MECHANISMS OF STEROID ACTION AND RESISTANCE IN INFLAMMATION

Glucocorticoid action and novel mechanisms of steroid resistance: role of glucocorticoid receptor-interacting proteins for glucocorticoid responsiveness

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Abstract

Glucocorticoids are widely used to treat inflammatory and malignant diseases. However, many individuals show a lack of therapeutic response and unwanted side-effects. Various known and unknown parameters determine glucocorticoid responsiveness, among them glucocorticoid receptor (GR)-interacting proteins. Several of the proteins interacting with GR also participate in other signal transduction pathways such as the AP-1 pathway and the nuclear factor-κB pathway. We suggest that a closer study of GR-interacting proteins may shed new light on mechanisms determining glucocorticoid sensitivity. In this commentary, the general mechanisms of GR action will be addressed and a proteomic-based method to study GR-interacting proteins will be described in brief.
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Introduction

Glucocorticoids and the glucocorticoid receptor

The nuclear receptor family includes receptors for glucocorticoids, mineralocorticoids, androgens, progestins, oestrogens, vitamin D, thyroid hormones, retinoic acid and a growing number of orphan receptors, i.e. receptors, yet lacking a known high-affinity binding ligand (Mangelsdorf *et al.* 1995). These intracellular receptors can bind to DNA under defined conditions and act as ligand-dependent transcription factors.

Glucocorticoids function as modulators of carbohydrate, lipid and protein metabolism. They also regulate signal transduction in immune and inflammatory systems as well as in growth and development. Glucocorticoids bind to the glucocorticoid receptor (GR) also denoted NR3C1 and hereby a glucocorticoid effect is obtained. GR is a 94 kDa protein which upon activation dissociates from a chaperone multi-protein complex, dimerizes and enters the nucleus (Fig. 1), where it binds to specific DNA regions, i.e. glucocorticoid response elements (GREs) (for review see Newton 2000). GR mediates glucocorticoid hormone action in a complex interplay with chaperone proteins, specific DNA sequences, basal and specific transcription factors, adaptors and co-regulators. GR also takes part in a cross-talk with other signal transduction pathways (for review see Horwitz *et al.* 1996). This complex protein interplay may result in activation or repression of cognate target genes. GR can also regulate transcriptional activity independent of DNA interaction, by interfering with other transcription factors in direct protein–protein interactions. Studies of GR have been valuable in elucidating the mode by which specific transcription factors such as hormone–receptor complexes regulate gene function.

GR domain structure

Based on studies employing limited proteolytic digestions, a domain structure for the glucocorticoid receptor was suggested (Fig. 2) (Carlstedt-Duke *et al.* 1987). This domain structure was later corroborated by molecular cloning of rat and human GRs (Hollenberg *et al.* 1985, Miesfeld *et al.* 1986). The domains have specific functions and these functions are independent and maintained if a domain is grafted to another protein or expressed on its own. The N-terminal domain harbours transactivation functions especially within the so-called τ1 region and is

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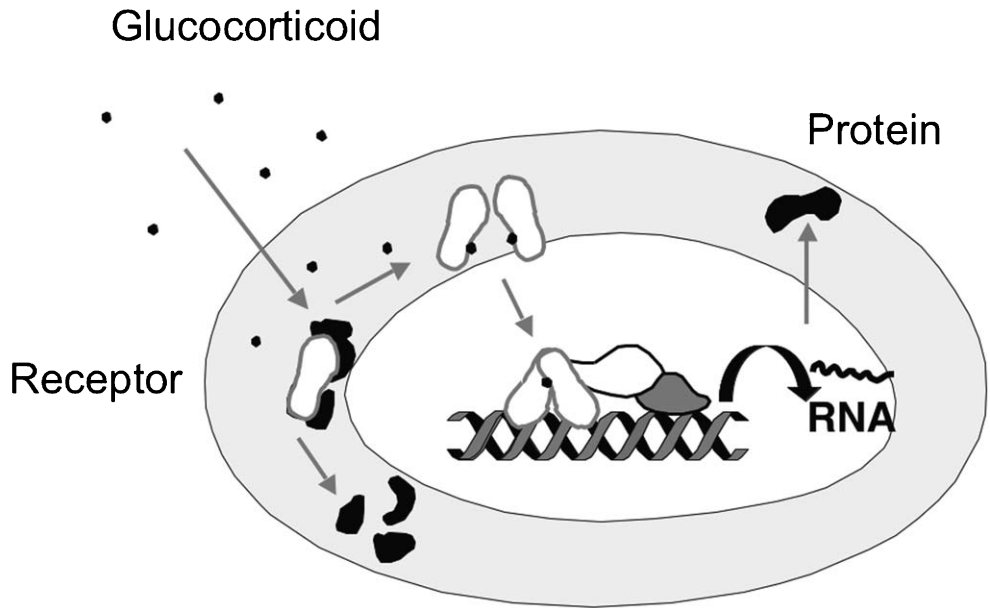

Figure 1 Transactivation by GR. Glucocorticoids enter the cell and bind to the GR which is maintained in an inactive conformation by chaperone proteins. After ligand binding, GR becomes activated and dissociates from the chaperones, dimerizes and translocates. In the nucleus, GR binds to GREs and hereby regulates glucocorticoid-responsive genes. The transactivation requires interaction with co-activators, adaptors and general transcription factors.

important for diverse biological effects. The middle part of the protein is well conserved among the nuclear hormone receptors and contains the amino acids that specifically interact with DNA. This domain is referred to as the DNA-binding domain or DBD. Within the DBD resides a motif common for DNA-interacting proteins, the zinc

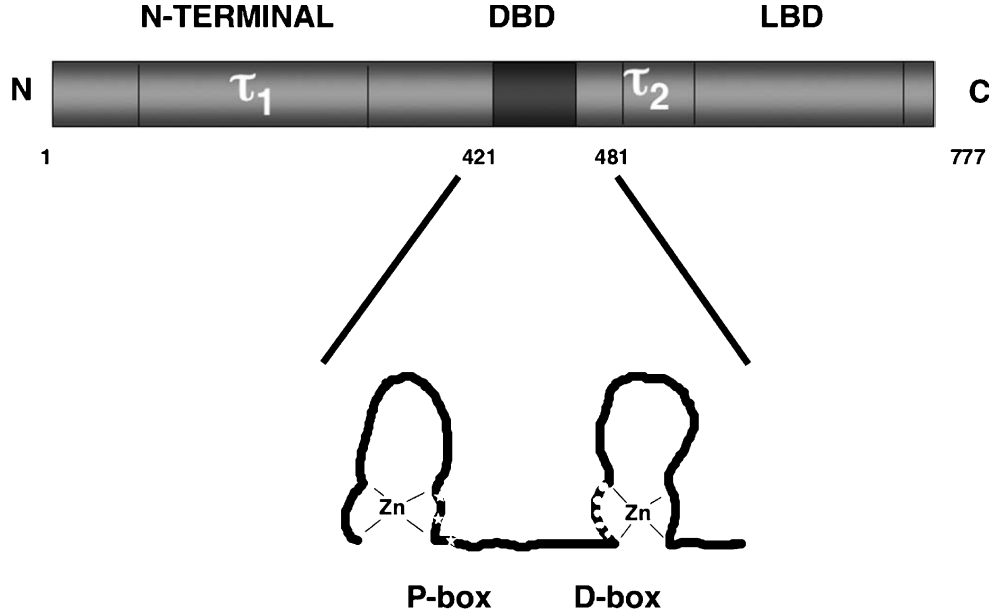

Figure 2 Domain structure of GR. The Figure shows a schematic representation of the human GR. GR consists of three major domains, N-terminal domain, DBD and LBD. GR contains two major transactivating areas, τ1 and τ2. The DBD contains two zinc finger motifs that in turn respectively harbour a proximal box (P-box) and a distal box (D-box). The numbers refer to amino acid number in human GR.

finger motif, which is found twice. The most C-terminal part of GR is the ligand-binding domain, also called the LBD. In addition, the LBD contains a transactivation region, which is called τ2.

GR and heat shock proteins

GR forms a multi-protein complex with several heat shock proteins (hsps) including a dimer of hsp90 (dimer), hsp70, hsp56 and hsp40. Furthermore, GR also interacts with the immunophilins FKBP51 and FKBP52 and a low molecular weight protein, p23 and most likely also with other hitherto uncharacterized proteins. The interaction with all these chaperones is important for the function of GR. An ordered sequence of assembly/maturation of the complex is necessary for high-affinity hormone binding and further transport and activation of GR. It has also been suggested that the chaperones may have an important role in inactivation and disassembly of the activated GR (for review see Freeman & Yamamoto 2001).

Ligand binding and GR activation by nuclear receptor–DNA interaction

Structural determination of the GR LBD, by X-ray crystallography (Bledsoe *et al.* 2002) has revealed large structural similarities to the LBD of other steroid receptors which have been crystallized. The LBD of these receptors consist of 12 α-helices, of which several take part in forming a hydrophobic ligand-binding pocket. However, the GR LBD adopts a modified dimer configuration involving formation of an intermolecular β sheet and an additional charge clamp as compared with the other steroid hormone receptor LBDs. These structural modifications help to explain the binding selectivity of co-activators and a distinct selectivity for endogenous glucocorticoids. In general, GR adheres to the overall structure of previously described steroid hormone receptors, where the binding of agonists and antagonists leads to different conformational changes of the receptor, especially with regard to the folding of the most C-terminal part of the receptor, encompassing a terminal α-helix, assigned number 12.

Upon ligand binding, GR sheds most of the proteins in the multi-protein complex, particularly hsp90. This in turn leads to the exposure of nuclear localization signals and the translocation of GR to the nucleus. During this process GR dimerizes and GR also binds the GRE as a dimer. The consensus binding motif consists of an inverted palindrome (AGA ACA nnn TGT TCT) of six specific nucleotides separated by any three nucleotides. Amino acids within the two zinc fingers in each of the dimer forming the DBDs of GR have discrete functions. The most N-terminal zinc finger contains a so-called proximal box (P-box) that contacts nucleotides in one half site, usually situated in a major groove of the DNA. The more C-terminal zinc finger contains amino acids in a so-called distal box (D-box) that constitutes a dimerization interface with the other GR subunit taking part in the homodimeric complex.

Co-activator and co-repressor effects on nuclear receptor transcriptional activities

During the last 10 years it has become clear that gene activation or gene repression by steroid hormone receptors also involves receptor interaction with co-activators and co-repressors of various kinds. There are several interaction surfaces within the receptors for such molecules and for GR there are two major areas that can interact with co-factors, the τ1 and the τ2. The τ1 is reported to be non-structured in aqueous solution, whereas the τ2 is an amphipathic α-helix identified as helix 12 in the LBD. Structural studies have also revealed that agonists allow proper folding of helix 12 to allow co-activator interaction, whereas antagonists prevent proper folding of helix 12 and co-activator interaction or alternatively allow co-repressor interaction. Among the co-regulators reported to interact with GR are both general transcription factors, ATP-dependent chromatin remodelling complexes and histone acetyl transferase co-activators (for general reviews see Collingwood *et al.* 1999, McKenna & O'Malley 2002).

Transcriptional effects exerted by the GR

Transcriptional activation by GR is generally subsequent to ligand binding, conformational changes, shedding of chaperones, dimerization, translocation and contact with both general transcription factors, adapter proteins and various co-activators as described above. As a result of this process, transcriptional activation of specific target genes occurs and subsequently levels of regulated proteins change. Furthermore, post-transcriptional effects including effects on mRNA may occur. Inherent in this process is a feedback regulation of GR itself; in most cells and tissues this feedback is negative, leading to down-regulation of GR. In certain cases, GR up-regulation has also been described, most notably prior to an apoptotic event (Eisen *et al.* 1988, Tonko *et al.* 2001) or as a consequence of a viral infection (Erlandsson *et al.* 2002).

As important, or even more important than transcriptional activation by GR, is transcriptional repression. Targeted deletions of GR in an *in vivo* mouse model leads to high perinatal mortality, mainly due to deficient lung maturation (for review see Tronche *et al.* 1998). If a knock-in experiment is performed in these mice, replacing wild-type GR with a dimerization deficient form of GR, as performed in the so called GR $^{dim-/dim-}$ mouse model (Reichardt *et al.* 1998), the mice are viable and only have moderate functional impairments. The GR $^{dim-/dim-}$ mouse model is based on the assumption that transactivation by GR requires receptor dimerization, whereas transcriptional repression in many cases would only

depend on monomeric GR. Repression, exerted by GR, on the activation of other transcription factors may be via DNA-dependent mechanisms, such as displacement of an activator by the GR, due to overlapping binding sites, or binding of GR to a contiguous negative GRE. DNA-independent mechanisms (in the sense that GR itself is not in direct contact with DNA) have also been described, including binding of GR to a DNA-bound activator, referred to as tethering. Furthermore, formation of an abortive complex between GR and another transcription factor may result in the repression of this particular activator by preventing its DNA binding, a mechanism that has been called squelching (Miner & Yamamoto 1991).

Therapeutic effects of glucocorticoids

Glucocorticoids are used in combination with other drugs for the treatment of various malignancies, most notably acute lymphatic leukaemias of children, but also in other cancer forms, both in cases where an apoptotic effect is desired, and as an adjuvant treatment of pain or oedema. Glucocorticoids are also among the first-line therapeutics in several inflammatory diseases, i.e. asthma, inflammatory bowel disease and dermatological disorders of various kinds. The anti-inflammatory effects of glucocorticoids are exerted in several different ways. Glucocorticoids down-regulate the transcription of many pro-inflammatory cytokines. These effects can occur via direct interaction with the promoter elements of the various cytokine genes. The down-regulation may also take place via interference with the activity of AP-1 response elements and via down-regulation of nuclear factor-κB (NF-κB) activity. The down-regulation of NF-κB activity is exerted mainly via a direct protein–protein interaction of GR and NF-κB but in some cells is also mediated via up-regulation of the NF-κB inhibitor I-κB. Apart from these effects caused by classical GR–DNA interactions and known protein–protein interactions, many rapid glucocorticoid effects are unexplained and the reasons for a varying degree of responsiveness to glucocorticoids are not well understood.

GR interaction with other proteins

As stated above, GR is part of a multi-protein complex where several of the participating proteins are important for glucocorticoid signalling. However, knowledge about the protein composition of the complex, and the stoichiometry of its subunits and their function is not complete. To determine which proteins GR interacts with may be important for the understanding of a number of rapid effects of glucocorticoids, i.e. so rapid that they do not allow time for transcriptional and translational events. Among such effects are, for instance, glucocorticoid-induced phosphorylation-dephosphorylation of GR, Ca^{2+} signalling-related effects of glucocorticoids and also some effects assumed to be due to membrane events (Brann *et al.* 1995, Buttgereit & Scheffold 2002, Sutter-Dub 2002).

Materials and Methods

In order to study GR-interacting proteins we have developed an immuno-affinity chromatography purification method using a monoclonal antibody against a defined epitope of GR and we have applied this method to isolate the GR complex from rat liver cytosol (Widén *et al.* 2000). Subsequent to the immuno-affinity purification we have used two approaches to study GR-interacting proteins. In the first case, we have specifically probed with antibodies, using Western blotting, for proteins that have been reported to interact with GR in various studies. Several of the interacting proteins that we have studied have, in turn, been reported to interact with other proteins important for signal transduction. Based on this information we have tested whether the interaction with GR also allows for interaction with previously described secondary interaction partners (Widén *et al.* 2000, Widén *et al.* 2003) and have sometimes found this to be the case (see below).

We have also used a more unbiased approach, employing two-dimensional gel electrophoresis with iso-electric focusing in the first dimension and SDS-PAGE in the second. GRs purified in the presence or absence of glucocorticoid ligand give rise to distinct patterns of co-immunopurified proteins. The protein spots on the two-dimensional gels have been identified by matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry. Using this method we can now identify potential GR-interacting proteins (E Hedman, C Widén, A Asadi, W Schröder, J Å Gustafsson & A-C Wikström, unpublished observations). Initial studies with different salt washing steps have allowed us to discriminate non-specifically bound proteins from true subunits of the GR multi-complex. Further studies are in progress to verify the interactions, to map the interacting domains and to investigate the functional consequences of the interactions. Part of this work will be described in more detail below.

Results

GR interactions with 14-3-3 and Raf-1

Previous work by Wakui *et al.* (1997), using yeast two-hybrid analysis and glutathion-s-transferase (GST) pull-down experiments had demonstrated that 14-3-3 is a ligand-dependent GR LBD-interacting protein. 14-3-3 is known as an adapter protein, reported to interact with a large array of proteins, among them protein kinase C and Raf-1. Using the immuno-affinity method that we have developed, we were interested to see if GR–14-3-3

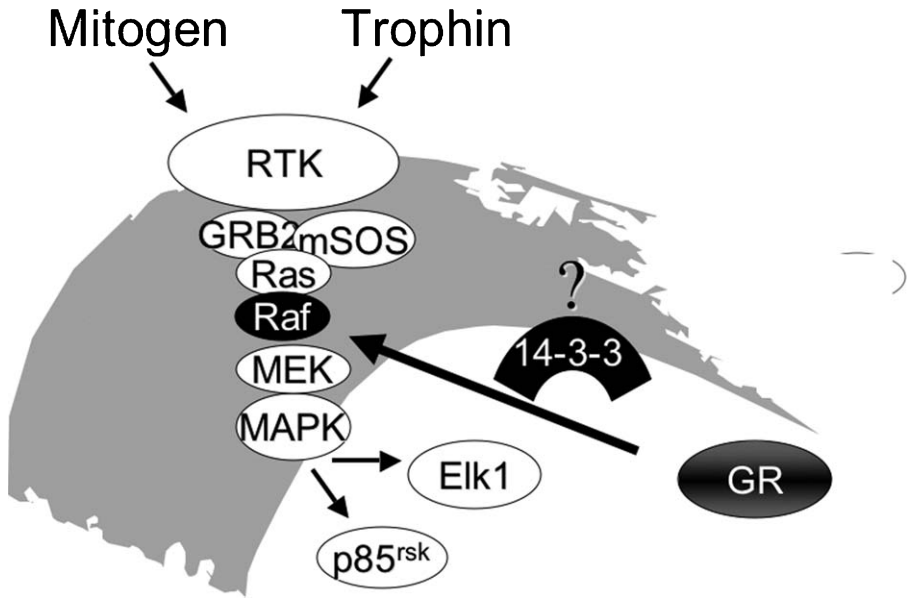

Figure 3 GR interaction with the MAPK–kinase cascade. GR can interact with Raf-1, which is a kinase downstream of Ras in the MAPK–kinase cascade. Whether this interaction occurs independently of 14-3-3 is not yet clear. RTK, receptor tyrosine kinase; GRB2, growth factor receptor bound protein 2; SOS, son of sevenless; MEK, mitogen activated protein kinase/extracellular signal related (ERK) kinase.

interactions occurred *in vivo* at physiological protein concentrations. We also studied whether some of the 14-3-3-interacting proteins other than GR were included in the GR receptosome. We found that 14-3-3 co-purified with GR, especially with liganded/activated GR, but also with non-liganded /nonactivated GR (Fig. 3). Furthermore, we found that Raf-1 co-purified with liganded GR (Widén *et al.* 2000). Raf-1 acts downstream of Ras in the mitogen-activated protein kinase (MAPK)–kinase cascade and is reported to be important for mitogen-induced cell proliferation. The interaction occurs to a larger extent when GR is liganded. Stepwise salt washes indicated that the Raf-1 interaction may not only be mediated via 14-3-3 but could also occur as a direct interaction with GR. Further studies of the importance of this finding, in relation to the function of GR and Raf-1, are in progress. The interaction of GR and Raf-1, regardless of whether it is mediated via 14-3-3 or is direct, is interesting in terms of how crosstalk between the MAPK–kinase pathway and nuclear receptors can occur. Indications of such crosstalk have mainly been demonstrated for the oestrogen (for review see Kato *et al.* 2000) and progesterone receptors (Lange *et al.* 2000). The molecular basis for GR–MAPK–kinase crosstalk has not been demonstrated previously and the GR and Raf-1 interaction may provide one explanation for the effects of glucocorticoids on the Raf-1–Ras signalling pathway. Another explanation may involve the glucocorticoid-induced leucine zipper (GILZ) protein, whose expression is augmented by dexamethasone treatment. GILZ interacts with Raf-1 and contributes, through protein–protein interaction with Raf-1, to the inhibition of Raf–MEK–ERK activation (Ayroldi *et al.* 2002).

Studies of GR and NF-κB proteins

NF-κB is a major pro-inflammatory mediator, which encompasses a protein family with several members, of which the protein RelA or p65 and its partner p50 are relevant to the experiments described below. p65 and p50 form a complex that is maintained in an inactive form in the cytoplasm by the inhibitor I-κBα (Li & Verma 2002). Mutual antagonistic effects of glucocorticoid and NF-κB signalling have been extensively demonstrated. A ligand-dependent interaction of the second zinc finger in the DBD of GR with RelA has been reported (Liden *et al.* 1997), and interacting domains in p65 have been identified, most recently by Nissen & Yamamoto (2000).

Contrary to current beliefs, we found that p65, p50 and I-κBα were co-immunopurifying with cytosolic, non-liganded, non-activated GR (Widén *et al.* 2003). The current understanding of GR and NF-κB interaction favours the model that glucocorticoids induce the GR–NF-κB interaction and that this interaction would be one of two possible mechanisms for how glucocorticoids counteract inflammation mediated by NF-κB. The observed cytosolic interaction indicates a tighter connection between GR signalling and NF-κB signalling than previously appreciated.

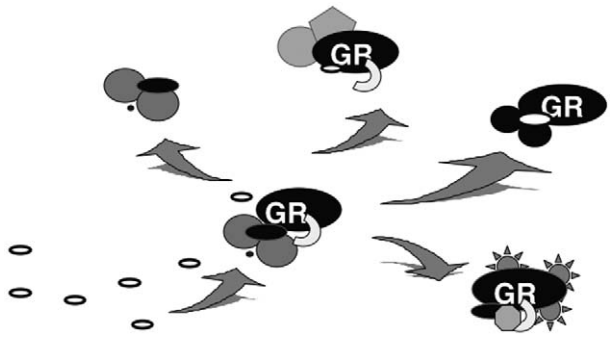

Figure 4 Multiple GR receptosomes? We speculate that GR may be present in diverse multi-protein complexes with different functions. Some of these protein interactions may be induced by glucocorticoids.

A proteomic approach to identification of GR-interacting proteins

The proteins of the isolated GR multi-protein complex were separated by two-dimensional electrophoresis, and their protein pattern was analyzed. This approach allows the determination of the molecular weights and the isoelectric points of the proteins of the GR multi-complex. In the next step, the proteins that were obtained by two-dimensional electrophoresis were digested with trypsin and analyzed by high resolution MALDI-TOF mass spectrometry (Shevchenko *et al.* 1996*a*,*b*). A peptide mass fingerprint is usually sufficient to clearly identify a protein. If further information is required to find the identity of a protein, a fragmentation analysis can be carried out to produce sequence tags for specific database searches. In certain situations *de novo* sequencing is required. Among the proteins that we have identified by MALDI-TOF mass spectrometry, forming part of the GR complex is hypoxanthine-guanine phosphoribosyltransferase, hsp70, a DNA/J-like protein, haemoglobin and DNA/K-type molecular chaperone grp75 precursor as well as several other proteins. However, using this approach, it is important to verify the interactions by other methods such as reverse immunoprecipitation, GST pull-down experiments, yeast or mammalian two-hybrid screening and also to determine the functional relevance of the interactions that were found.

Conclusions

Cytosolic interaction of GR with proteins in other signalling pathways ('receptosomes') are emerging as an alternative important way for both steroid signalling and integration of signals in different pathways (Fig. 4). Proteomic and functional analysis of the cytosolic proteins that interact with the GR may shed light on the mechanisms of glucocorticoid-regulated events that do not occur via the classical nuclear receptor activation pathway. Levels and the functional state of such GR-interacting proteins may have important effects on glucocorticoid responsiveness. A deeper understanding of the interplay of GR and its interaction partners may lead to improved treatment strategies for diseases where glucocorticoid therapy is used.

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