together, we have found that if both GR and NF- κ B reside in the same cellular compartment in a liver cell, they can associate with each other regardless of TA and TNF α treatments. Figure 7 is an attempt to summarize the various GR receptosomes containing NF- κ B that we have identified in liver cells. We believe that in a cell, the antagonistic GR- and NF- κ B-signalling pathways are so important for the fate of a cell that the two pathways need to be in continuous contact. Probably, transrepression of GR and

NF- κ B occurs at several different levels within a cell. Our studies suggest a strong physical interaction between GR and NF- κ B in the cytosol, and we propose that this direct interaction constitutes an integral part of GR–NF- κ B cross-talk.

We acknowledge Marika Rönnholm for skilful technical assistance. We also thank Dr Sam Okret for critical reading of the manuscript. This work was supported by grants from the Swedish Science Council (nos. 12557 and KI 13X-2819), and by grants from the Karolinska Institutet, the Magnus Bergvall Foundation and the Åke Wiberg Foundation.

REFERENCES

- 1 Caldenhoven, E., Lidén, J., Wissink, S., Van de Stolpe, A., Raaijmakers, J., Koenderman, L., Okret, S., Gustafsson, J.-Å. and Van der Saag, P. T. (1995) Negative cross-talk between RelA and the glucocorticoid receptor: a possible mechanism for the antiinflammatory action of glucocorticoids. *Mol. Endocrinol.* **9**, 401–412
- 2 McKay, L. I. and Cidlowski, J. A. (1998) Cross-talk between nuclear factor- κ B and the steroid hormone receptors: mechanisms of mutual antagonism. *Mol. Endocrinol.* **12**, 45–56
- 3 McKay, L. I. and Cidlowski, J. A. (1999) Molecular control of immune/inflammatory responses: interactions between nuclear factor- κ B and steroid receptor-signaling pathways. *Endocrine Rev.* **20**, 435–459
- 4 Pratt, W. B. and Toft, D. O. (1997) Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocrine Rev.* **18**, 306–360
- 5 Nemoto, T., Ohara-Nemoto, Y., Denis, M. and Gustafsson, J.-Å. (1990) The transformed glucocorticoid receptor has a lower steroid-binding affinity than the nontransformed receptor. *Biochemistry* **29**, 1880–1886
- 6 Newton, R. (2000) Molecular mechanisms of glucocorticoid action: what is important? *Thorax* **55**, 603–613
- 7 Adcock, I. M. (2001) Glucocorticoid-regulated transcription factors. *Pulmon. Pharmacol. Ther.* **14**, 211–219
- 8 Baeuerle, P. A. and Baltimore, D. (1988) I κ B: a specific inhibitor of the NF- κ B transcription factor. *Science* **242**, 540–546

- 9 Li, Q. and Verma, I. M. (2002) NF- κ B regulation in the immune system. *Nat. Rev. Immunol.* **2**, 725–734
- 10 Ray, A. and Prefontaine, K. E. (1994) Physical association and functional antagonism between the p65 subunit of transcription factor NF- κ B and the glucocorticoid receptor. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 752–756
- 11 Scheinman, R. I., Gualberto, A., Jewell, C. M., Cidlowski, J. A. and Baldwin, Jr, A. S. (1995) Characterization of mechanisms involved in transrepression of NF- κ B by activated glucocorticoid receptors. *Mol. Cell Biol.* **15**, 943–953
- 12 Lidén, J., Delaunay, F., Rafter, I., Gustafsson, J.-Å. and Okret, S. (1997) A new function for the C-terminal zinc finger of the glucocorticoid receptor. Repression of RelA transactivation. *J. Biol. Chem.* **272**, 21467–21472
- 13 Doucas, V., Shi, Y., Miyamoto, S., West, A., Verma, I. and Evans, R. M. (2000) Cytoplasmic catalytic subunit of protein kinase A mediates cross-repression by NF- κ B and the glucocorticoid receptor. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 11893–11898
- 14 Widén, C., Zilliacus, J., Gustafsson, J.-Å. and Wikström, A. C. (2000) Glucocorticoid receptor interaction with 14-3-3 and Raf-1, a proposed mechanism for cross-talk of two signal transduction pathways. *J. Biol. Chem.* **275**, 39296–39301
- 15 Okret, S., Wikström, A. C., Wrangé, O., Andersson, B. and Gustafsson, J.-Å. (1984) Monoclonal antibodies against the rat liver glucocorticoid receptor. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1609–1613
- 16 McEwan, I. J., Almlof, T., Wikström, A. C., Dahlman-Wright, K., Wright, A. P. and Gustafsson, J.-Å. (1994) The glucocorticoid receptor functions at multiple steps during transcription initiation by RNA polymerase II. *J. Biol. Chem.* **269**, 25629–25636
- 17 Carrasco, D., Cheng, J., Lewin, A., Warr, G., Yang, H., Rizzo, C., Rosas, F., Snapper, C. and Bravo, R. (1998) Multiple hemopoietic defects and lymphoid hyperplasia in mice lacking the transcriptional activation domain of the c-Rel protein. *J. Exp. Med.* **187**, 973–984
- 18 Ding, G. J., Fischer, P. A., Boltz, R. C., Schmidt, J. A., Colaienne, J. J., Gough, A., Rubin, R. A. and Miller, D. K. (1998) Characterization and quantitation of NF- κ B nuclear translocation induced by interleukin-1 and tumor necrosis factor- α . Development and use of a high capacity fluorescence cytometric system. *J. Biol. Chem.* **273**, 28897–28905
- 19 Hache, R. J., Tse, R., Reich, T., Savory, J. G. and Lefebvre, Y. A. (1999) Nucleocytoplasmic trafficking of steroid-free glucocorticoid receptor. *J. Biol. Chem.* **274**, 1432–1439
- 20 Wissink, S., van Heerde, E. C., Schmitz, M. L., Kalkhoven, E., van der Burg, B., Baeuerle, P. A. and van der Saag, P. T. (1997) Distinct domains of the RelA NF- κ B subunit are required for negative cross-talk and direct interaction with the glucocorticoid receptor. *J. Biol. Chem.* **272**, 22278–22284
- 21 Nissen, R. M. and Yamamoto, K. R. (2000) The glucocorticoid receptor inhibits NF- κ B by interfering with serine-2 phosphorylation of the RNA polymerase II carboxy-terminal domain. *Genes Dev.* **14**, 2314–2329
- 22 Lidén, J., Rafter, I., Truss, M., Gustafsson, J.-Å. and Okret, S. (2000) Glucocorticoid effects on NF- κ B binding in the transcription of the ICAM-1 gene. *Biochem. Biophys. Res. Commun.* **273**, 1008–1014
- 23 Wikström, A. C., Bakke, O., Okret, S., Bronnegard, M. and Gustafsson, J.-Å. (1987) Intracellular localization of the glucocorticoid receptor: evidence for cytoplasmic and nuclear localization. *Endocrinology* **120**, 1232–1242
- 24 McNally, J. G., Muller, W. G., Walker, D., Wolford, R. and Hager, G. L. (2000) The glucocorticoid receptor: rapid exchange with regulatory sites in living cells. *Science* **287**, 1262–1265
- 25 Akner, G., Mossberg, K., Sundqvist, K. G., Gustafsson, J.-Å. and Wikström, A. C. (1992) Evidence for reversible, non-microtubule and non-microfilament-dependent nuclear translocation of hsp90 after heat shock in human fibroblasts. *Eur. J. Cell Biol.* **58**, 356–364
- 26 Csermely, P., Schnaider, T., Soti, C., Prohászka, Z. and Nardai, G. (1998) The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review. *Pharmacol. Ther.* **79**, 129–168
- 27 De Bosscher, K., Vanden Berghe, W., Vermeulen, L., Plaisance, S., Boone, E. and Haegeman, G. (2000) Glucocorticoids repress NF- κ B-driven genes by disturbing the interaction of p65 with the basal transcription machinery, irrespective of coactivator levels in the cell. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 3919–3924

Received 28 January 2003/1 April 2003; accepted 2 April 2003

Published as BJ Immediate Publication 2 April 2003, DOI 10.1042/BJ20030175