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Studies of glucocorticoid receptor interacting proteins

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Contents

Abstract

The glucocorticoid receptor (GR) functions as a ligand dependent transcription factor. Nonliganded GR resides mainly in the cytoplasm bound to a complex containing heat shock proteins (hsp). Upon ligand binding, GR dissociates from the hsp-complex and translocates to the nucleus where it regulates the transcription of specific genes by binding to glucocorticoid response elements (GREs). Within the nucleus, GR is known to interact with other transcription factors via protein-protein contacts thereby affecting their transcriptional action via so called transcriptional cross-talk. GR is thought to be able to interact with other signaling pathways also in the cytoplasm although this is not as well studied as the transcriptional cross-talk in the nucleus. Therefore, we wanted to study GR interactions with proteins in the cytoplasm and to define conditions when specific GR-protein interactions occurred.

We have developed a specific immunoaffinity chromatographic purification of GR to identify hitherto unknown cytosolic GR interacting proteins. Briefly, by using the anti-GR monoclonal antibody (mAb) 250, GR is purified from the liver cytosol of adrenalectomized rats and peptide eluted. The elution of GR allows GR-interacting proteins to co-purify with the receptor as intact complexes. Using Western Blotting we have identified 14-3-3, Raf-1 and NF-κB and IκBα as GR co-purifying proteins in the rat liver cytosol. We identified Flt3 among the GR co-purifying proteins using Edman based N-terminal sequencing. Flt3 was found to interact with both the non-liganded and the liganded form of GR and the DNA-binding domain of GR is sufficient for Flt3 interaction as shown by GST-pulldown experiments using human GR and Flt3.

Using two-dimensional gel electrophoresis in combination with MALDI-TOF mass spectrometry, we have been able to identify 36 individual proteins, among which 28 are novel GR interacting proteins, for example: Major Vault Protein, TATA binding interacting protein 49 and glycoprotein PP63. Using Blue Native gel electrophoresis, we demonstrated that GR exists in several separate protein complexes.

This study shows that GR interacts with a number of different proteins within rat liver cells. Proteins, regulating such a vast number of vital biological functions as GR does, perhaps need to integrate their functions with those of other proteins via protein-protein interactions. We hypothesize that cytosolic GR, besides the well-known hsp-complex, also interacts with other specific proteins in dynamic multiprotein complexes, "receptosomes" which may form the biochemical basis for cross-talk between GR and other signaling pathways.

Main references

This Thesis is based on the following scientific publications and manuscripts, which will be referred to by their Roman numerals

I. Christina Widén, Johanna Zilliacus, Jan-Åke Gustafsson and Ann-Charlotte Wikström, *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 (50): 39296-301, 2000
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II. Christina Widén, Jan-Åke Gustafsson and Ann-Charlotte Wikström, *Mechanistic studies of Glucocorticoid Receptor Interaction with NF-κB proteins,* Biochem J. 373(Pt1): 211-20, 2003
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III. Abolfazl Asadi, Christina Widén, Erik Hedman, Johanna Zilliacus, Jan-Åke Gustafsson, and Ann-Charlotte Wikström, *FLT3 interacts with the glucocorticoid receptor complex and affects glucocorticoid dependent signaling*, Submitted to Blood

IV. Erik Hedman, Christina Widén, Abolfazl Asadi, Wolfgang P. Schröder, Jan-Åke Gustafsson and Ann-Charlotte Wikström, *Proteomics of glucocorticoid receptor interacting proteins*, Resubmitted to MCP

Abbreviations

AA	arachidonic acid
ACTH	adrenocorticotrophic hormone
AP-1	Activator protein 1
CBP	CREB (cAMP response element binding protein) binding protein
CDK	cyclin-dependent kinase
CRH	corticotrophin releasing factor
eNOS	endothelial nitric oxide synthase
EGF	epidermal growth factor
ER	estrogen receptor
ERK	extracellular-signal-regulated kinase
FL	Flt3 ligand
Flt3	Fms-like tyrosine kinase 3
Flk2	fetal liver kinase 2
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HAT	histone acetyltransferase
HOP	heat shock organizing protein
HPA axis	hypothalamus-pituitary-adrenal axis
hsp	heat shock protein
IκB	Inhibitory protein IκB
IKK	IκB kinase
IRS-1	insulin receptor substrate-1
JNK	c-Jun N-terminal kinase
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MEK	MAPK/ERK kinase
NF-κB	nuclear factor κB
NIK	NF-κB inducing kinase
NMIgG	normal mouse IgG
PLA	phospholipase A
PLC	phospholipase C
PR	progesterone receptor
RTK	receptor tyrosine kinase
STAT	signal transducer and activator of transcription
STK-1	stem cell kinase 1
SWI/SNF	Switch/Sucrose non fermenting
TA	triamcinolone acetonide
TAF	TBP associated factors
TBP	TATA binding protein
TF	transcription factor
TBP	TATA-box-binding protein
TNFα	tumor necrosis factor α
TR	thyroid receptor
VDR	vitamin D receptor

General introduction

Steroid hormones and their receptors

Steroid hormones are small, lipophilic compounds that are mainly produced by endogenous endocrine organs such as the adrenals, ovaries, testes and placenta. There are five classes of steroid hormones: androgens, estrogens, glucocorticoids, mineralocorticoids and progestins. The steroid hormones regulate a variety of metabolic and developmental events, for example sexual differentiation, reproduction, carbohydrate metabolism and electrolyte balance. After synthesis, steroid hormones circulate via the bloodstream to their target tissues where they bind to their respective intracellular steroid hormone receptor. Steroids are considered to pass over the cellular membrane by passive diffusion. The binding of a steroid hormone to its receptor leads to a series of events ending with modulation of gene transcription. The steroid hormone receptors belong to a large superfamily of nuclear receptors where also receptors for thyroid hormones, retinoids and vitamin D_3 are found (71). The superfamily also consists of so-called orphan receptors *i.e.* receptors for which the ligand, if any, so far is unknown (71).

The steroid receptors share a common three-domain structure, which consists of an N-terminal transactivating domain, a central DNA-binding domain and a C-terminal ligand-binding domain. Another common feature of the steroid receptors is that they in their nonliganded, inactive state are bound to a multiprotein complex consisting of heat shock protein (hsp) 90, hsp70, heat shock organizing protein (HOP), hsp40, and p23 (87). Upon ligand binding, the steroid receptor dissociates from the multiprotein complex and translocates to the nucleus where it binds as a homodimer to specific hormone response elements on the DNA and thereby regulates gene transcription.

Gene regulation by steroid receptors

In steroid hormone signaling, the gene regulation itself has been intensively studied and most research, so far, has focused on regulation of gene transcription, especially of the promoter region of steroid hormone regulated genes. With the help of multiple co-activators and co-repressors the steroid receptors can inhibit or promote gene transcription. GRIPs (GR interacting proteins) have been reported to stimulate ligand-dependent activation of GR *in vitro* (25). The TRAP (TR-associated polypeptides) (30) and DRIP (Vitamin D receptor interacting proteins) (90) stimulate the activity of TR and VDR in a ligand-dependent manner *in vitro*. The gene transcription process, of course, involves steroid receptor interactions with general transcription factors such as TATA-box-binding protein (TBP), TBP associated factors (TAF), the transcription factors TFII B, -D -E, -F and -H, and the enzyme RNA polymerase II (104). The steroid receptors also interact with co-factors that remodel the chromatin structure of promoters. The SWI/SNF complex remodels the chromatin in an ATP-dependent manner (118) and histone acetyltransferases (HATs) by transferring acetyl groups from acetyl-coenzyme A to lysine residues in histone proteins. SRC-1 (steroid receptor co-activator) and p300/CBP are examples of HATs that interact with steroid receptors (83,15). In addition to direct transcriptional regulation, it has during recent decades become obvious that the steroid receptors can also interfere with other transcription factors for example, Activator protein 1 (AP-1), Nuclear factor κB (NF-

κB) and signal transducer and activator of transcription 5 (STAT5) and thereby affect their transcriptional action via so called transcriptional cross-talk (38).

Cross-talk between signal transduction pathways

Traditionally, cellular signaling pathways are often described as linear events from A to B where the activation, at a start-point A, leads to a cellular event B. In the case of steroid hormones, their signaling pathways are classically considered as a sequence of steps, starting with an extra-cellular signal leading to activation of a specific intracellular receptor which in turn leads to gene regulation. Today, one knows that proteins from one signaling pathway are able to interact with other signaling pathways not only in the nucleus of a cell but at several levels within a cell: at the cell membrane, in the cytoplasm and in the nucleus. Such interactions of different signaling pathways allow fine tunings of regulation at multiple steps. The regulation of signaling pathways can be described as networks where separate signaling pathways interact with other signaling pathways at any level. This phenomenon is referred to as cross-talk or cross-signaling and has opened up the idea that, in theory, everything could interact with everything everywhere (24). In reality, this is probably not totally true since many reported cross-signaling events are restricted to a few cell types and circumstances. Also, many reported interactions may be artifacts depending on the experimental system used (24). For example, using merely *in vitro* experiments to verify a protein-protein interaction is not 100% reliable since these types of experiments often involve overexpressed proteins, up to 100-fold or more, and at these concentrations, weak and/or non-physiological interactions and effects may well take place. Therefore, protein-protein interaction studies using endogenous proteins are preferred but not always easy to perform.

Introduction

Glucocorticoids

Glucocorticoids, e.g. cortisol in humans, were originally named for their ability to influence glucose metabolism. During fasting, glucocorticoids see to that plasma glucose levels are maintained by increasing for example gluconeogenesis, glycogen release, lipolysis and protein catabolism (39). Glucocorticoids are essential for survival and they affect cell growth and differentiation, they have effects on mood and cognitive functions and they are important for inflammation and immune responses (7). Glucocorticoids are produced by the adrenal cortex from cholesterol. The nervous system, influenced by physical, emotional or chemical stress, regulates the release of a peptide hormone, corticotrophin releasing hormone (CRH), from the hypothalamus which stimulates the release of another peptide hormone, adrenocorticotrophic hormone (ACTH) from the pituitary, which in turn stimulates the adrenals to produce and release glucocorticoids. In the resting state, CRH, ACTH and cortisol are released in a pulsatile and circadian fashion. The highest levels of cortisol are observed in the morning, just before awakening, and the levels decrease during the day to low levels in the evening. The cortisol levels continue to decline during the first hours of sleep before starting to increase again early in the morning. The so-called hypothalamo-pituitary-adrenal (HPA) axis (Fig. 1) is kept in balance by a negative feedback loop of cortisol acting on the secretion of both ACTH and CRH (3).

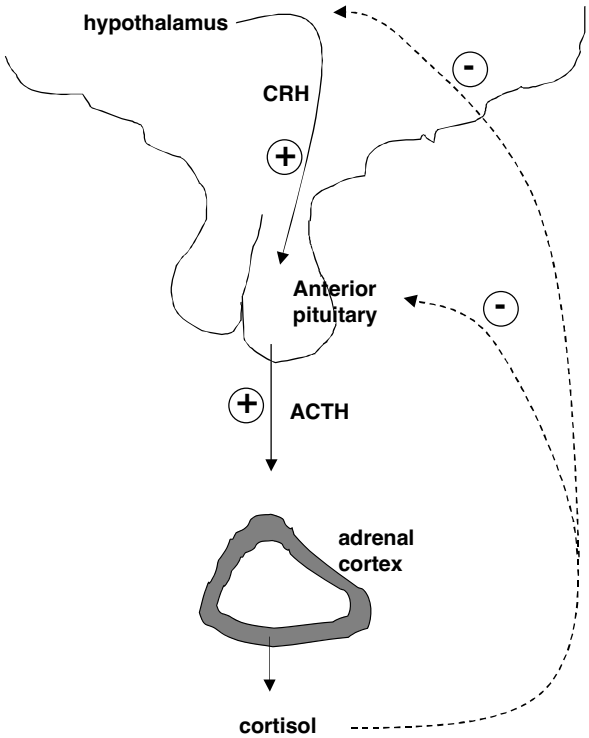

Figure 1. Hypothalamo-pituitary-adrenal (HPA) axis. The secretion of cortisol is controlled by the central nervous system. Stress (e.g., injury, fasting, surgery) induces the hypothalamus to release corticotrophin-releasing hormone (CRH), which stimulates the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary. Via the blood stream, ACTH reaches the adrenal cortex where it increases the synthesis and release of cortisol. The secreted cortisol exerts its effect on target organs and also negatively feedbacks on CRH and ACTH secretion.

In 1948, it was found that hydrocortisone (cortisol) suppressed the clinical manifestations of rheumatoid arthritis and since then, glucocorticoids have become a widely and commonly used drug in the clinic. Glucocorticoids are used to treat inflammatory diseases like rheumatoid arthritis, asthma, allergy, dermatological diseases, inflammatory bowel diseases etc. Glucocorticoids are also used as immuno-suppressive agents after transplantation (99) and as chemotherapeutic agents in the treatment of cancers, especially cancers of hematological origin (4).

The glucocorticoid receptor

The biological effects of glucocorticoids are mediated via the 94-kDa glucocorticoid receptor (GR). GR belongs to a super-family of nuclear receptors that function as ligand dependent transcription factors (27). The three-domain structure of GR (Fig. 2) includes an N-terminal domain, a central DNA-binding domain (DBD), and a C-terminal domain or ligand binding domain (LBD) (101). This division into domains was originally defined by protease cleavage (14).

Figure 2. The domain structure of GR. The human GR consists of 777 amino acids divided into three main domains: an N-terminal domain, a DNA binding domain and a ligand binding domain. GR also contains two transactivational domains τ1 and τ2. The figure also shows the location of regions responsible for dimerization, binding to hsp90 and nuclear localization.

The N-terminal domain harbors the main transactivation function (τ1) that interacts with the general transcription machinery either directly or via bridging co-factors (31,44). In steroid receptors, the N-terminal region is the most variable in terms of

length and variability. The N-terminal part of GR contains many antigenic sites that have been used for developing antibodies against GR (82,116). The N-terminal domain is also the major target for ligand-dependent phosphorylation at multiple serine residues (50,8).

The DBD is highly conserved among the steroid receptors and contains two zinc finger motifs essential for DNA-binding at specific promoter sites (33). This domain is also important for receptor dimerization and contains a nuclear localization signal (85).

The LBD is folded into a hydrophobic pocket that binds the ligand with high specificity (3,35). Besides ligand binding, the LBD has several functions, for example it contains a nuclear localization signal (85), transactivation functions (τ2) (46,113) and binding sites for hsp90 (19,21). In studies where the LBD has been removed, the receptor becomes constitutively active showing that the LBD also prevents activation of the receptor (37). Recently, the LBD structure of GR was solved (5,54) and the GR LBD was found to have a very flexible structure that helps the receptor to bind a variety of ligands (6).

Mechanism of GR action

The nonliganded GR forms a multiprotein complex in which, among other proteins, a dimer of hsp90, as well as monomeric hsp70, Hop, hsp40 and p23 are present (for a review see Ref. (87)). The nonliganded GR-multiprotein complex resides mainly in the cytoplasm of cells (Fig. 3), although a small fraction of GR associated with hsp's may reside in the nucleus (115), or recirculate to the nuclear compartment (40,76). The proteins in the GR-multiprotein complex presumably play a role in the function of GR. For example, the association of the dimer of hsp90 to the LBD of GR, suppresses GR activation and also keeps GR in a conformation optimal for ligand binding (80,11). Hsp90 also helps to anchor GR to the cytoskeleton (78). Upon ligand binding GR is activated *i.e.* GR dissociates from the hsp complex and the nuclear localization signals of GR become exposed (85). The receptor then migrates into the nucleus where it regulates gene transcription by binding as a homodimer to glucocorticoid response elements (GRE) in glucocorticoid regulated genes, thereby inducing or repressing gene transcription (3,101). Five years ago, it was shown for the first time that the binding of GR to GRE is not static but highly dynamic and that GR moves on and off its regulatory elements very rapidly (76). This phenomenon has been called the "hit-and-run" model (for a review see (79)). The factors responsible for this fast nuclear trafficking have so far been unknown but in a recent report several chaperones (among others: hsp90, hsp70, and hsp40) were found to function as nuclear mobility factors of both GR and PR (26).

Most of the endocrine and metabolic effects of glucocorticoids are thought to be mediated via binding to GRE, whereas the anti-inflammatory effects of glucocorticoids are mainly mediated by repression of other transcription factors (for a review see (4)).

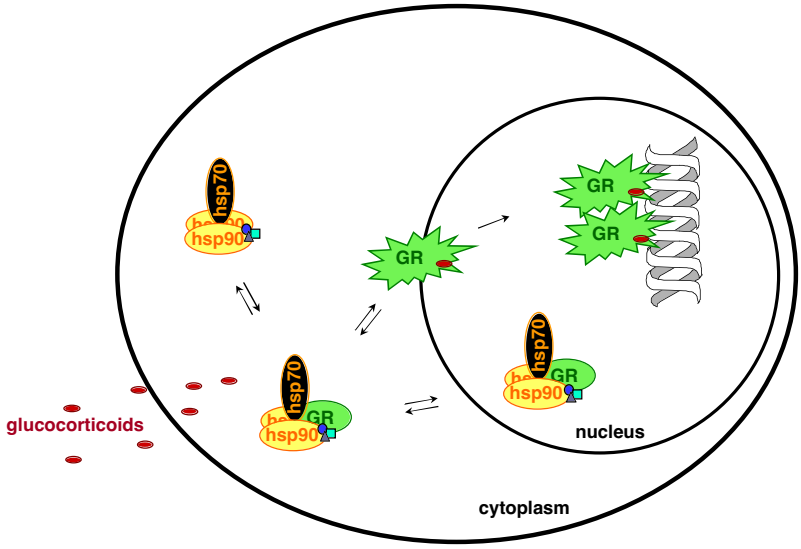

Figure 3. Mechanism of GR action. When non-active, GR resides mainly in the cytoplasm bound to a multiprotein complex containing a dimer of hsp90, hsp70, Hop, hsp40 and p23. A small fraction of GR associated to hsp's may reside in or recirculate to the nuclear compartment. Upon ligand binding, GR dissociates from the multiprotein complex and translocates into the nucleus where it binds as a dimer to specific GRE sites in glucocorticoid regulated genes.

GR phosphorylation

Phosphorylation of a protein can modulate various protein functions, for example regulate enzymatic activity, subcellular localization, protein-protein interactions, DNA binding or ubiquitination (50). The phosphorylation state of GR is regulated both by the cell cycle and by the ligandation state of GR (9). Nonliganded GR has a basal phosphorylation level and upon ligand binding GR becomes hyperphosphorylated. Most phosphorylated residues are serines and threonine in the N-terminal part of the receptor (9,50). Phosphorylation of GR has been suggested to affect receptor turnover, subcellular trafficking and transcriptional regulatory functions. The mitogen-activated protein kinases (MAPK), the cyclin-dependent kinases (CDK) and c-Jun N-terminal kinase (JNK) are responsible for phosphorylation of GR (59,51) and probably different phosphorylation sites in GR can be phosphorylated by multiple kinases depending on distinct cell signaling (50). Differentially phosphorylated GR has been shown to reside in unique subcellular compartments (112). Little is known about the phosphatases involved in regulating GR dephosphorylation. It has been suggested that GR dissociation from hsp90 and subsequent translocation to the nucleus requires dephosphorylation (34). GR binding to other proteins can depend on the phosphorylation state of GR. For example, association of a particular GR phospho-isoform with p85, a subunit of phosphatidylinositol 3-kinase (PI3K), stimulates the formation of PIP3 (50) indicating

that GR phosphorylation may also play a role other than in the context of direct transcriptional effects of GR.

The progesterone receptor (PR) is also phosphorylated by multiple protein kinases. The phosphorylations occur mainly on serine residues in the N-terminal part of the PR (106). The role of PR phosphorylation is known to influence interactions with co-regulators, receptor turnover and nucleo-cytoplasmic shuttling. MAPK-dependent phosphorylation of unliganded PR leads to rapid nuclear translocation to the nucleus but the consequence is unclear (61). Phosphorylated PR binds progestin with a higher affinity as compared to its less phosphorylated counterpart (89).

The estrogen receptors α and β (ERα and ERβ) are phosphorylated in response to estradiol binding and the phosphorylation results in enhanced ER-mediated transcription (62). Also, activation of the MAPK pathway increases phosphorylation of both ERα and ERβ (107,62). Both PKA and PKC are known to phosphorylate ERα and thereby increase the genomic effects of estrogens (23).

The precise role of steroid receptor phosphorylation and the functional significance remain elusive. Intracellular protein kinases are emerging as possible mediators of steroid receptor action. Cross-talk between steroid receptor- and growth factor-initiated signaling might serve to coordinate the different responses to steroid hormones and growth factors (61).

Rapid non-genomic effects of GR

There are reports that GR, besides its well-documented functions as a ligand dependent transcription factor, also influences processes in the cell that do not require gene transcription or gene regulation (65). These transcription independent reactions occur very fast, often within minutes after administration of glucocorticoids and indicate that the effects of GR are much more complex than previously anticipated. For example, Hafezi-Moghadam *et al*. have shown that liganded GR activates endothelial nitric oxide synthetase (eNOS) in a rapid, non-transcriptional manner that leads to nitric oxide dependent vasorelaxation (41). eNOS is activated by the PI3K and protein kinase Akt (Akt) pathway. The exact mechanism behind liganded GR activation of eNOS is unknown but the process is thought to involve a particular phospho-isoform of GR that activates the PI3K-Akt pathway (41,50). Estrogen is also known to have rapid effects on the vasodilation of blood vessels and this effect is believed to involve calcium and PI3K/Akt-mediated stimulation of eNOS activity (55,103). Very recently, this effect was shown to be mediated via the G protein-coupled receptor GPR30, an intracellular 7-transmembrane receptor that is localized predominantly in the endoplasmic reticulum where it specifically binds estrogens (91).

In a study by Croxtall *et al*. in 2000, it was found that glucocorticoids inhibited epidermal growth factor (EGF) activated release of arachidonic acid (AA) within

minutes and that this effect was transcription-independent. AA is cleaved from the phospholipid layer in the cell membrane by phospholipase A (PLA), which in turn is activated by extracellular signal-regulated kinase (ERK) which itself is activated by MAPK/ERK kinase and Raf. The effect of glucocorticoids on PLA activity and AA release was claimed to be mediated via the protein annexin 1 (17) (for a review on annexin 1 see (52)).

Mineralocorticoids and glucocorticoids have been reported to have effects on Na^+ uptake in the kidneys, independent of transcription or translation. This effect is thought to involve stimulation of the calmodulin-dependent serine-threonine phosphatase calcineurin activity by a pathway that involves the release of hsp's from the GR complex (108).

Also, progestin treatment leads to rapid and transient activation of the MAPK signaling pathway and this effect is PR dependent but independent of PR transcriptional activity (10).

GR interacting proteins

GR interacts with many different proteins, hsp's, co-regulators, co-repressors, basal transcription factors and chromatin remodeling proteins. GR also interacts with several transcription factors, for example, the NF-κB transcription factor (13), AP-1 (43) and STAT5 (105). Cross-talk between steroid receptors and receptor tyrosine kinases has been reported. For example, estrogen-like effects of epidermal growth factor (EGF) require ER since EGF failed to induce transcription and DNA synthesis in the uterus of the ER knock-out mice (18). A similar relationship has been reported for the insulin-like growth factor 1 (IGF-1) and ERα (57). ERα and PR as well, have been found to physically interact with the cytoplasmic tyrosine kinase molecule, Src (102). Src seems to be responsible for integrating the signaling pathways of growth factors and estrogens (102). Src is thought to be involved in the dexamethasone inhibition of EGF induced release of AA reported by Croxtall *et al*. (17).

GR-interacting proteins identified in Papers I-IV in the present thesis

14-3-3

Proteins of the 14-3-3-family are highly conserved and widely distributed in all eukaryotic cells studied so far. In mammals there are seven isoforms of the 14-3-3 protein, β, γ, ε, η, σ, τ and ζ (1). The 14-3-3 proteins have been found to associate with more than 200 cellular proteins (97) and most often the 14-3-3 proteins bind to the phosphorylated forms of these proteins (110,70). Different isoforms of the 14-3-3 family have a distinct subcellular pattern and tissue distribution in both plants and animals and expression of 14-3-3 also changes during development and in response to extracellular signals (70). 14-3-3 exists as a dimer *in vivo* and has three major structural functions upon binding: 14-3-3 can function as an adaptor protein *i.e*. it binds to and connects two different proteins at the same time; 14-3-3 can cause a

conformational change in target protein; 14-3-3 can also mask a specific region on a target protein (12).

Members of the 14-3-3 protein family are involved in many different cellular processes such as signal transduction, cell-cycle control, apoptosis, stress response and malignant transformation (110,70). To mention a few examples, 14-3-3 may affect the cell cycle by binding to the mitotic inducer Cdc25 and thereby functions as an attachable nuclear export signal for Cdc25 (67). The 14-3-3 interaction with the insulin receptor substrate-1 (IRS-1) interrupts the association between the insulin receptor and IRS-1 (81), whereas the reported 14-3-3 interaction with Raf-1 has been shown to be a prerequisite for plasma membrane recruitment and activation of Ras (96).

In a yeast two-hybrid screening, 14-3-3η was found to associate with the LBD of GR in a ligand dependent manner (111). 14-3-3 has been proposed to act as a chaperone for GR thereby facilitating the release of the liganded, monomeric GR from hsp90. 14-3-3 is also proposed to facilitate the dimerization and/or translocation of GR to the nucleus (111). More recent research published in JBC 2003 shows that 14-3-3 helps localize GR in the cytoplasm and that 14-3-3 contributes to nuclear export of GR after withdrawal of ligand (56). Also, 14-3-3 was found to suppress ligand-activated GR-induced transactivation of a glucocorticoid-responsive promoter (56).

Raf

Raf is a key regulator of the MAPK pathway (Fig. 4). The MAPK signaling cascade mediates many different signals within a cell and its regulation is complex and still not fully elucidated. The MAPK pathway is used by several membrane-bound receptors (*i.e.* growth factor receptors and cytokine receptors (47)) to transmit different signals involved in proliferation, differentiation, survival and apoptosis (86). The MAPK pathway starts with the activation of the small membrane-bound G protein Ras. Activated Ras recruits Raf from the cytosol to the cell membrane. Raf thereby becomes activated and phosphorylates and activates MAPK/extra-cellular-signal-regulated kinase (ERK) kinase (MEK), which in turn phosphorylates and activates ERK. Upon activation through phosphorylation, ERK can translocate to the nucleus and activate transcription. ERK has several cytoplasmic substrates, which in turn can also affect gene expression (47,58,45).

Raf consists of a family of three kinases, A-Raf, B-Raf and Raf-1. Knockout studies in mice have shown that the different Rafs seem to have different functions: Knockout of the A-Raf gene results in neurological defects and the mice die 7-21 days post partum (88); B-Raf knockout mice die in midgestation due to haemorrage caused by massive apoptosis of endothelial cells (117); Raf-1-deficient mice die in midgestation due to widespread apoptosis throughout the embryo but especially the liver and placenta showed anomalies (48,77). The studies of Hüser and Mikula indicate that Raf-1 plays a crucial role in preventing apoptosis.

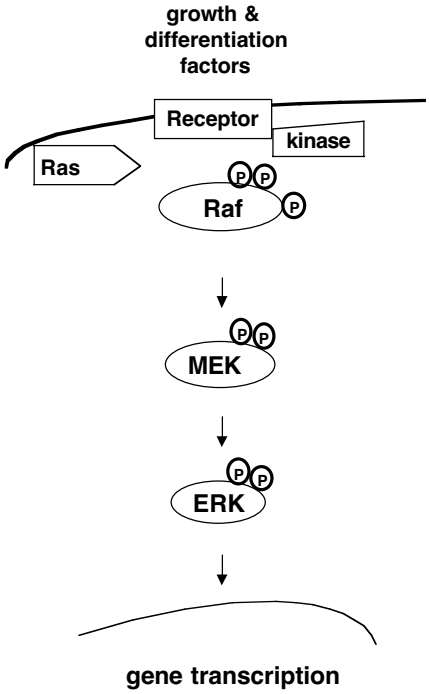

Figure 4. The Ras/Raf/MEK/ERK-signaling pathway.

Many proteins interact with various components within the MEK pathway and the interacting proteins are important for the function and regulation of the pathway. Among these interacting proteins are hsp90 and hsp70 and their role is thought to enhance the physical interaction between the components of the pathway, thereby rendering the signal transmission more efficient (22). It is also known that Raf-1 binds to 14-3-3 and that this binding leads to activation of Raf-1 (28,32,49).

NF-κB proteins

NF-κB is an inducible transcription factor that regulates more than 100 genes involved in the immune and inflammatory responses (for an updated list of NF-κB regulated genes: http://people.bu.edu/gilmore/nf-kb/). NF-κB is activated by a wide variety of stimuli including the pro-inflammatory cytokine tumor necrosis factor α (TNFα), oxidative stress, bacterial and viral proteins. Nonactivated NF-κB resides in the cytoplasm associated with an inhibitory protein IκB (2). IκB covers a nuclear localization signal of NF-κB and inhibits NF-κB activation. Activation and nuclear translocation of NF-κB is preceded by phosphorylation of IκB by an IkB kinase complex (IKK), followed by ubiquitinylation by a ubiquitin ligase and proteolytic degradation of IκB (53) (Fig. 5). Almost all signals that lead to activation of NF-κB converge on the activation of IKK. The IKK complex consists of at least three subunits: IKKα and IKKβ, which both contain kinase activity and IKKγ, a regulatory unit that assembles the IKK complex (16,95). Several kinases are thought to be

involved in the activation of the IKK-complex, for example PKC (60), Akt (84), NIK (NF-κB inducing kinase) (66).

NF-κB is a dimer, and the classical and most studied dimeric NF-κB transcription factor is the p65/p50 heterodimer of which p65 is the transcriptionally active subunit (for reviews on NF-κB, see Ref. (75,63,20)). Many of the genes that are upregulated by NF-κB are suppressed by GR. For example, NF-κB positively regulates the transcription of several cytokines, cell adhesion molecules and complement factors that are suppressed by glucocorticoids (109).

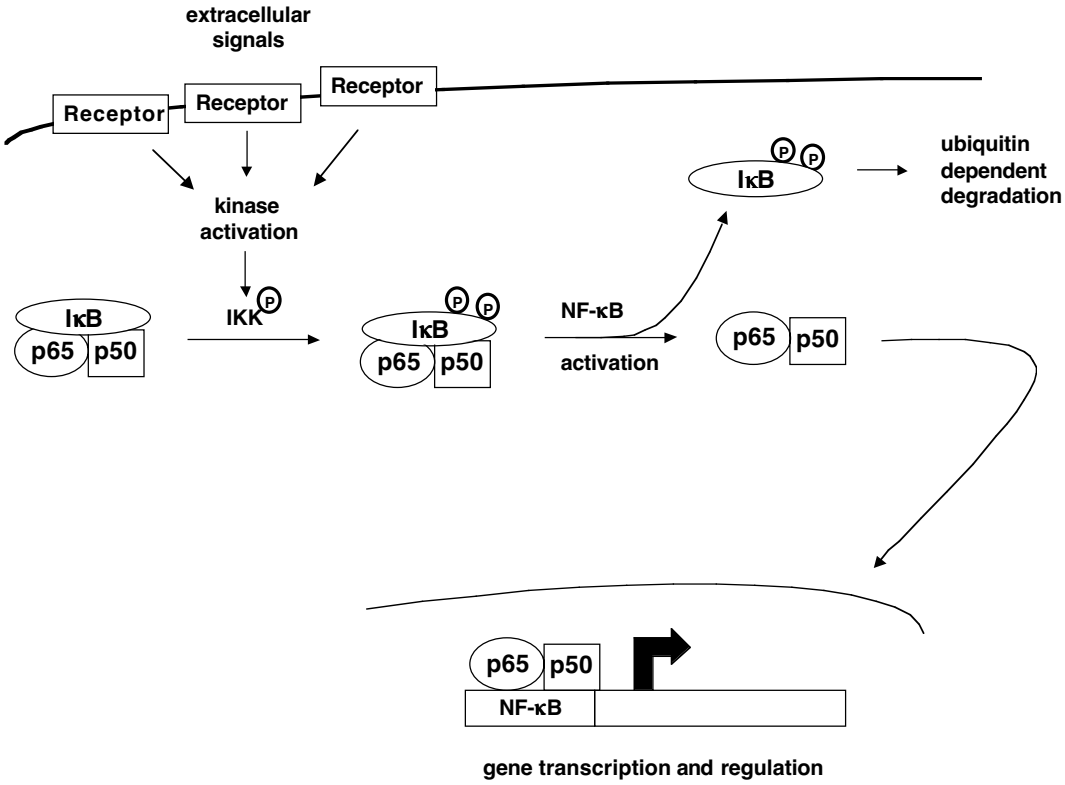

Figure 5. NF-κB activation pathway. NF-κB is activated by many stimuli and the different pathways converge on the activation of IKK which phosphorylates IκB which leads to ubiquitin dependent degradation of IκB. The degradation of IκB renders nuclear translocation of NF-κB possible. In the nucleus, NF-κB binds to NF-κB response elements on DNA and thereby regulates gene transcription.

In mammals, there are five members of the NF-κB family: p65 (RelA), c-Rel, RelB, NFKB1 (p50 and its precursor p105) and NFKB2 (p52 and its precursor p100). These proteins share a Rel homology region, which is necessary for dimerization, DNA binding, nuclear localization and interaction with IκB (75).

The IκB family consists of several members of which IκBα, IκBβ and IκBε are the most important in mammals. All IκBs share multiple ankyrin repeat motifs that are involved in the protein interaction between NF-κB and IκB (75).

Recent research has indicated that GR and NF-κB interact physically and that they function as mutual transcriptional antagonists. In various experimental *in vitro* systems, GR has been found to associate with the p65 subunit of NF-κB. The interaction and subsequent cross-talk is considered to occur in the nucleus of cells upon stimulation of GR and NF-κB (75) and is known to involve the Rel homology domain of p65 and the DNA binding domain of GR (74,13,100,64).

Flt-3

Flt-3 (Fms-like tyrosine kinase 3), also known as FLK-2 (Fetal liver kinase-2) and STK-1 (human stem cell kinase-1) was cloned independently by two groups in 1991 from cDNA libraries (93) and from murine fetal liver cells (73). The receptor tyrosine kinase Flt3 belongs to the class III receptor tyrosine kinase (RTK) receptor family. The RTK III family also includes KIT, FMS and the PDGF receptors. The RTK III family is characterized by an extracellular domain comprised of five immunoglobulin-like domains, a juxtamembrane domain and a cytoplasmic domain with a split tyrosine kinase motif (92). In mice, *flt3* mRNA is expressed ubiquitously in most tissues. Strong expression is found in hematopoietic organs (bone marrow, spleen, thymus, lymph nodes and fetal liver) as well as in neural and placental tissues (72). In humans the *flt3* gene expression shows a more restricted pattern. The *flt3* gene is predominantly expressed in immature lymphoid and myeloid cells, but *flt3* has been detected in fetal liver, spleen and thymus (94) (for a review see also (69)). Mutations of Flt3 have been detected in about 30% of patients with acute myeloid leukemia (AML) (36) and these mutations have been associated with a poorer disease prognosis.

The ligand for Flt3 (FL) is a transmembrane protein that can be released as a soluble homodimeric protein (42,98). Both variants of the ligand can activate the tyrosine kinase activity of the receptor and thereby stimulate proliferation and survival of hematopoietic progenitor cells, as well as differentiation of early B, NK and dendritic cell progenitors (for a review see (29)). However, FL does not efficiently work on its own but strongly synergizes with other hematopoietic growth factors and interleukins (68). The binding of FL to the extracellular Flt3-domain, leads to conformational changes that induce and stabilize receptor dimerization (114). Receptor dimerization brings the kinase domains together and this induces tyrosine autophosphorylation and further kinase activity. In both human and mouse, the expression of *fl* is widespread and particularly abundant in stromal cells, endothelial cells and fibroblasts from hematopoietic tissues (29).

Aims of the thesis

In our efforts to further understand other modes of GR signaling than the classical transcriptional signaling, we set out to investigate if GR also interacts with other proteins, besides the hsp-complex, in the cytoplasm of cells. Hence, the aims of this study were as follows:

- To identify proteins interacting with GR in the cytosol of rat liver

- To characterize conditions when specific protein interactions occur

- To examine if/how the GR interacting proteins could affect the function of GR

Methodological comments

GR purification using immunoaffinity chromatography

To identify hitherto unknown GR-interacting proteins in rat liver cytosol, we have developed a GR immunoaffinity chromatographic purification method (Fig.6). The starting material for purification was rat liver, usually taken from adrenalectomized rats. After adrenalectomy, the amount of GR in the livers is upregulated and more GR can be purified from each animal. The adrenalectomy also assures that GR is purifed in its nonliganded/nonactivated state only. To obtain GR in a liganded/activated state, ligand was added to the cytosol followed by dilution (1:2) and heat activation. By using the anti-GR monoclonal antibody (mAb 250) with a known epitope for binding, the corresponding peptide can be used for elution of GR. The elution is mild and specific and GR and its interacting proteins are eluted in their native state. The disadvantage of using peptide elution is that not all GR comes off the column. To accomplish a complete elution of GR, it was necessary to use 2.5 M NaSCN.

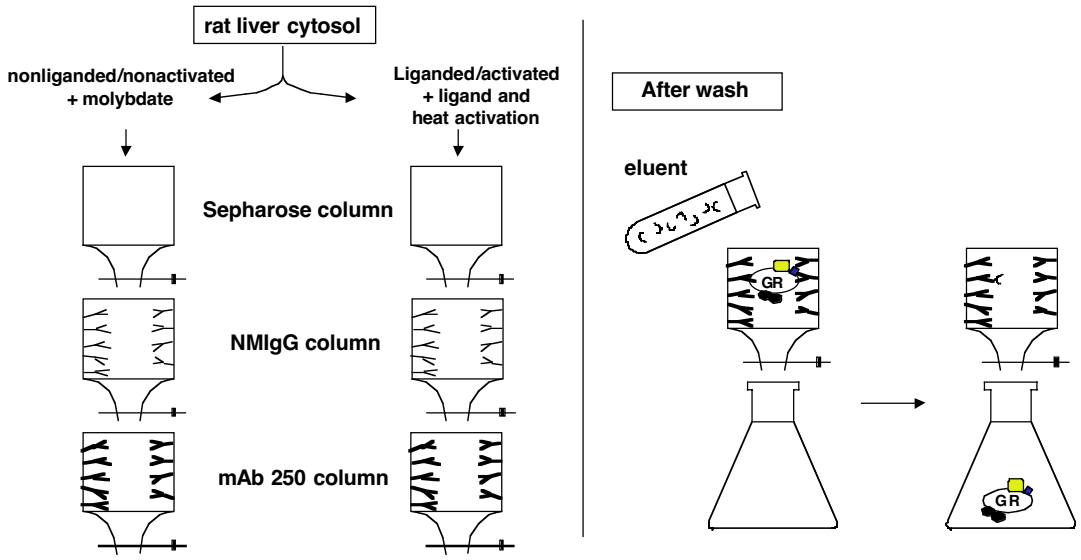

Figure 6. Schematic picture showing the GR immunoaffinity chromatographic purification. Rat liver cytosol was prepared from adrenalectomized rats and divided into two equal parts. Molybdate was added to obtain GR in its nonliganded/nonactivated state and ligand was added followed by heat activation to obtain GR in liganded/activated state. The cytosols were prepurified first on a Sepharose column followed by a column containing normal mouse IgG (NMIgG) immobilized on a Sepharose matrix. The prepurified cytosols were subsequently applied to columns containing the anti-GR monoclonal antibody 250 coupled to a sepharose matrix. After washing, the epitope-corresponding peptide was used for elution of GR.

To identify the GR co-purifying proteins, the proteins were analyzed by SDS-PAGE followed by Western blot (Paper I and Paper II). By using Edman based N-terminal sequencing, a previously unknown GR co-purifying protein was identified (Paper III). Edman based N-terminal sequencing was less suitable to identify novel GR interacting proteins, since endogenous mammalian proteins often are N-terminally modified. Instead, 2-D gel electrophoresis followed by in-gel tryptic-digestion and MALDI-TOF mass spectrometry analysis proved to be a better alternative to identify GR co-purifying proteins (Paper IV) since the method demands less protein, is quicker and not hampered by N-terminally blocked proteins.

Western blot

Standard protocol for the Western blot assays was used. Worth noting is that three types of substrate, SuperSignal® Substrate, SuperSignal© WestFemtoMaximum Sensitivity Substrate or ECL™ Advanced Western Blotting Detection Kit were used. The two latter substrates are more sensitive and they were used when the protein amount in the experiment was limited.

Salt gradient analysis

To investigate the binding affinity of GR to the mAb 250 and to investigate the strength of the interaction between GR and its associated proteins, salt gradient analyses were performed. Salt breaks electrostatic protein-protein bonds and the principle is that the higher concentration of salt a protein bond can withstand, the stronger is the binding. Already 300 mM and 600 mM NaCl can be enough to interfere with weak electrostatic protein bonds. A protein bond that withstands >1 M NaCl is strong.

Rat hepatoma cells

In Paper II, the effect of a synthetic glucocorticoid, triamcinolone acetonide (TA) and the inflammatory stimulus, TNFα, on GR/NF-κB interaction was studied in a rat hepatoma cell line. The rat hepatoma cell line expresses native levels of both GR and NF-κB. As a starting point, we had to modify our original GR-purification protocol and evaluate if it was possible to scale down the experiment for purification of GR from small cell-pellet extracts. We also established a method for purification of GR from nuclear extract.

In Paper III we also used the rat hepatoma cells to investigate the cellular distribution of GR and Flt3 after treatment with dexamethasone and/or FL. First of all, we investigated if Flt3 was expressed in these cells and we detected endogenous Flt3 both in the cytosolic and the nuclear extract of rat hepatoma cells.

GST pull-down

GST pull-down is used to identify or verify direct protein interactions *in vitro*. A protein or a domain of a protein, fused to GST and overexpressed in *E. Coli*, can easily be purified using glutathione-agarose beads. The GST-fusion proteins bound to beads are incubated with *in vitro* translated ^{35}S-methionine labeled proteins and direct

protein–protein interactions can be detected by SDS-PAGE and subsequent autoradiography. The method is widely used and a valuable tool when studying protein-protein interactions. However, one should bear in mind that weak and/or non-physiological interactions may well take place using overexpressed proteins.

2-D gel electrophoresis

To obtain a general overview of GR and its co-purifying proteins, a broad range 2-D electrophoresis system was used covering isoelectric points between 3 to 10 and protein masses from 10 to 150 kDa. We were initially unable to identify the protein spot(s) representing GR on the silver stained 2-D gels in the theoretical region of GR (94 kDa, pI 6.1). In a Western Blotting experiment, we did not detect full length GR, only smaller GR-fragments. We hence draw the conclusion that GR was probably degraded during the 12-hour long rehydration step preceding the isoelectric focusing. The in-gel rehydration step was changed to sample cup loading of the sample in the first dimension, to decrease the time at room temperature in order to minimize degradation of GR. After changing the protocol, GR spots were detected in the predicted region (94 kDa, pI 6.1).

2-D DIGE

The 2-D fluorescence difference gel electrophoresis (DIGE) technology used in Paper IV is based on the labeling of biologically different samples with fluorescent dyes (CyDyes) of different emission wavelengths but with similar mass and charge. The 2-D DIGE technique allows a simplified image analysis of 2-D gels, as two samples can be separated in one and the same gel and distinguished by their different colors. Furthermore, the CyDye labeling leads to a broader range of linearity of staining intensity in relation to protein amounts, as compared to conventional Coomassie and silver staining.

Mass spectrometry

To identify the GR interacting proteins we used MALDI-TOF (matrix assisted laser desorption ionization – time of flight) mass spectrometry. All proteins that matched fingerprint spectra with a Mascot score above 90 were considered significant. Protein-hits with Mascot scores below 90 were confirmed by post source decay analysis of single peptides.

Results and Discussion

GR interacts with 14-3-3 and Raf-1 (Paper I)

In the first paper, we established and described the immunoaffinity chromatography of GR from rat liver cytosol. By using the anti-GR mAb 250, a peptide corresponding to the identified epitope for mAb 250 was used for elution of GR. The method is specific and when cytosol was purified on a normal mouse IgG (NMIgG) column no GR was detected using Western blotting.

It had previously been demonstrated *in vitro* that GR interacted with 14-3-3 in a ligand-dependent manner (111). We studied if the previously reported GR-14-3-3 interaction found *in vitro* could also be verified *in vivo* using co-immunoaffinity chromatography. Indeed, by using this method followed by Western blotting we detected 14-3-3 among the GR co-purifying proteins. Thus, we concluded that our method was suitable for verifying already known GR interactions found *in vitro*. The method is valuable since it is based on endogenous proteins and on *in vivo* like conditions. We also examined if Raf-1 and IRS-1 which are known 14-3-3 interacting proteins were also GR co-purifying proteins. Indeed, we identified Raf-1 among the GR co-purifying proteins.

To evaluate the GR immunoaffinity chromatography we performed a salt gradient analysis that showed that the mAb 250 binding to GR is very stable and despite extensive washing with increasing salt concentrations the column still retained GR that could be eluted later using the specific peptide. We studied the interaction of GR and Raf-1 as well as of GR and 14-3-3 using the high salt gradient experiment. The result showed that the associations of 14-3-3 and Raf-1 to liganded/activated GR was strong enough to withstand a 2.4 M salt gradient washing.

Using the immunoaffinity chromatographic purification of GR it is not possible to establish whether GR and its co-purified proteins interact directly or indirectly. Possibly, the GR-Raf-1 association is mediated via 14-3-3 or other GR interacting proteins, for example hsp90 which has also been reported to interact with Raf-1.

Based on our studies, we speculate that cytosolic GR, besides occurring in the well-known hsp-complex, also interacts with other specific proteins in the context of dynamic multiprotein complexes, "receptosomes". Since the GR-14-3-3 and GR-Raf-1 interactions were strengthened following ligand activation of GR, we also speculate that introduction of glucocorticoids into the GR-receptosome might trigger changes in the GR-protein interactions leading to rapid non-genomic effects.

Studies of Glucocorticoid Receptor Interactions with NF-κB proteins (Paper II)

In this paper, we continued the investigation of cytosolic GR interacting proteins. Again, we investigated proteins previously found to associate with GR *in vitro*, in this case the NF-κB proteins. The GR-NF-κB interaction has been studied extensively

using *in vitro* experiments and this interaction is known to lead to a mutual transcriptional antagonism.

We found that, using immunoaffinity chromatographic purification of GR from liver cytosol from adrenalectomized rats, the NF-κB proteins p65 and p50 both co-purified with GR. p65 and p50 co-purified with GR both when GR was in its nonliganded/nonactivated state as well as in its liganded/activated state. The same thing was true for the NF-κB inhibiting protein, *i.e.* IκBα co-purified with GR independently of the functional state of GR. Both the GR-p65 and GR-IκBα interactions withstood high salt washing, indicating that the interactions between GR-p65 and GR-IκBα are strong.

To further investigate the interaction between GR and p65 we wanted to study the GR-p65 interaction both in the cytosol and in the nuclear extract after treatment with TA and/or TNFα. To be able to perform experiments where we added different ligands we used rat hepatoma cells, expressing endogenous GR, p65, p50 and IκBα. We modified the GR immunochromatographic purification protocol to assure that we still could detect GR and GR co-purifying proteins in a small scale cell experiment.

We found that p65 and IκBα co-purified with cytosolic GR from the rat hepatoma cell line independently of TA and TNFα. We also found that the combined treatment with TA and TNFα for 15 min did not result in as much GR nuclear translocation as did treatment with TA only. We hence speculated that activated p65 hinders or delays GR from entering the nucleus. Upon TNFα treatment for 30 min, p65 and p50 entered the nucleus and p65 and p50 appeared as GR co-immunoprecipitating proteins in the nucleus of rat hepatoma cells. Following combined treatment with TA and TNFα, p65 and p50 were still associated with GR in the nucleus but to a lesser extent than after treatment with TNFα only. We speculate that this is due to either a weaker association of p65 and p50 to liganded/activated GR in the nucleus than to nonliganded/nonactivated GR. Alternatively, a lower amount of nuclear p65 and p50 was available in the nucleus after treatment with TA and TNFα in combination as compared to treatment with TNFα alone.

Taken together, we have found that if both GR and NF-κB reside in the same cellular compartment in a liver cell, they can associate with each other regardless of TA and TNFα treatments. Our studies suggest a strong physical interaction between GR and NF-κB in the cytosol, and we propose that this direct interaction constitutes an integral part of GR-NF-κB cross-talk.

FLT3 interacts with the glucocorticoid receptor complex and affects glucocorticoid dependent signaling (Paper III)

To be able to identify hitherto unknown GR interacting proteins we analysed the GR co-purifying proteins by Edman based N-terminal sequencing. We obtained one interesting sequence that corresponded to the receptor tyrosine kinase Flt-3. This finding was also confirmed by Western blotting.

Flt3 was found to interact with both the nonliganded and the liganded form of GR. In this study, we have also analyzed which part of GR that is responsible for the interaction with Flt3. We found that the DNA-binding domain of GR is sufficient for Flt3 interaction as shown by GST-pulldown experiments.

Studies of the effects of Flt3 and its ligand FL in a glucocorticoid driven reporter-gene assay in Cos7 cells (African green monkey kidney cells), shows that cotransfection with Flt3 and FL potentiates glucocorticoid effects. Using the rat hepatoma cell line H-II-4-E that contains endogenous GR and Flt3, treatment with FL leads to a decreased GR stability, prevented by dexamethasone. On the other hand, dexamethasone induced translocation of GR was unaffected by FL.

In summary an interaction between GR and Flt3 has been observed and this interaction has functional consequences for GR-signaling and stability. The protein-protein interaction of GR and Flt3 is an example of how cross talk between glucocorticoid/GR signaling and a hematopoietic growth factor may take place.

Proteomics of glucocorticoid receptor interacting proteins (Paper IV)

In this paper, we continued the investigation of the cytosolic GR interacting proteins employing a proteomic approach. Conventional proteomic techniques, such as two dimensional gel elctrophoresis and MALDI-TOF mass spectrometry, were adjusted and developed to be used in combination with the immunoaffinity chromatography of GR. The purification of GR from rat liver cytosol was performed with GR in the two functional states, i.e. nonliganded/nonactivated as well as liganded/activated. We showed a reproducible pattern of GR interactions with other cytosolic proteins, also when stringent washing conditions were employed.

The GR interacting proteins identified so far by these methods include some of the proteins previously identified as GR-interacting proteins, but also a number of hitherto unreported interaction partners for example: Major Vault Protein, TATA binding interacting protein 49 and glycoprotein PP63. Furthermore, using two dimensional difference gel electrophoresis we observed differences in the protein composition of the two functional states of the GR complex, notably for some acute phase proteins that showed a lower degree of interaction with liganded GR. We speculate that the introduction of glucocorticoids into the GR-receptosome triggers changes in the GR-receptosome that might lead to rapid non-genomic effects.

By using two dimensional blue native gel electrophoresis, we could demonstrate several GR receptosomes. This fits our hypothesis that different GR molecules interact with different protein complexes in a cell. Since we detect so many GR co-purifying proteins, it is hard to conceive that every GR co-purifying protein interacts with all GR molecules at the same time. In the previous papers we have also seen that only a fraction of both p65 and Flt3 interacts with a fraction of GR, a fact that also strengthens this theory. At the present time we do not know the number and specific composition of the various GR receptosomes, a topic that would merit further investigation in the future.

Conclusions

This study shows that GR interacts with a number of different proteins within rat liver cells. Proteins, regulating a vast number of vital biological functions in the way GR does, perhaps need to integrate their functions with other proteins via protein-protein interactions. We propose that cytosolic GR, besides occurring in the well-known hsp-complex, also interacts with other specific proteins in dynamic multiprotein complexes, "receptosomes" which may form the biochemical basis for cross-talk between GR and other signaling pathways.

This study shows that GR immunoaffinity chromatographic purification followed by proteomic techniques can be used for screening of cytosolic GR interacting proteins.

The earlier reported interactions between GR and 14-3-3 and GR and the NF-κB proteins have been verified using GR immunoaffinity chromatographic purification followed by Western Blotting. We conclude that our method is suitable for verifying already known GR interactions found *in vitro*

Raf-1 and Flt3 have been found to interact with GR in liver cytosol.

Using 2D-DIGE, we observe differences in the protein composition of nonliganded as compared to liganded GR complexes.

Different GR receptosomes were demonstrated using Blue Native PAGE.

Future directions

One straight forward mission is to further verify the GR protein interactions identified using the 2-D/Mass Spectrometry technique. Verifications can be done using, for example, Western blotting, reversed immunoprecipitations and/or glutathione *S*-transferase (GST) pulldown assay. When a protein is identified as a true GR co-purifying protein, functional effects of interesting candidates can be studied by using for example transfection assays. Presently, interesting candidates are MVP and glycoprotein PP63.

It would be very interesting to further investigate the content of different GR receptosomes and to study how addition of glucocorticoids changes the receptosome content. It would also be interesting to evaluate if/how the receptosome contents change upon variations of the phosphorylation status of GR.

It would also be interesting to continue studying the GR/NF-κB interaction, for example, to further investigate how GR and NF-κB affect the nuclear translocation of one another after treatment with TA and TNFα. This could be addressed by transfecting cells with constructs where different fluorophors were fused to GR and p65 and studying the kinetics of the movements of the proteins in living cells, by fluorescence microscopy.

Using GST pulldown experiments, one could continue to study the GR and Raf-1 interaction to find out if this interaction is direct or not. It would also be interesting to study how/if Raf and Ras/Raf/Mek/Erk signaling pathway and GR signaling pathway influence each other.

In the light of reported steroid receptor interactions with different tyrosine kinase receptor pathways, it would be interesting to further examine how GR and Flt3 signaling pathway influence one another. Do, for instance, glucocorticoids influence the differentiation of various stem cells, perhaps via Flt3 signaling?

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