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MOLECULAR MECHANISMS OF GLUCOCORTICOIDS – ANTI-INFLAMMATORY IMPLICATIONS

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To My Family

ABSTRACT

Glucocorticoids (GCs) play important roles in many biological processes including inflammatory responses. The study of this process has involved investigations of cross-talk abilities by GC receptor (GR) with cellular signaling pathways that have been associated with inflammatory disorders or implicated as contributory for the adverse effects observed with GC therapy. This thesis aims to provide additional insights into this area of research and contains studies made to investigate aspects of GC-cross-talk with some of these signaling pathways, in particular the MAPK, NF-κB and Wnt signaling pathways.

MKP-1, an anti-inflammatory feed-back inhibitor of MAPK-signaling, has also been shown to be up-regulated by GCs. In **paper I**, we demonstrate that GCs stimulate expression of MKP-1 through a positive "tethering" mechanism involving the GR and the promoter-bound transcription factor C/EBP, without GR itself contacting the DNA. This study emphasizes the multiple mechanisms that exist by which agonist-bound GR can affect gene expression, also in the anti-inflammatory response.

GR mutants are powerful investigative tools that can be used to study cross-talk between GCs and the pro-inflammatory canonical NF-κB pathway. In **paper II**, we demonstrate that a "loss of function" GR mutant is able to repress NF-κB activated by TNFα, but not by TPA, whereas the wild-type receptor inhibits NF-κB in both cases. This study highlights that the ability of the GR to repress NF-κB not only relies on gene and cell context but also depends on the signaling pathway that is employed to activate NF-κB.

The non-canonical NF-κB pathway is emerging as a key player in various inflammatory diseases, but a cross-talk between this pathway and GCs have not been described. In **paper III**, we demonstrate for that GCs inhibit non-canonical NF-κB signaling and that the GR physically interacts with RelB and inhibits the transcriptional activity of RelB/p52 heterodimers in a hormone-dependent manner.

GC-induced hippocampal damage and osteoporosis have been suggested to be caused by the inhibition of the Wnt-signaling pathway by GCs. In **paper IV**, we demonstrate that GC stimulation of DKK-1, a Wnt-antagonist, inhibits proliferation of neuronal progenitor cells. The agonist-bound GR binds to a GRE in the regulatory promoter region of the DKK-1 gene and induces its expression. Restoring the activity of Wnt-pathways that are inhibited by GCs may be valuable to limit some of the side-effects observed with GC therapy.

In conclusion, the studies in this thesis give additional insight into the complexity, reciprocity and variety of cross-talk mechanisms involving GR and several signaling pathways, thereby providing an increased understanding of the molecular mechanisms behind GC effects with emphasis on their anti-inflammatory actions.

LIST OF PUBLICATIONS

I. **Krishan Johansson-Haque**, Elanchelian Palanichamy and Sam Okret. *Stimulation of MAPK-phosphatase 1 gene expression by glucocorticoids occurs through a tethering mechanism involving C/EBP*. J Mol Endocrinol. 2008 Oct;41(4):239-49.

II. Lars-Göran Bladh, **Krishan Johansson-Haque**, Ingalill Rafter, Stefan Nilsson and Sam Okret. *Inhibition of extracellular signal-regulated kinase (ERK) signaling participates in repression of nuclear factor (NF)-kappaB activity by glucocorticoids.* Biochim Biophys Acta. 2009 Mar;1793(3):439-46.

III. **Krishan Johansson-Haque**, Elanchelian Palanichamy and Sam Okret. *Hormone-dependent Glucocorticoid receptor interaction and transcriptional repression of NF-kappa B RelB*. Manuscript

IV. Michaela Moors, Raj Bose, **Krishan Johansson-Haque**, Karin Edoff, Sam Okret and Sandra Ceccatelli. *Dickkopf 1 mediates glucocorticoid-induced changes in human neural progenitor cell proliferation and differentiation.* Toxicol Sci. 2012 Feb;125(2):488-95.

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
AF	Activator function
BAFF	B-cell activating Factor
C/EBP	CCAAT Enhancer binding protein
CBG	Corticosteroid-binding globulin
CBP	CREB-binding protein
COX-2	Cyclooxygenase 2
ChIP	Chromatin immunoprecipitation
CREB	cAMP response element-binding protein
CRH	Corticotropin-releasing hormone
DBD	DNA-binding domain
DKK-1	Dickkopf-1
ERK	Extracellular signal-regulated kinase
GC	Glucocorticoid
GILZ	Glucocorticoid-induced leucine zipper
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
HPA	Hypothalamic-pituitary-adrenal
HSD	Hydroxysteroid dehydrogenase
IL	Interleukin
IKK	IκB kinase
JNK	c-Jun N-terminal kinase
LBD	Ligand-binding doain
LPS	Lipopolysaccharide
LTβR	Lymphotoxin beta receptor
MAPK	Mitogen-activated protein kinase
MEKK	MAPK kinase
MKP-1	MAPK phosphatase-1
NF-κB	Nuclear Factor kappa B
NTD	N-terminal domain
NPC	Neuronal Progenitor Cells
PEPCK	Phosphoenolpyruvate carboxykinase
RHD	Rel homology domain
SMRT	Silencing mediator for retinoid or thyroid-hormone receptors
SRC	Steroid receptor coactivator
STAT	Signal transduction and activator of transcription
TAD	Transcriptional activation domain
TAK-1	Transforming growth factor β–activated kinase
TAT	Tyrosine aminotransferase
TLR	Toll-like receptor
TNF	Tumor Necrosis Factor
TPA	12-O-tetradecanoylphorbol-13-acetate

1 BACKGROUND

In 1948 the therapeutic effects of glucocorticoids (GCs) were discovered when Compound E (later named cortisone) was successfully tested on arthritic patients. The impact of this event in the history of modern medicine has been profound (1). Due to their powerful ability to weaken the activity of the immune system and dampen inflammatory responses, cortisone and other GC equivalents have been extended to treat a wide spread of chronic inflammatory conditions. Today, exogenous GCs are the most commonly used drugs to treat autoimmune disorders, including rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, inflammatory bowel disease, respiratory disorders such as asthma and chronic obstructive pulmonary disease, sepsis and multiple hypersensitivity and allergic reactions (2, 3). In addition to inflammatory diseases, GCs are commonly used for their immunosuppressive properties in transplantation, and for their anti-proliferative effects in lymphoid malignancies. However, prolonged use of GCs for therapeutic purposes has since the initial implementation been associated with multiple crippling side effects that significantly limits GC employment in the clinic. Osteoporosis is one of the most disabling side effects observed with long-term use of GCs, which leads to an increased fracture risk of the spine, hips and ribs (4). Other side effects accompanied with GC therapy include diabetes mellitus, hypertension, affective disorders, impaired wound healing, skin atrophy, central obesity and muscle wasting (5). Also, as a consequence of GCs immunosuppressive activity, immunodeficiency and increased sensitivity to pathogenic infections is a vital concern with long-term GC therapy. Indeed, even after more than half a century, the clinical use and potential for misuse of GCs is a matter of debate but despite the problems associated with their long-term use, GCs still remain the mainstay treatment of multiple inflammatory conditions (6). In addition, GC-resistance, characterized by either a progressive, partial or complete inability of inflamed tissues to respond to GCs, represents additional problems in current treatment of immune disorders such as asthma, inflammatory bowel disease and multiple sclerosis (2). The obstacles connected to GC therapy need to be overcome in order to improve the specificity and safety of GCs in the clinic. For this purpose, attention has for a long time been focused on elucidating aspects important for the biological effects of GCs such as regulation of inflammatory responses at the molecular level. This has involved, among many things, the study of GC receptor (GR) interactions or “cross-talk” with

cellular signaling pathways that have been implicated in the pathology of inflammatory disorders (such as NF-κB and MAPK signaling pathways) or implicated as contributory for the adverse effects observed with GC therapy (e.g. metabolic and Wnt signaling pathways). This work contains studies (**Paper I-IV**) made to address aspects of the cross-talk ability of GR with some of these signaling pathways and presents some new findings in this area of research.

2 INTRODUCTION

2.1 GLUCOCORTICOID SIGNALING

2.1.1 Glucocorticoids and their functions

GCs are small fat-soluble steroid hormones produced from the adrenal cortex of vertebrates that influence the functions of basically all organs and tissues throughout life, with key roles in a broad range of biological processes, including metabolism, immune responses, growth, development, differentiation and apoptosis (2, 7). GCs, in humans mainly cortisol, are the secreted end-effectors of the hypothalamic-pituitary-adrenal (HPA) axis, which is a set of integrating and reciprocal interactions between the endocrine and nervous system that is controlled by the circadian rhythm and stressful events (8, 9). Stress from diverse sources including deranged metabolism, inflammation or physical or emotional stressors stimulates the HPA-axis towards increased production of GCs. Upon exposure to stressful stimuli, the hypothalamus increases its secretion of the hormones vasopressin and corticotropin-releasing hormone (CRH), which trigger the pituitary gland to release adrenocorticotropic hormone (ACTH) thereby initiating the HPA-axis. ACTH is carried in the blood to the zona faciculata of the adrenal cortex, which in response to stimulation by ACTH produces GCs through the enzymatic processing of cholesterol**,** the precursor molecule of steroids, by the cytochrome P450 family of steroid hydroxylases and 3-β-hydroxysteroid dehydrogenase (3-β-HSD). The principal role of GCs secreted from the adrenals during the HPA-initiated stress response is to limit the effectors of the stress response and restore homeostasis. For instance, in the fasted state, GCs activates several processes to increase blood levels of glucose. These include increasing the stimulation of gluconeogenesis in the liver, lowering insulin release from pancreatic β-cells, and preventing glucose uptake in peripheral muscle and adipose tissue (7, 9, 10). At the molecular level, GCs stimulate hepatic gluconeogenesis by increasing the rate of transcription of genes that encode key gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) (7). Thus, the elevation of blood glucose follows the surge of GC secretion. Importantly, these metabolic functions of GCs are believed to form the basis for the adverse metabolic effects that is accompanied with long-term GC treatment e.g. steroid-induced diabetes.

Another central function of GCs is balancing the immune response to infection and to prevent excessive inflammatory conditions caused by overreaction of the immune system. Circulating messengers of inflammation, including the cytokines tumor necrosis factor (TNF)α, interleukin (IL)-1 and IL-6 stimulate the HPA-axis during inflammation to facilitate increased blood levels of GCs (11, 12). Elevated GCs in turn, suppress the activity of cells in the innate and adaptive immune system and modulate anti-inflammatory responses (13). Importantly, GCs decrease the global production of numerous mediators of inflammation, including pro-inflammatory cytokines, prostaglandins, and reactive oxygen and nitrogen species. They reduce leukocyte migration to sites of inflammation by inhibiting the expression of adhesion molecules, and promote apoptosis of leukocytes, thereby limiting the magnitude of the inflammatory response (14, 15). At the molecular level, a pivotal mechanism for the immunosuppressive and anti-inflammatory effects by GCs is through inhibition of NF-κB (Nuclear Factor kappa B), a key regulator of inflammation and a transcription factor critically involved in the synthesis of pro-inflammatory mediators, including cytokines (IL-1β, TNFα, IL-4, IL-5), chemokines (IL-8, RANTES, eotaxin), enzymes (inducible nitric oxide synthase, iNOS; cyclooxygenase-2, COX-2) and adhesion molecules (Intercellular Adhesion Molecule-1, ICAM-1; Vascular Cell Adhesion Molecule-1, VCAM-1) (16-19). Global inhibition of this transcription factor and its target genes by GCs, therefore, blunts the capacity of the immune system to mount an inflammatory response. In addition, GCs concomitantly stimulate the expression of multiple important anti-inflammatory genes including IκBα (inhibitor kappa B-alpha), Annexin-1, GILZ (Glucocorticoid-induced leucine zipper), MKP-1 (MAPK phosphatase-1) and IL-10 contributing to a multifaceted suppression of inflammation by GCs (20). Because of their potent inhibitory effect on inflammation, exogenous GCs are among the most important drugs used today to treat various inflammatory and autoimmune disorders.

Importantly, GCs prevent their excessive production and HPA hyperactivity by acting back on the hypothalamus and pituitary in a negative feed back cycle, which results in the decline of elevated GC concentration back to basal levels (7, 8). Patients with Cushing's syndrome have sustained and pronounced blood levels of GCs due to pituitary or adrenal tumors that continuously produce ACTH or GCs. Similarly, administration of exogenous GCs may lead to Cushing's syndrome overrunning the normal feed-back regulation and giving rise to adverse effects including hypertension, hyperglycemia, obesity, muscle and bone catabolism, and impaired wound healing (7,

21) and could be considered to be normal but undesirable physiological effects of endogenous GCs.

The majority of GCs in the blood is bound to corticosteroid-binding globulin (CBG) and serum albumin and since the biologically active GCs are the unbound or free fractions, CBG binding regulates the availability of GCs to target tissues (22). For instance, during inflammation the CBG level decreases and as a consequence, the free fraction of hormone increases, which in turn suppresses inflammatory responses (23). GC availability is also regulated at the tissue level by the 11-β hydroxysteroid dehydrogenase (11-β HSD) system, which consists of the two enzymes 11-β HSD1 and 11-β HSD2. 11-β HSD1 increases the concentration of active GCs by converting biologically inert cortisone to biologically active cortisol, whereas 11-β HSD2 converts cortisol to cortisone and thereby decreases intracellular concentrations of biologically active GCs (24, 25).

Moreover, there is increasing evidence for extra-adrenal synthesis of GCs in a variety of tissues, including the thymus, intestine, skin and brain (26, 27). Whereas adrenal GC secretion has coordinated effects on multiple organ system, the local extra-adrenal synthesis of GCs results in tissue confined effects of GC action. For instance, in thymus there is a a role for endogenous thymic-derived GCs in regulating the homeostasis of thymocytes (28). Also, GC synthesis in intestinal epithelial cells was induced after immune cell activation, which in turn had an immunoregulatory effect on the activation of local T cells (29).

2.1.2 Glucorticoid receptor mechanisms of action

The GC receptor (GR) is the intracellular receptor to which GCs bind and elicit their effects and, as the other members of the nuclear receptor (NR) superfamily, the GR functions as a ligand-activated transcription factor. In the absence of GCs, GR resides predominantly in the cytosol where it is held in an optimal high affinity configuration for its steroid ligands by large multi-protein complexes consisting of several chaperone proteins such as heat shock proteins 70 and 90 (Hsp70/90) and co-chaperones such as immunophilins FK506 binding proteins (FKBPs) (30). Upon ligand binding, the GR becomes activated and hyperphosphorylated and initiates substitution of one immunophilin (FKBP51) for another (FKBP52) and recruitment of the transport protein dynein (31, 32). The conformational changes of GR lead to the exposure of its nuclear localization signals (NLSs), followed by subsequent translocation of GR in complex

with hsp90, FKBP52 and dynein into the nucleus where the complex is dissociated and GC-activated GR affects target gene expression (Fig. 1).

Three mechanisms of GC action are known: 1) Transcriptional activation or repression of target genes that requires a direct DNA-binding by GR, 2) transcriptional activation or repression of target genes that is independent of DNA-binding by GR and 3) transcription-independent non-genomic effects (33, 34). Transcriptional activation involving GR binding to the DNA occurs when translocated GR homodimerizes and binds to specific enhancer elements in regulatory regions of target genes called GC response elements (GREs), which can either be "simple" (or primary) GREs, where GR binds as the sole component of a regulatory complex or "composite" GREs, where GR interacts with at least one additional DNA-bound factor to synergistically mediate transcriptional activation (35, 36). The binding of GR to GREs is accompanied by a recruitment of transcriptional co-regulators including the p160 co-activator family members steroid receptor coactivator 1 (SRC-1), GRIP1 (SRC2/TIF2), and SRC-3 (37-39). The p160 members interact directly with liganded GR (and other NRs) through specific LXXLL motifs (L, Leucine; X, any amino acid) in their nuclear receptor interaction domain (NID) and recruit additional co-activators of transcription to the complex including histone acetylases p300 or its close homolog cAMP response element-binding protein (CREB)-binding protein (CBP). This generates a regulatory complex that stabilizes and initiates the basal transcription machinery to facilitate transcription, and ultimately, mediates the response of a target gene to GCs. Contrary to the positive GREs, which act as enhancers of transcription, negative GREs (nGREs) are suppressors of gene regulation and mediate DNA-dependent transrepression by GR. Agonist-bound GR interacts with GREs and can suppress the production mRNA by preventing activating transcription factors from accessing their binding sites via competition for DNA binding (40, 41). Additionally, GR has also recently been shown to associate with either transcriptional co-repressor proteins SMRT/N-CoR (Silencing mediator for retinoid or thyroid-hormone receptors/ nuclear receptor corepressor) or histone-deacetylase (HDAC)-6 at nGREs to mediate transrepression (42, 43). The presence of nGRE in GC-regulated genes has previously been rarely described but a recent study suggests that as many as thousand mouse/human ortholog genes contain transrepression-mediating nGREs (42). For instance, some nGREs has been found in genes involved in feedback mechanisms of GC activity (e.g. Pro-opiomelanocortin, POMC; CRH, GR, ACTH/MC_2- receptor and 11-β HSD2) and others in genes involved in glucose regulation (insulin precursor gene and insulin receptor gene) (42, 43).

Additionally, an interesting idea is the possible existence of so called "nonproductive" GR binding sites to which the GR interacts but has no apparent effects on transcriptional regulation (35, 44, 45). The genomic presence of such binding sites or of "occasionally" productive sites, dependent on the cell context, raises the questions of when or if GR binding to DNA is translated to an effect on transcription.

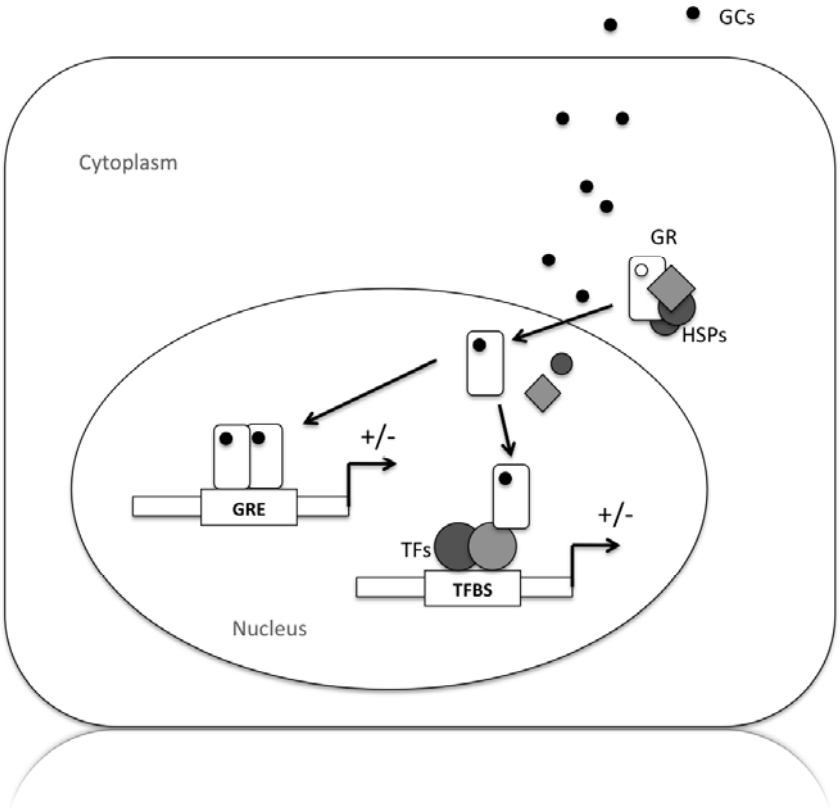

Figure 1. *GCs act via the intracellular GR, which can regulate gene expression by binding to GREs in the DNA or independently of GRE-binding through protein-protein "tethering" interactions with promoter-bound TFs. TF = Transcription factor, TFBS = TF binding sites, HSP = Heat-shock protein*

GR also regulate transcription through DNA-independent mechanism, where it does not itself bind specifically to DNA, but is recruited through interaction with another DNA-bound factor. This mechanism of transcriptional regulation is often referred to as "tethering" and can either enhance or suppress gene transcription depending on the activating and promoter-bound transcription factor with which GR interacts and gene context (46)(Fig. 1). For instance, GR interacts with signal transduction and activator of transcription 5 (Stat5) independently of its DNA binding function to stimulate expression of the β-casein gene (47), whereas the anti-inflammatory gene product IL-10 was recently shown to be activated by GCs through GRE-independent mechanisms

involving Stat3 (48). Additionally, the GR induces expression of the anti-inflammatory protein MKP-1 through a tethering mechanism involving the promoter-bound transcription factor C/EBP (CCAAT Enhancer binding protein) (**Paper I**). Interestingly, other studies have demonstrated a cooperative ability of GR and C/EBP for transactivation (49), but GR has also been shown to inhibit transcription driven by C/EBP of certain genes by recruiting SMRT (50). This highlights a level of complexity of the transcriptional regulation by GCs that is above the mere identity of transcription factors interacting with GR at target genes.

In addition to positive tethering, agonist-bound GR transcriptionally inhibits genes involved inflammation and proliferation through negative tethering interactions with transcription factors pivotal for these processes such as NF-κB and Activator Protein (AP)-1 (51). To date, several mechanisms for transcriptional repression by GR of NF-κB have been proposed, many of which describe a direct protein-protein interaction by GR with the NF-κB subunit p65 (Fig. 2) (52). Early observations suggested that GR was able to represses NF-κBs (p65, p50 and c-Rel) by affecting their ability to bind to the DNA (53). More recent studies however, indicate that GR mainly interacts with NF-κB at a variety of inflammatory genes and imposes transrepression without affecting the ability of NF-κB to access the DNA. For instance, GR may repress a subset of NF-κB target genes that require the physical interaction between promoter-bound NF-κB and co-activator IRF3 (Interferon regulatory factor 3) for transactivation by binding to p65 and thus blocking the formation of p65-IRF3 complexes (54). Conversely, when signals induce p65 to act as the critical co-activator of promoter-bound IRF3, GR binding to p65 prevents recruitment of p65 to IRF3 target genes and thus terminates the NF-κB response of these genes (54). Furthermore, GR has been suggested to sequester GRIP1 from IRF3 and use it as a co-repressor in GC-mediated inhibition of NF-κB (14). The GR/GRIP1 recruitment to the p65 DNA complex was recently demonstrated for the GC repression of bacterial lipopolysaccharide (LPS)-dependent induction of the COX-2 (55). Moreover, GR have been reported to act as a repressor of NF-κB transactivation by manipulating the basal transcription machinery at inflammatory promoters. For instance, GR has been shown to inhibit NF-κB transcription of IL-8 by by blocking p65 recruitment of P-TEFb (Positive Transcription Elongation Factor b) and consequently impairing the phosphorylation of the serine 2 residue of the RNA polymerase II necessary to initiate transcription (56). Also, GR associates with HDAC2 *in vivo* and represses p65 histone acetylase (HAT) activity (57) demonstrating that GCs can inhibit NF-κB access to its DNA binding sites through GR-

mediated deacetylation of histones. In addition, competition for limited amounts of co-regulators necessary that are shared by NF-κB and GRs to regulate transcription, including CREB and CBP, have been suggested to lead to a decreased gene transcription of inflammatory gene products (58-60), but remain controversial as a valid mechanism for NF-κB repression by the GR (61-63). Furthermore, GR may also inhibit the activity of NF-κB signaling by interacting with MSK-1 (mitogen- and stress-activated protein kinase-1), a potentiating kinase for NF-κB activity, and block its ability to phosphorylate NF-κB at serine residue 276 (64). Conversely, NF-κB has been shown to mutually inhibit transactivation of promoter-bound GR by p65 tethering to the GR-bound co-activator nTrip6 (nuclear isoform of Thyroid receptor-interacting protein 6) (65, 66). Interestingly, GR and NF-κB have under some conditions also been shown to cooperatively transactivate genes, e.g. Toll-like receptor 2 (TLR2) (67). Thus, a large body of literature highlights an intricacy in the cross-talk mechanisms between GR and NF-κB ranging from mutual antagonism through various transrepression mechanisms to cooperation that is dependent on gene and cell context.

Lastly, GCs also exert their effects via rapid nongenomic mechanisms that are independent of GC-regulation of gene transcription. These nongenomic actions of GCs may occur without receptor involvement, via cytosolic GR, or through interactions with membrane-bound GRs (50, 68, 69). In some cases, GC action at the post-transcriptional level also appears to be independent from *de novo* gene expression, such as GC-mediated acceleration of macrophage chemotactic protein -1 (MCP-1) mRNA degradation (70). Interestingly, the cytoplasmic GR has recently been demonstrated to function as a RNA-binding protein that promotes mRNA degradation (71). In addition, GCs have been shown to rapidly inhibit the release of the inflammatory molecule prostaglandin E2 (PGE2) regardless of the presence or absence of protein synthesis inhibitors, indicating this to be a transcription-independent nongenomic mechanism by GCs (72).

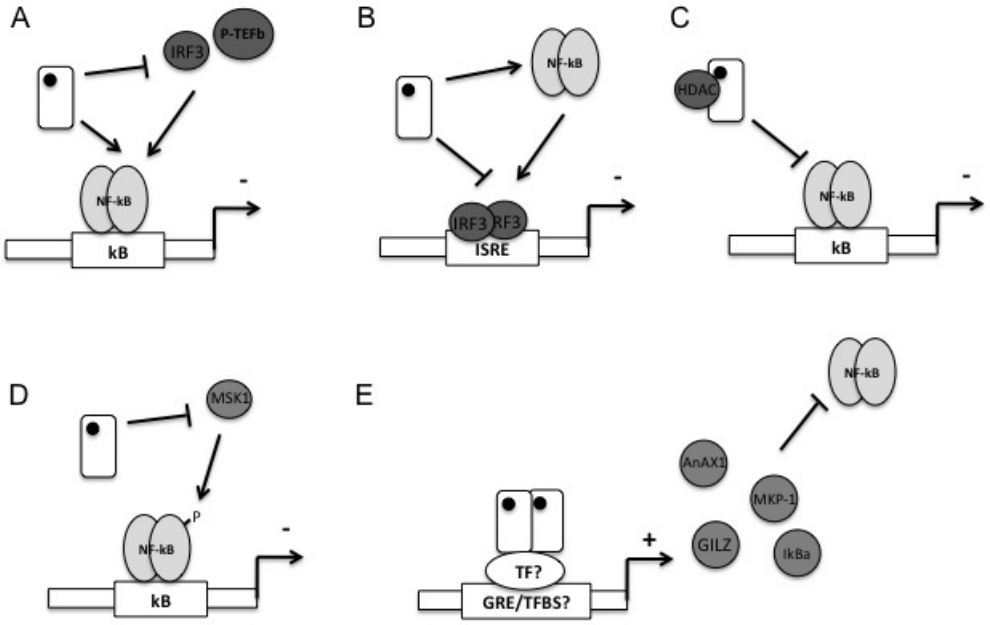

***Figure 2**. A selection of mechanisms proposed for GR inhibition of NF-κB activity. A. Blocking recruitment of co-regulators by NF-κB. B. Blocking the recruitment of NF-κB to promoter-bound IRF3. C. Recruitment of HDACs by GR to NF-κB controlled genes. D. Blocking phosphorylation of NF-κB by interfering with NF-κB kinases e.g. MSK-1. E. GR transactivation of inhibitory gene products that block NF-κB activity*

2.1.3 Transcriptional regulation by the GR and its relevance for the anti-inflammatory effects of GCs

The significance of the various transcriptional regulatory capabilities by GR with regards to the anti-inflammatory effects of GCs is under debate. The fact that numerous anti-inflammatory actions by GCs such as cytokine gene repression and immune cell proliferation have been shown to involve the transrepression capability of GR whereas the clinically undesired effects induced by GCs, such as increased glucose production in the liver, predominantly involve transactivation has provided a rationale tilted towards dissociating these two functions completely from each other in order to achieve selective effects with GC therapy (5, 73). Considerable support for this hypothesis came from *in vivo* experiments in mice in which the wild-type GR had been replaced by the dimerization-deficient mutant (GR^{dim}) (74). Mutations in the dimerization interface of GR had previously been shown to cause a loss of ability by GR to transactivate via GREs while sparing the capability of GR to transrepress (75-77). Indeed, in these mice induced pro-inflammatory gene expression was still inhibited by GC treatment (75, 78). Importantly, while GC-inhibition of inflammatory processes

(e.g. 12-O-tetradecanoylphorbol-13-acetate (TPA) -induced skin inflammation or LPS-induced endotoxemia) where comparable between wild-type and mutant mice, the GR^{dim} mutant poorly induced expression of model genes considered as representative of GC-induced side effects, including the gluconeogenic enzymes TAT (Tyrosine aminotransferase) and PEPCK.

From this premise, "dissociated" GC ligands (termed e.g. selective GR agonists; SEGRAs, or selective GR modulators; SGRMs), capable of favoring transrepression over transactivation, have been developed to provide the breakthrough gateway to safer GCs that preserve their anti-inflammatory properties but have fewer side effects (79-81). Although these compounds demonstrate dissociation *in vitro* using reporter gene assays (e.g. GRE-luc and NF-κB-luc) and at some biomarker genes (e.g. IL-6 and PEPCK) the results *in vivo* are less clear-cut. While some dissociated compounds have been shown to inhibit the progression of certain inflammatory models to a reasonable extent as compared with "classic" GCs (82-84), a reoccurring problem for many selective GCs *in vivo*, ultimately, includes either a poor anti-inflammatory efficacy or a limited loss of adverse effects (85-89). Similarly, GR^{dim} mice have in later studies been shown to be GC-insensitive in experimental models of contact allergy (89) and arthritis (90) and are not protected from GC-induced muscle athropy (91) or osteoporosis (92). The reduced therapeutic efficacy of dissociated compounds could be because transactivation, which is the function that is purposely avoided with the use of these dissociated GC agonist, is required for the full anti-inflammatory effects of GCs. Indeed, GCs stimulate the expression of multiple genes with distinct anti-inflammatory properties (20, 93). For instance, in addition to transrepression of NF-κB through protein-protein interaction between GR and p65, induction of IκBα by GCs has been implicated as relevant for NF-κB repression by GCs (94-96). Moreover, GCs induce expression of GILZ and MKP-1 in order to block inflammatory gene transcription. Whereas MKP-1 targets and inhibits pro-inflammatory MAPK signaling pahways (97), GILZ have been shown to interact directly with AP-1 and NF-κB and impair their functions (98). Furthermore, GCs stimulate the expression of IL-10 and Annexin A1, the former a potent anti-inflammatory cytokine and the latter a calcium-dependent phospholipid binding protein that inhibits production of pro-inflammatory mediators such as prostaglandins and leukotreines (99, 100). Interestingly, Annexin A1, induced by GCs, was recently discovered to directly interact with the NF-κB p65 subunit and suppress its transcriptional activity by preventing NF-κB binding to DNA (101). Indeed, several recent studies have implicated both GR homodimerization and

transactivation, along with transrepression, as relevant for the complete anti-inflammatory effects of GCs (89, 102). Furthermore, GR mutants, including GR^{dim}, along with a selection of dissociated GC compounds have been shown to still be capable of stimulating the expression of genes such as IL-10, MKP-1 and phenylethanolamine N-methyltrasferase (PNMT), which all have been shown to contain atypical binding sites for GR (86, 97, 103-105), thereby demonstrating that the GR^{dim} mouse strain does not display a complete separation of transactivation and transrepression. Thus, both anti-inflammatory and adverse effects still observed with some dissociated GCs and in GR^{dim} mice might be due to a residual transactivation capability by GCs of target genes regulated through GRE-independent mechanisms. In particular, MKP-1 may embody both wanted and unwanted effects of GCs. On one hand, MKP-1 has been implicated as a potent agent for the anti-inflammatory actions of GCs (see MAPK section). On the other hand, MKP-1 has also been shown to mediate GC-inhibition of osteoblast proliferation (106, 107), which could relate it to GC-induced osteoporosis. When forcefully expressed, MKP-1 induces muscle atrophy (108), which is also a side effect observed with prolonged and high-dose use of GCs. Importantly, like the anti-inflammatory action of GCs, side effects of GC therapy could also involve both transactivation and transrepression capabilities of GR. For instance, both GC-inhibition of cytokines, including IL-11, via transrepression by monomeric GR of AP-1 (92) as well as transactivation of e.g. the Wnt signaling antagonist DKK-1 (Dickkopf-1) (109) (see Wnt section) by GCs have been shown to inhibit proliferation and differentiation of osteoblasts and have both been suggested to be underlying mechanisms for GC-induced osteoporosis. In addition to GRE –mediated transactivaton of hepatic PEPCK and TAT by GCs (7), transrepression of the insulin precursor gene in pancreas and the insulin receptor gene in the liver via their recently discovered nGREs might concomitantly contribute to insulin resistance and the development of steroid induced diabetes (42). Thus, the uncoupling of wanted GC effects from unwanted effects through separation of transactivation and transrepression functions of GR may be difficult to achieve since an effect may involve both these functions to various extent (Fig. 3).

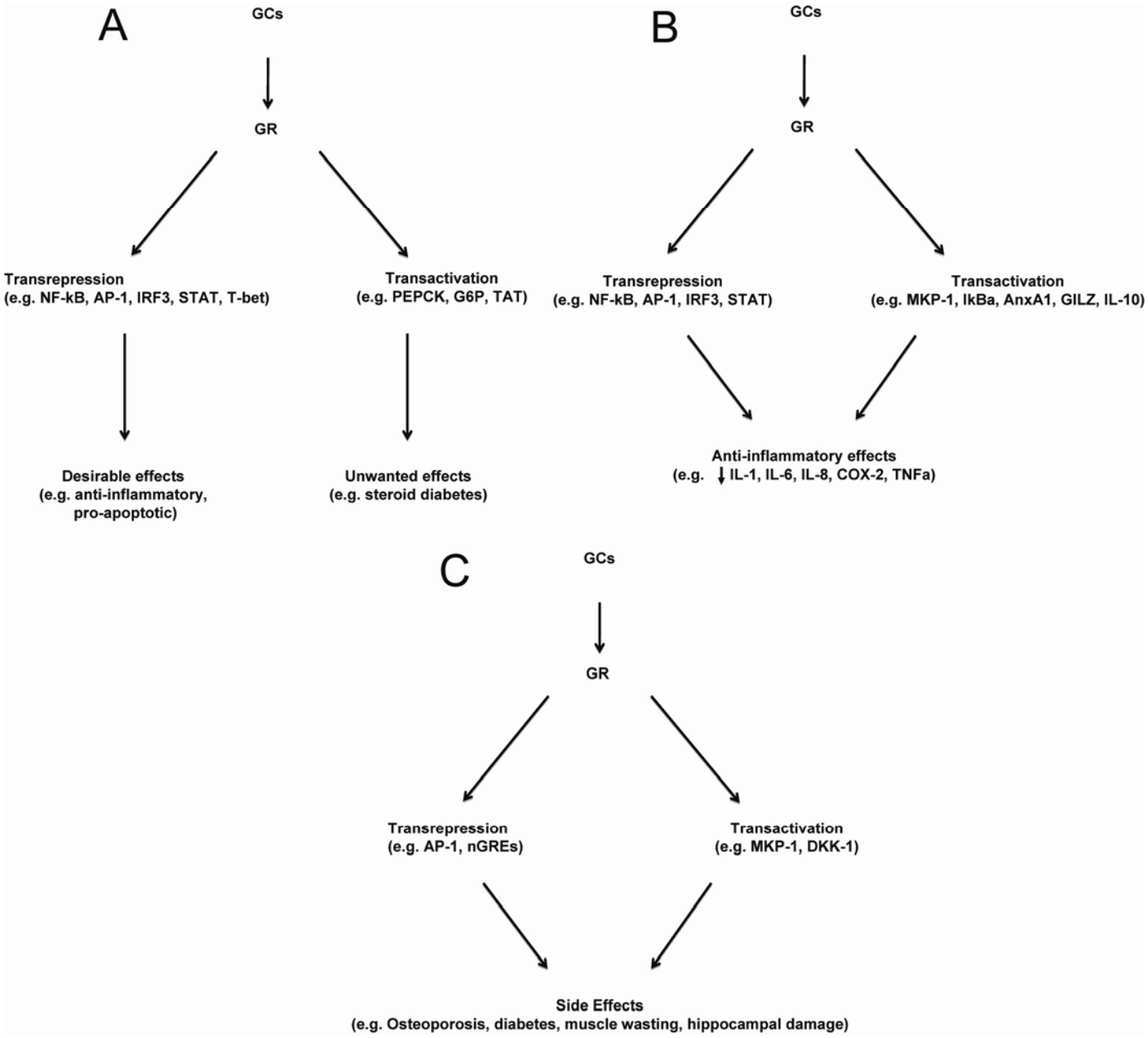

Figure 3. *Models proposed for GC-effects. A. A model suggesting dissociated GR functions for separate GC effects. B. A model suggesting a contribution of both transactivation and transrepression functions of GR for anti-inflammatory effects by GCs. C. A model highlighting an involvement of both GR functions for adverse effects by GCs*

2.1.4 Glucocortiocid receptor structure and function

The GR belongs to the superfamily of of ligand-regulated nuclear receptors and is produced from a single GR gene located on chromosome 5 (5q31-32), which consists of 9 exons. Alternative splicing events can give rise to GR isoforms that differ at the C-terminus (GRβ) or within the central structure of GR (GRγ) (110). Furthermore, alternative translation initiation generates isoforms that lack parts or all of AF-1 (111). The full length GRα isoform (777 amino acids in human) is widely expressed and is responsible for GC transactivation and transrepression (throughout this work referred to as GR) whereas the relative expression of the other GR isoforms can modulate GC sensitivity in a cell-specific manner. For instance, GRβ is a GC-independent negative regulator of GR that targets GR for heterodimerization and has been implicated in GC-resistance (112). Like other members of the NR family, GR is a modular protein that contains three major domains including the N-terminal domain (NTD), the central DNA-binding domain (DBD), and the C-terminal ligand-binding domain (LBD) (Fig. 4) (46). A flexible region of the molecule, termed the hinge region, separates the DBD and LBD. Two nuclear localization signals, NL1 and NL2, are located at the DBD/hinge region junction and within the LBD, respectively.

The GR DBD is a highly evolutionary conserved region and contains two zinc finger motifs crucial for the DNA binding of GR to GREs in GC-target genes. These similar zinc finger structures, each with a Zn ion held in center by four cystein residues have been shown to have separate functions. The N-terminal zinc-finger contains three amino acids that form the P-box, which allows GR to interact with GREs in the DNA of target genes. The C-terminal zinc finger contains the D-box, which plays an important role in homodimerization of GR and together the two zinc finger structures promote transactivation by facilitating coordinated binding of both DBD motifs of homodimerized GRs to palindromic GREs in GC responsive target genes. Interestingly, recent studies have shown that the GRE itself, depending on its sequence composition, may influence the three-dimensional structure of the DBD resulting in the expression of certain GC genes by liganded GR to be allosterically fine-tuned and this could in part explain the gene-specific transcriptional effects of GR (113).

The NTD contains a strong ligand-independent transcriptional activation function (AF1) that binds various co-regulators, including chromatin remodeling factors (e.g. Brahma-related gene-1 (BRG1), histone modifiers (e.g. HDAC-6, CREB and p300/CBP-associated factor (P/CAF)), and components of the basal transcription

machinery proteins of the transcription intitiation complex, such as RNA polymerase II, TATA-binding protein (TBP) and TBP-associated factors (TAFs) (114). Furthermore, the NTD bears the most amino acid residues of GR prone to post-translational modifications, which allows other signaling pathways to influence GC signaling at the post-translational level (115). The human GR has phosphorylation sites at serines 113, 141, 203, 211, 226 and 404 and the status of GR phosphorylation at these sites alters its transcriptional activity. Phosphorylation of residues S203 and S211 by kinases such cyclin-dependent kinase (CDK) 2 and CDK5 has been shown to increase GR transactivation (115). In contrast, phosphorylation of GR at S404 by Glycogen synthase kinase 3 β (GSK3β) enhances nuclear export of GR and diminishes its ability to regulate transcription. Other post-translational modifications of the GR NTD that alter the function of GR have been confirmed by the use of GR mutants and include SUMOlaytion (modification by Small Ubiquitin-like Modifier) at lysine 293, 277 and 703, ubiquitination at lysine 419 and acetylation at lysine 480, 492, 494 and 495 (115). Moreover, since alternative translation initiation of the GR mRNA generates naturally occurring isoforms of GR that have varying lengths of the NTD, this may contribute to the pleiotropic effects of GR such as the ability to interact with other proteins and regulate transcription (116).

The LBD, contains 12 α-helices and four β-sheets that form a hydrophobic pocket that binds GCs (117). A second activation function (AF2) that interacts with coregulators in a ligand-dependent manner is embedded in the LBD. The AF-2 region, like the AF-1 region in NTD, interacts with histone modifiers p300/CBP, p300/CBP-associated factor (p/CAF) and members of the p160 family of co-activators (e.g. SRC1, GRIP1) on GC target genes to drive transactivation (114). Many of these co-activators contain several specific LXXLL sequence motifs that interact with helix 12 in the AF-2 region of the LBD in response to GC ligand binding. Importantly, different GC agonists may influence co-regulator interaction with the AF-2 region. Dexamethasone, a potent GC agonist, strongly promotes binding of GR to the TIF2 co-activator protein, which mediates the activating functions of GR by acetylating histones making the target gene DNA more accessible for transcription (117). Dexamethasone has also been shown to prevent GR recruitment of nuclear receptor co-repressor N-CoR, whereas RU-486, a GC antagonist, increases GR interaction with co-repressors N-CoR and SMRT (118). Furthermore, several dissociated GC ligands have demonstrated capabilities to alter GR interaction with its co-regulators. GR when bound to AL-438, a non-steroidal alternative to classical GCs, is incapable to interact with co-activator PGC-1, a GR co-

activator important for hepatic gluconeogenesis, while it remains fully capable of binding to TIF-2 (119). Since the LBD contributes to GR dimerization, some of these dissociated GCs are also aimed to prevent the required GR dimer formation for transcriptional activation from GREs in target genes. Compound A (Cpd A), a non-steroidal plant-dervied phenyl phenyl aziridine precursor that has been shown to induce GR monomer formation, does not enhance expression of genes including Glucose-6-phosphatase, PEPCK, GILZ but maintains its transrepression capabilities such as the down-regulation of TNFα-induced and NF-κB driven expression of pro-inflammatory markers (IL-6 and IL-8) (120).

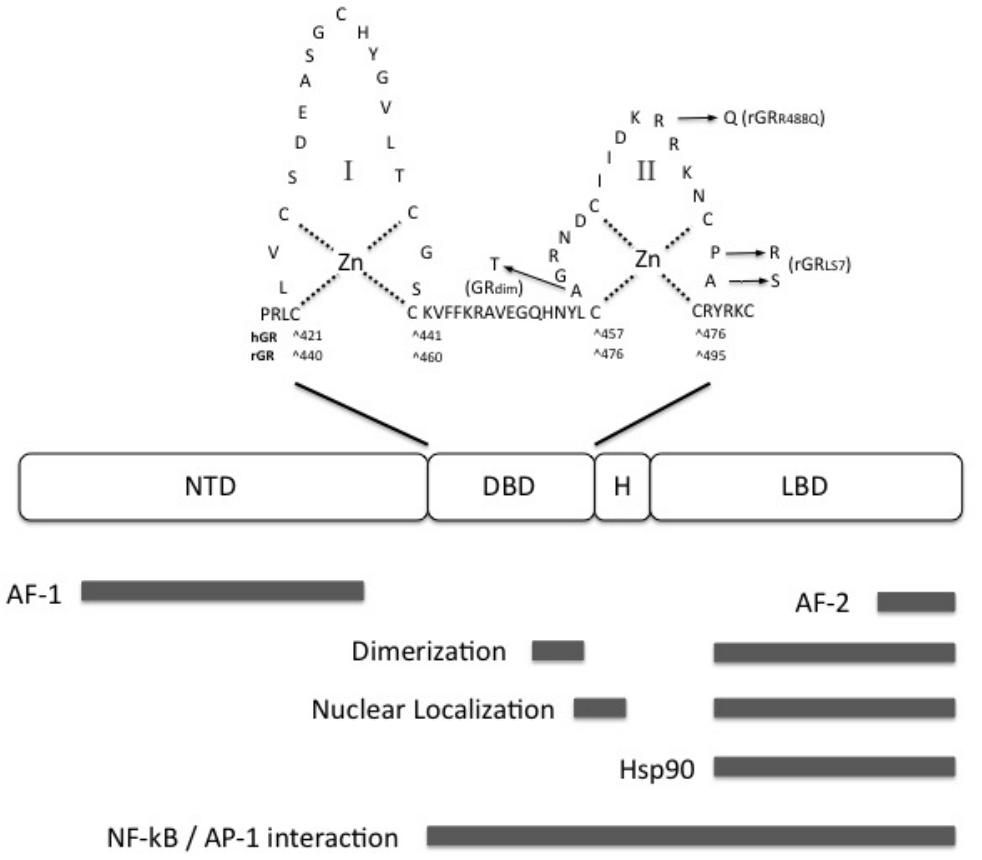

Figure 4. *Schematic overview of the GR protein structure.*

2.1.5 Glucorticoid receptor mutants

Since the cloning of full length GR in 1985, the core functions and relevance of the GR regions for the GC effects has been determined by subjecting the GR to mutagenesis

analysis (Fig. 4). Indeed, several GR mutants (a selection of them described below) have been used as investigative tools to explore GR function (121).

A point mutation, E755A, in the LTD was shown to inhibit the ligand-dependent interaction of GR with LXXLL-containing co-activators and led to a decrease in transactivation, but had little effect on transrepression (61), implicating these functions to be involving separate functions of the GR. Similarly, a specific point mutation, A465T, in the second zinc finger of the DBD generates the dimerization deficient mutant, GR^{dim}, which, although it is not completely transcriptionally inactive, does not bind to or transactivate via GREs on the DNA of GC-sensitive genes (74). However, the GR^{dim} is still capable of transrepressing NF-κB and AP-1-mediated gene regulation *in vitro* and *in vivo*, suggesting that transcriptional repression by GR can occur independently of both GRE-binding and dimerization. Whereas mutations in the dimerization box in the DBD or in regions necessary for full transcriptional GR activity do not affect GC repression of p65-dependent transcription, mutations of arginine or lysine (Arg-488 or Lys-490 in the rat GR) in the second zinc finger abolished the ability of GR to inhibit p65 activity (76). Deletions in the second zinc finger result in a loss of GC-mediated repression of IL-6 (122), which further illustrates the importance of the DBD for the transrepression mechanism. Other GR DBD mutants, such as GR_{LS7}, which carries a double point mutation (P493R/A494S, rat nomenclature), does not transactivate through GREs but inhibits the transcriptional activity of NF-κB and AP-1 (76, 123) while a similar rat GR DBD mutant, GR_{R488Q}, is able to transrepress AP-1 but not NF-κB regulated transcription activated by TPA (123). Interestingly in **paper II**, we show that the GR_{R488Q} is capable of inhibiting NF-κB activated by TNFα, but no activated by TPA, implicating that repression of NF-κB by GR, through the DBD, may depend highly on the signaling pathway that is employed to activate NF-κB.

Although GR DBD mutants have been shown to be deficient in their capability to homodimerize and transactivate via GREs, some of them are still capable of transactivating genes that do not require GREs for their activation by GCs. For instance, the GR^{dim} mutant has in studies been shown to induce gene expression of the anti-inflammatory protein MKP-1 (97), suggesting that both transrepression and transactivation can occur independently of homodimerization and DNA-binding by GR.

2.2 NF-κB

The NF-κB family of inducible transcription factors is the target of multiple pro-inflammatory and extracellular stimuli, such as stress, cytokines, UV radiation, bacterial and viral antigens, and is a key participant in various biological processes, including proliferation, development and most notably regulation of innate and adaptive immunity and inflammatory response (17). There are five family members of NF-κB, named NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), p65 (or RelA), c-Rel and RelB, which all carry the highly conserved 300 amino acid long signature motif, N-terminal Rel homology domain (RHD). This domain is responsible for homo- and heterodimerization of the NF-κB subunits and sequence-specific DNA-binding to kB enhancer element in the regulatory regions of NF-κB target genes. In addition, p65, c-Rel and RelB contain a C-terminal transcriptional activation domain (TAD), whereas p100 and p105 contain C-terminal ankyrin repeats that become proteasomally processed to generate p52 and p50, respectively. In their mature form, p52 and p50 heterodimerize with TAD-containing NF-κB family members and normally, RelB predominantly form heterodimers with p52/p100 and p65 and c-Rel preferentially binds to p50 (124).

In resting cells, NF-κB is kept inactive through its association with inhibitory IκB proteins in the cytoplasm, which interacts with NF-κB through its ankyrin repeats domain and blocks the exposure of the nuclear localization signal (NLS) motifs of NF-κB. Following cell stimulation, the IκB inhibitory proteins become phosphorylated by IκB kinase (IKKs) complexes, which leads to the IκBs becoming polyubiquitinated and proteolytically processed. Consequently, the release of NF-κB from IκB unmasks its NLS, which results in a nuclear entry of transcriptionally active NF-κB. Inside the nucleus NF-κB binds to and stimulates expression of genes involved in immune regulation and inflammatory response (124).

Two distinct NF-κB pathways, the canonical (or classical) and the non-canonical (or alternative) NF-κB pathway, have been characterized. Although these two NF-κB pathways follow the general pattern of activation outlined above, they are activated by distinct stimuli, use different IKK complexes to promote nuclear accumulation of separate NF-κB heterodimer complexes that, ultimately, stimulate the expression of individual subsets of target genes (124-126).

In the canonical NF-κB pathway, IκBα holds the p65/p50 heterodimer complex inactive in the cytoplasm. When cells are triggered by pro-inflammatory signals, such

as cell surface binding of TNF ligands to TNFRs, engagement of TLR signaling and ligation of antigen receptors on lymphocytes, an IKK complex comprising of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, NEMO becomes activated (124). Through the catalytic activity of IKKβ, the IKK complex phosphorylates IκBα on serine residues 32 and 36. This leads to a polyubiquitination of IκBα and its degradation by the 26S proteasome and allows nuclear translocation of liberated p65/p50. In the nucleus p65/p50 can bind to and transcriptional activate a broad range of pro-inflammatory mediators, such as cytokines (TNFα, IL-1β, IL-6, GM-CSF), chemokines (CCL2, RANTES, IL-8, CXCL2), growth factors (epidermal growth factor, EGF; fibroblast growth factors, FGF), cell surface receptors (TNFR, TLR), adhesion molecules (ICAM-1, VCAM-1, E-selectin) and enzymes (iNOS, COX-2) (127). With the wide battery of pro-inflammatory mediators under its direct control, the canonical NF-κB signaling pathway plays an essential role in mounting inflammatory responses. Importantly, the canonical NF-κB pathway limits its own activity and prevents uncontrolled expansion of inflammation by regulatory feedback mechanisms. Crucially, NF-κB up-regulates the expression of IκBα, which binds to the active p65/p50 complexes released by previous degradation of IκBα, retains them back in the cytoplasm and thereby renders them inactive (124, 125, 128).

The "alternative" or non-canonical NF-κB pathway is, unlike the canonical NF-κB pathway, activated by a narrow subset of predominantly cell developmental stimuli, including ligation of the Lymphotoxin β receptor (LTβR) by LTα1β2 and LIGHT, stimulation of BAFF (B-cell activating factor) on B cells, RANKL (Receptor activator of NF-κB ligand), CD40 and TWEAK (TNF-like weak inducer of apoptosis) (129). This pathway, in contrast to the canonical NF-κB pathway, does not involve NEMO and IKKβ mediated degradation of IκB, but relies upon homodimers of IKKα for its signaling activity (124). Importantly, the activation of IKKα in non-canonical NF-κB signaling requires the stabilization and activation of its direct upstream regulator, NIK (NF-κB inducing kinase), which is kept at low levels by constitutive proteasomal degradation in resting cells. The activation of the non-canonical NF-κB pathway results in the inhibition of degradation of NIK allowing the NIK protein to reach the sufficient threshold level to activate IKKα by phosphorylation. NIK-activated IKKα targets inactive RelB/p100 heterodimers in the cytoplasm and phosphorylates the p100 on serine residues 865, 869 and 871, which initiates the process of polyubiquitination and proteolytic cleavage of p100 to generate p52. The RelB/p52 complex, consequently,

translocates into the nucleus where it activates immune regulatory genes, including homeostatic chemokines CXCL12 and13, CCL19 and 21, immune growth factor BAFF and anti-apoptotic gene products B-cell lymphoma (BCL)-xl and BCL-2 (125, 130, 131). Through the up-regulation of these and other organogenic chemokines, growth factors and anti-apopoptic regulators, the non-canonical NF-κB pathway controls specific immune functions, including lymphoid organogenesis (splenic organization and germinal center formation), trafficking of lymphocytes to lymphoid organs, and B-cell proliferation, maturation and survival (125, 129-132).

2.3 MAPK SIGNALING PATHWAY

The mitogen-activated protein kinase (MAPK) signaling pathways are evolutionary conserved signal transduction pathways that relay information from the extracellular environment, into cellular compartments to generate a cellular response. MAPKs respond to a wide array of extracellular stimuli (mitogens, stress, cytokines, phorbol esters, ultraviolet radiation, and growth factors) and influence many key biological processes such as proliferation, development, differentiation, apoptosis, and inflammation through the activity of intracellular signaling cascade events (133, 134). Importantly, the MAPK pathways can also coordinate and amplify signals from other activated signaling pathways to generate various cellular response patterns (115, 134). There are at least three known MAPK signaling pathways, including the extracellular signal-regulated kinase (ERK) pathway, c-Jun N-terminal kinase (JNK) pathway and p38 MAPK pathway and all three members of the MAPK family are serine/threonine specific protein kinases that share a hallmark feature in their requirement of a dual threonine and tyrosine phosphorylation to display maximal activity. MAPKs become activated in a three-step regulatory intracellular phosphorylation "cascade" involving MAPK kinase kinase (or MEKK), MAPK kinase (or MEK) and MAPK. This MAPK cascade is initiated when a MEKK is activated by extracellular stimuli and phosphorylates serine and threonine residues of its substrate MEK, which becomes activated. The MEK in turn targets and phosphorylates the threonine and tyrosine residues of MAPK. Activated MAPKs relay the signal by phosphorylating other downstream kinases or, as in the case of ERK1/2, translocate into the nucleus and directly phosphorylate regulators of transcription (e.g. AP-1) and thereby critically influencing their transcriptional activity (135, 136).

The MAPKs respond to many inflammatory signals and mediate inflammatory responses by regulating transcription and stabilizing the mRNA of key pro-

inflammatory mediators, including COX-2, IL-6, IL-1β, TNFα and IL-12 (115, 137-140). Furthermore, MAPKs can influence other inflammatory pathways including the canonical NF-κB pathway. For instance, the p38 MAPK have been shown to mediate IκB phosphorylation/degradation and NF-κB activation by various stimuli (141, 142). Additionally, the serine residue 276 of p65 has been shown to be a substrate target for ERK- and p38-activated MSK-1 and inhibition of activated p38 MAPK and ERK1/2 results in a decrease of phosphorylation of the p65 NF-κB subunit (143). The phosphorylation of the serine residue 276 of p65 by MAPK-activated MSK-1 has been demonstrated to be important for the transcriptional regulation of target genes by the canonical NF-κB signaling pathway. The p38 MAPK has also been implicated in the phosphorylation of the NF-κB co-activator p300, which results in its increased activity and association with NF-κB (144). Also, the MEKK TAK1 (Transforming growth factor β–activated kinase-1) has been shown to phosphorylate NIK in response to IL-1 stimulation and, by acting upstream on the NIK-IKK pathway, influence NF-κB activity (145).

Importantly, the limiting of duration and amplitude of MAPK signaling pathways is profound for normal cell function and since these pathways are strong conductors of the inflammatory response, strict control mechanisms exist to prevent their excessive activity. Inactivation of MAPKs occurs through dephosphorylation at their phosphothreonine and phosphotyrosine residues by specific dual-specificity (Thr/Thy) MAPK phosphatases (MKPs) (146). The archetypical member of the MKP family is MKP-1 (or DUSP1), which has been shown to interact with and dephosphorylate active ERK1/2, JNK1/2 and p38MAPK and thus regulate the activity of MAPK-dependent effects on gene transcription (147). MKP-1 expression is induced by pro-inflammatory stimuli through MAPK signaling pathways and plays an important role in the negative feedback inhibition of various cellular responses mediated by p38 MAPK, JNK and in some cells ERK1/2 (148, 149). The up-regulation of MKP-1 has been suggested to contribute to the destabilization of pro-inflammatory mRNAs (150) and to inhibit transcription factors that regulate the expression of these messengers (149, 151). MKP-$1^{-/-}$ mice have been shown to have elevated MAPK signaling activities and be hypersensitive to inflammatory insults, such as LPS, where an already low dose challenge with LPS results in significantly increased amounts of serum cytokines, severe hypotension, multi-organ failure and increased mortality due to sepsis (152). Furthermore, absence of MKP-1 increases systemic levels of pro-inflammatory cytokines and exacerbates disease development in a mouse model of rheumatoid

arthritis (153). Thus, MKP-1 appears to be an important negative regulator of inflammatory responses, and its induction has a critical role for the restraint of pro-inflammatory cytokine production by stimulated MAPKs. Additionally, MKP-1 expression is stimulated by GCs and provides GCs with a capability of regulating MAPK signaling (154, 155). Since MAPKs regulate inflammatory responses, the inhibition of these signaling molecules through the increased expression of MKP-1 by GCs has been studied for the ability of GCs to exert their anti-inflammatory effects. For instance, GCs was shown to inhibit LPS-induced JNK and p38 MAPK in MKP-$1^{+/+}$ but in not MKP-$1^{-/-}$ bone marrow derived macrophages (97). In the MKP-1 knock out cells inhibitory effects of GCs on distinct inflammatory genes where shown to be either strongly MKP-1 dependent (e.g. IL-1α), independent of MKP-1 (e.g. colony stimulating factor 2 or CSF2), or partially dependent on MKP-1 (e.g. TNFα, COX-2, and others), which demonstrated a partial and gene-specific dependency of MKP-1 up-regulation by GCs for their inhibitory activity. In addition to macrophages, GC-induction of MKP-1 has also been implicated in the inhibition of pro-inflammatory gene expression by GCs in microglia (156), vascular endothelial cells (157) and airway smooth muscle cells (158). *In vivo*, MKP-$1^{-/-}$ mice show enhanced mast cell degranulation and are highly susceptible to anaphylaxis, but were still shown to be sensitive to GCs (159). This suggested that some anti-inflammatory effects of GCs *in vivo* were independent of MKP-1, although they could involve GC-induction of other MAPK-phosphatases (159). In contrast, another *in vivo* study observed that GC-treatment completely protected wild-type $MKP^{+/+}$ mice from mortality caused by a high dose of LPS, whereas it only offered a partial protection in MKP-$1^{-/-}$ mice (160). These studies indicate that the anti-inflammatory effects of GCs can be partially dependent on GC-induction of MKP-1 expression and inactivation of MAPKs both *ex vivo* and *in vivo*. Furthermore, since MAPK-signaling has been demonstrated to influence transcriptional activity of NF-κB, MKP-1 induction by GCs might constitute an additional mechanism by which GCs may repress NF-κB activity. In endothelial cells, GC-induction of MKP-1 inactivated TNFα-activated p38 MAPK and inhibited expression of E-selectin, a gene that is largely regulated by NF-κB, whereas in endothelial $MKP^{-/-}$ cells, GCs did not inhibit the TNFα-activated E-selectin expression (157). Moreover, in human bronchial epithelial cells where MKP-1 was down-regulated using RNA interference, GC-inhibition of p38 MAPK-activated NF-κB was partially reversed (151). Furthermore, the p65 kinase MSK-1 is not turned off in the

absence of MKP-1, allowing pro-inflammatory pathways to continue in an uncontrolled manner (161). Thus, GC-induction of MKP-1 could provide an additional mechanism for MSK-1 and NF-κB control by GCs. However, the relevance for GC-up-regulation of MKP-1 for its ability to inhibit NF-κB may be cell dependent. For instance, we demonstrate in **paper II** that inhibition of TNFα or TPA-induced NF-κB activity in human embryonic kidney cells does not require MKP-1 up-regulation by GCs. Similarly, GCs were shown to inhibit TLR4-dependent COX-2 gene induction in macrophages via a combinatorial mechanism that involves both MKP-1-mediated inhibition of AP-1 and GR-GRIP1-mediated transrepression of p65 (55). Taken together, the relevance of the ability by GCs to induce MKP-1 expression for their capacity to inhibit NF-κB still remains somewhat contradictory and could depend on cell and gene context.

Moreover, MAPKs can reciprocally inhibit both GR and MPK-1 and thus protract their own signaling activity. For instance, JNK phosphorylates rat GR at position S246 (S226 conserved in human) and inhibits transcriptional activation by GR (162). In addition, cooperation of ERK and the E3 ubiquitin ligase SCF^{skp2} can initiate proteolysis of MKP-1 and thereby sustain MAPK ERK signaling (163). These mutually antagonistic cross-talk mechanisms, involving altered phosphorylation status of GR, decreased expression of MKP-1 and persistent activity of MAPKs, have been proposed to contribute to GC-insensitivity (164, 165).

2.4 WNT SIGNALING PATHWAY

Wnts constitute a large family of secreted glycoprotein ligands that are essential for a wide array of developmental and physiological processes, including proliferation, differentiation, apoptosis and survival, by activating multiple intracellular signaling cascades (166). Wnt signaling has been implicated in maintaining neuronal and hematopoietic stem cells in a self-renewing state and is central to bone development and homeostasis (167, 168). Wnt signals are transduced in at least two distinct ways, a well-established canonical Wnt pathway, which is dependent on the β-catenin, and a noncanonical pathway or pathways that are β-catenin independent. In the absence of Wnt proteins, β-catenin is constitutively degraded in the cytoplasm of cells by the β-catenin destruction complex, which consists of GSK3β, Axin, APC (adenomatous polyposis coli), and CKIa (casein kinase Ia). The continous degradation of synthesized β-catenin is achieved by phosphorylation of β-catenin by CKIa and GSK3B, which results in polyubiquitination of β-catenin by β-transducin and ubiquitinated β-catenins

are consequently degraded by proteasomes. Activation of canonical Wnt signaling occurs when a Wnt ligand binds to its receptor, the Frizzled (FZD) family of proteins, and then along with FZD binds to lipoprotein-related receptor (LRP)5 or LRP6. The Wnt-FZD-LRP5/6 complex activates Dishevelled (Dvl), which destabilizes and disrupts the destruction complex of β-catenin by removing Axin away from the complex. This blocks the degradation fate of cytosolic β-catenin, which translocates to the nucleus where it associates with transcription factors from the lymphoid enhancer factor (LEF)/T-cell factor (Tcf) family and enhance target gene transcription of genes associated with enhanced cell growth (e.g. cyclin D1, c-Myc, C-jun and VEGF) (169, 170). The non-canonical Wnt pathway uses common intracellular components as the canonical Wnt pathway to relay its signals, including Dvl, axin, and GSK3β, but is β-catenin-independent. This pathway has been shown to be involved in the regulation of intracellular calcium. Binding of noncanonical Wnt ligands such as Wnt5a causes a calcium flux that leads to the activation of Ca^{2+}-dependent effectors such as calcium/calmodulin dependent kinase II (CamKII), nuclear factor associated with T cells (NFAT), and proteins kinase C (PKC), which regulate a diverse battery of genes (171-173). Importantly, CAMKII activates TAK1 and NLK (Nemo-like kinase), which can antagonize the canonical Wnt pathway by regulating LEF1/TCF transcriptional factors (174)**.**

DKK-1 is a secreted antagonist of Wnt signaling that binds with high affinity to LRP6 and LRP5 and this binding is essential for DKK-1 inhibition of Wnt signaling (175-177). This interaction between DKK-1 and LRP6 has been suggested to result in an internalization and degradation of LRP6, thereby rendering the cells less responsive to Wnt signaling. Ultimately, the diverse biological roles for DKK-1 rely on its ability to inhibit β-catenin accumulation caused by activated Wnt signaling (178-180). Importantly, the Wnt signaling pathway stimulates DKK-1 expression as a negative feedback control mechanism for its activity (175). Also, GCs induce the transcription of DKK-1 through binding of GR to a GREs in the DKK-1 promoter (**Paper IV**) and cause GCs-mediated inhibition of Wnt dependent transcription and decrease of β-catenin levels (109, 181-184). Treatment of cells with anti-DKK-1 antibodies have been demonstrated to partially suppress the GC-dependent inhibition of Wnt-signaling (182). In addition, liganded GR have been shown to represses cyclin D1 expression by directly targeting the Tcf/β -catenin complex (185). Taken together, GCs can inhibit the activity of Wnt-signaling at multiple levels.

3 AIMS OF RESEARCH

Although GCs have been widely used since the late 1940s to treat inflammatory disorders, the molecular mechanisms responsible for their anti-inflammatory activity are still not completely resolved. This thesis contains studies (**Paper I-IV**) highlighting GR-cross talk abilities with other signaling pathways, including the two NF-κB pathways, the MAPK pathways and canonical Wnt signaling pathway. These pathways have either been described in pathology of inflammatory disorders or implicated as contributory for the adverse effects observed with GC therapy. In many cases, reported here and elsewhere, the use of GR mutants as investigative tools has been proven to be fruitful for the purpose of studying GC-cross-talk functions. More specifically, the aims of this thesis include:

- To investigate the transcriptional activation mechanism by which GCs induce expression of the MAPK inhibitor, MKP-1 (**Paper I**).
- To study GC crosstalk with canonical NF-κB activity and comparing the ability of wild-type versus mutant GR to transrepress NF-κB activated by different pro-inflammatory stimuli (**Paper II**).
- To study GC-regulation of the non-canonical NF-κB pathway in an endogenous context and, with the use of GR mutants, determine what functions of GR that might be relevant for this regulation (**Paper III**).
- To study the relevance of GC-induction of DKK-1 for neuronal progenitor cell (NPC) proliferation and differentiation and to study GR-binding to the DKK-1 gene (**Paper IV**).

4 RESULTS AND DISCUSSION

4.1 PAPER I

A large body of evidence supports an anti-inflammatory role for MKP-1, the prototype inhibitor of the MAPKs JNK, p38 and ERK1/2. GCs have been shown to stimulate expression of MKP-1 in a variety of cells and in absence of MKP-1, either by knock-out or siRNA knock-down, GCs have partially impaired anti-inflammatory responses. Thus, the overall anti-inflammatory effects of GCs may, in addition to transcriptional repression of NF-κB, also involve GC-stimulation of inhibitory gene products e.g. MKP-1. Furthermore, since some dissociated GCs and transactivation-deficient GR mutants that are unable to operate through GREs still induce expression of MKP-1, transactivation of this gene by GR has been speculated to involve GRE-independent mechanisms.

To study the transcriptional activation mechanism by which GCs induce MKP-1 expression we first sought to confirm GR binding to the MKP-1 gene. Using a chromatin immunprecipitation (ChIP) scanning assay covering the proximal 3kb of the MKP-1 promoter, we demonstrated agonist-bound GR to bind to a single region approximately -1.4kb upstream from the transcription initiation site. Interestingly, so did the GR DBD mutant GR_{LS7} as well, which neither binds to nor transactivates GRE-containing genes. Thus, we speculated that the GC-responsive region would be located in proximity to the binding site located at -1.4kb of the MKP-1 promoter. Indeed, 5' promoter deletions of the 3kb MKP-1 promoter plasmid constructs had no effects on GC-responsiveness until position -1380bp while deletions beyond this point resulted in a loss of GC-responsiveness. A minimal GC-responsive fragment between position -1380 and -1266 was identified. Sequence analysis of this 114bp fragment did not reveal any GRE-like sequences in this fragment but did however identify a binding site for C/EBP that was highly conserved among species. Using EMSA we confirmed that the protein complex formed on the 114bp minimal GC-responsive region included C/EBP. ChIP assay further revealed the concomitant binding of C/EBP and GR to the same region in the MKP-1 promoter. Importantly, GC-responsiveness of MKP-1 was significantly impaired when the C/EBP site was subjected to point mutations. Taken together, these results demonstrate that agonist-bound GR induces the expression of MKP-1 trough a positive tethering interaction involving promoter-bound C/EBP at a

C/EBP-binding site located -1.4kb upstream of the transcription start site, without the GR itself contacting the DNA (see also below).

Since our published study in 2008, two later papers describing a similar GC regulation of MKP-1 have appeared (45, 186). Both studies provide interesting additions to the GC regulation of MKP-1. In the first study a longer stretch of the MKP-1 promoter (4.8kb) is used in promoter deletions studies (45). Deletions from -4834 to -2726 impaired but did not eliminate the GC-response. A second drop in GC-response was observed on deletions between -1495 and -1095, which is similarly observed in our study. This suggests that that at least two regions in the MKP-1 promoter contribute to MKP-1 gene expression. In the second study, p300 was shown to be a necessary transcriptional co-activator for GR-regulated MKP-1 gene transcription (186). In line with our results, both these studies confirmed GR binding to the -1.4kb region by ChIP assay. In contrast to our study however, *in silico* analysis in both these studies indicated a GR binding site between positions -1337 and -1323 matching a loose consensus sequence XGXACXxxxXGTXCX. This site was not recognized as a GR binding site in our sequence analysis even at very low stringency settings of the sequence analysis tools we used. In one of the studies, Tchen et al. isolated a 130bp minimal GC-responsive region between positions -1366 to -1237, which overlaps significantly with the 114bp minimal promoter construct found in our study (-1380 to 1266) (45). In Hela cells, mutations made in this context of the putative GRE resulted in impairment of the GC response, whereas mutations of the C/EBP-site had no effect on GC-responsiveness of this construct. In contrast to the situation in Hela cells, we observed that mutation of the C/EBP-site resulted in a significant decrease of GC-responsiveness in A549 cells. Furthermore, in our experiments we demonstrated that when a labeled consensus GRE-sequence was incubated with recombinant GR DBD, this DNA-protein complex was not competed with an excess of our GC-responsive 114bp fragment, arguing against a direct GR binding to this part of the MKP-1 promoter. In contrast, using dexamethasone-treated nuclear extract from Hela cells incubated with a consensus GRE sequence, Tchen et al observed a relative, but not complete competition for GR-binding with an excess of their 130bp GC-responsive MKP-1 fragment (45). Thus, Tchen et al suggested MKP-1 expression by GCs to be independent of C/EBP activity and to involve a direct binding of GR to MKP-1, contradictory to what we observed in our study. The reason for the differences is unclear but may relate to the use of different cells for the respective studies.

Supporting our suggestion of a tethering mechanism between GR and C/EBP, we demonstrated that in a native chromatin context the GR_{LS7} which does not bind to a classical GRE still is able to bind and induce MKP-1 expression.

In summary, the discovery of GC-stimulation of MKP-1 involving GR-tethering with promoter-bound C/EBP (**paper I**), the possibility of additional regions (45) and additional transcription factors (187) being important for the GC-responsiveness and a hormone-dependent recruitment of certain co-activators (186) provides an increased understanding about the mechanisms behind GC-induced MKP-1 expression that could provide insights into new therapeutic approaches against inflammatory conditions. Interestingly, down-regulation of both C/EBPβ (188, 189) and MKP-1 (164) has been implicated separately in the GC-resistance of lung conditions such as asthma and chronic obstructive pulmonary disorder.

4.2 PAPER II - III

GR targets activated p65/p50 by various transcriptional repression mechanisms that inhibit gene expression of pro-inflammatory mediators regulated by the canonical NF-κB signaling pathway. The activation of canonical NF-κB signaling by multiple inflammatory stimuli, including TNFα and TPA, is inhibited by GCs.

To study if inflammatory cues might influence the ability of GCs to inhibit canonical NF-κB, we used a GR DBD mutant, GR_{R488Q}, stably expressed in HEK293 cells to study aspects of this inhibition. We observed that while GR_{R488Q} inhibited TNFα-activated NF-κB, the GR_{R488Q} was not capable of inhibiting NF-κB activated by TPA. The wild-type GR however, repressed NF-κB activity in both cases. Interestingly, the ability of GR_{R488Q} to repress TPA-stimulated NF-κB activity was restored when TPA-activated ERK1/2 was blocked either using a dominant negative MEK-1 mutant or the chemical inhibitor ERK-inhibitor U0126. In contrast to the wild-type GR, the GR_{R488Q} did not induce expression of MKP-1. However, the differential ability by the wild-type and mutant GR to induce MKP-1 expression was not found to be relevant for their dissimilar ability to repress NF-κB activity in these cells. This concluded that GR repression of NF-κB in these cells occur independently of MKP-1 induction by GCs. Thus, our study provides evidence demonstrating that GR-mediated repression of activated NF-κB requires separate functions of the GR and, importantly, that repression

of canonical NF-κB signaling by GR may not only depend on the particular gene and cell context, but can also depend on what signaling pathway is employed to activate NF-κB. This is in line with previous results from the laboratory of Glass and co-workers, who showed that the GR only represses NF-κB dependent target genes following activation by the TLR4 and TLR9 but not following NF-κB activation by TLR3 (54). In this case, the different signal transduction pathways used to activate NF-κB resulted in differential co-activator utilization by NF-κB, which constituted the basis for the differential GC response of NF-κB target genes. In summary, this highlights the complexity and diversity in the mechanisms involved in GR repression of the canonical NF-κB pathway.

With regards to GC interaction with the non-canonical NF-κB pathway, little is known. A recent study observed that co-treatment with dexamethasone potentiated the ability of 1,25(OH)vitamin-D3 to decrease the de novo expression of RelB protein in dendritic cells (190). Although this could indeed be one of the ways in which GCs limit the activity of non-canonical NF-κB signaling, no studies have yet demonstrated a capability of GCs to inhibit ReB/p52 driven transcription or a physical interaction between GR and RelB. In **paper III**, we demonstrate that agonist-bound GR can repress gene transcription stimulated by RelB/p52 in both endogenous and ectopic expression conditions. LTβR stimulation of mouse embryonal fibroblast (MEF) cells lacking p65, but not RelB, resulted in the increase of NF-κB reporter gene expression, which is in line of it being a known activator of the non-canonical NF-κB pathway. This LTβR activated RelB-dependent expression of the NF-κB reporter gene was inhibited by co-treatment with dexamethasone. Furthermore, in COS7 cells transiently co-transfected with expression plasmids for GR, RelB and p52, the GR transcriptionally inhibited RelB/p52 and physically interacted with the RelB subunit in response to GC treatment. Finally, wild-type GR, stably expressed in HEK293 cells, inhibited reporter gene expression driven by RelB/p52 whereas repression of RelB/p52 activity by GR DBD mutants GR_{R488Q} and GR_{LS7} was slightly impaired, which would suggests the GR DBD to be involved in the repression of non-canonical NF-κB signaling by GR. To our knowledge, this is the first evidence demonstrating an ability of GCs to repress the transcriptional activity of the non-canonical NF-κB signaling pathway and an ability of GR to interact with RelB in a hormone-dependent manner. In light of studies implicating a deregulated non-canonical pathway in inflammatory

disorders (129), the significance of the ability of GR to down-regulate RelB/p52 transcriptional activity for the overall inflammatory effects by GCs is interesting, but remains to be further explored.

Blocking defective activation of NF-κB by either targeting the classical and/or the alternative pathway may be therapeutically beneficial for many inflammatory diseases and hematopoietic malignancies (125, 129). The classical NF-κB pathway signals the production of pro-inflammatory cytokines and inflammatory mediators via the activity of p65/p50 heterodimers, whereas the alternative NF-κB pathway regulates genes important for lymphoid organ development and B lymphocyte maturation and survival via RelB/p52 heterodimers. Interrelationship studies between the NF-κB pathways demonstrate that a deletion of RelB in mice results in multi-organ inflammation and increased production of TNFα (191). This could imply that the NF-κB pathways act antagonistically against each other and a mechanism for a competitive interference binding with p65 by RelB has been proposed as a way of which RelB can regulate the production of TNFα (192). Moreover, studies have shown that p100 can function as an IκB and bind to canonical NF-κB heterodimers preventing their nuclear translocation (193, 194). Conversely, classical NF-κB signaling has been shown to inhibit the non-canonical pathway. A recent study demonstrated that classical NF-κB activation via TNFα stimulation negatively regulates alternative NF-κB dependent expression of the homeostatic chemokine CXCL12 in human umbilical vein endothelial cells (130). Also, RelB and p100 are inducible target genes of p65/p50 and have been implicated to serve as a feedback mechanism to limit both an undue p65/p50 and RelB/p52 driven gene transcription (195). Taking these observations together, the alternative NF-κB pathway has been branded as a balancing anti-inflammatory control pathway to the classical NF-κB pathway. However, this view may not fully explain the relationship between these two pathways. Studies show that certain inducers of NF-κB activity give rise to two waves of activation: first of the classical NF-κB pathway and then later followed by the alternative pathway to induce the expression of shared target genes. For instance, a recent study showed that expression of the polymeric immunoglobulin receptor (pIgR) in intestinal epithelial cells was regulated by both the classical and the alternative NF-κB pathway, while the induction of pro-inflammatory genes such as IL-8 required only p65 (196). Also, the alternative NF-κB signaling pathway was shown to be vital to mount a proper immune response against lymphocytic choriomeningitis virus (LCMV) in mice (197, 198). Thus seemingly, highly dependent on the cell type

and the nature of the stimulus, the classical and alternative NF-κB pathways can control target genes independently of each other, in synergy or antagonistically.

Recently various chronic inflammatory diseases, in particular those chiefly characterized by an abnormal B-cell activity and autoantibody production such as rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, and Sjögrens syndrome, have been proposed to be driven by an excess activation of the alternative NF-κB pathway (199-203). BAFF, a potent stimulator of the alternative NF-κB pathway, has been found in elevated levels in the sera of patients suffering various autoimmune conditions and neutralizing agents targeting BAFF has shown to have positive outcomes in clinical trials correlating to a depletion of mature peripheral B-cell subsets (204, 205). Moreover, patients with particular infectious diseases connected to autoimmune conditions (e.g. EBV, HPC, HIV) have elevated levels of selective inducers of the alternative NF-κB pathway, such as BAFF and APRIL, in their sera (200, 206). Additionally, many reports describe the survival of B-cells to be dependent on the up-regulation of anti-apoptotic factors (e.g. Bcl-2, Bcl-X) by the alternative NF-κB pathway (207). In line with this observation, the alternative NF-κB pathway has been implicated in the apoptotic resistance and uncontrolled activity of pre B lymphoma, myeloma, Hodgkin's lymphoma and chronic lymphocytic leukemia (CLL) cells (208). Interestingly, GCs induce cell cycle arrest and apoptosis of lymphoid cells and constitute an important part among the chemotherapeutic regimens for the treatment of many of the lymphoid malignancies where deregulated non-canonical NF-κB signaling has been implicated (209, 210). A possible cross-talk between GCs and non-canonical NF-κB signaling in lymphoid malignant cells might exist but has not yet been demonstrated.

4.3 PAPER IV

DKK-1 is a secreted protein that inhibits the Wnt/ β-catenin signaling pathway, which is a major signaling cascade in various cells. Recent evidence has suggested that deregulation of DKK-1 expression is causally related to the process of neurodegeneration in a number of disorders of the central nervous system, including Alzheimers disease (211), brain ischemia (212) and temporal lobe epilepsy (32). Targeting the increased expression of DKK-1 and restoring Wnt-signaling has been suggested for the treatment of these neurodegenerative disorders (213). Also, DKK-1 regulates bone development and elevated DKK-1 expression has been implicated in

causing bone resorption and erosive arthritis in multiple myeloma (214-216). GCs have been shown to stimulate the expression of DKK-1 in several cells most notably in osteoblasts (109, 182-184, 217) and, as recently discovered, in hippocampal neurons (218).

In **paper IV**, we demonstrated that GC-treatment of human neuronal progenitor cells (hNPCs) markedly decreased proliferation and neuronal differentiation while it promoted glia cell formation. Analysis of pathway-specific genes revealed that Dex induced DKK-1 in hNPCs. Dex- or DKK-1-treated hNPCs showed reduced transcriptional levels of the two canonical Wnt target genes cyclin D1 and inhibitor of DNA binding 2 (ID2). Furthermore, a putative GRE in regulatory promoter region of DKK-1 has been suggested by sequence analysis and using ChIP assay we demonstrated that GR binds to this GRE in the DKK-1 promoter and stimulates expression of DKK-1 in a hormone-dependent manner. Taken together, these findings show that GCs reduce proliferation and interfere with differentiation of hNPCs via inhibition of the canonical Wnt signaling pathway. Other reports support the observation made in **Paper IV** and show that both NPCs and immature neurons in the hippocampus are sensitive to the pro-apoptotic actions of GCs. For instance, induction of DKK-1 by GCs was demonstrated to cause stress-induced hippocampal damage (218) and is suggested to be involved in hippocampal athrophy observed in depressed patients. Also, the stimulation of DKK-1 by GCs in osteoblasts has been implicated as a mechanism for GC-induced osteoporosis. A recent study demonstrated that silencing DKK-1 expression rescued dexamethasone-induced suppression of primary human osteoblast differentiation (109). Importantly, clinical agents that antagonizes DKK-1 and rescues canonical Wnt signaling could therefore potentially attenuate adverse effects observed with GC therapy, such as hippocampal damage and osteoporosis.

Both canonical and non-canonical Wnt signaling has been implicated in inflammatory disease. For instance, β-catenin levels in macrophage-like microglia are elevated in Alzheimer's disease associated with chronic neuroinflammation and the canonical Wnt3a ligand induces the expression of pro-inflammatory cytokines including interleukin (IL)-6, IL-12 and TNFα (219, 220). Furthermore, Wnt5a, a representative ligand for the non-canonical Wnt pathway, has recently been implicated in both inflammatory response and disease. The expression of Wnt5a and frizzled 5, a possible receptor for Wnt5a, is enhanced in synoviocytes of rheumatoid arthritis (221) and the blockade of Wnt5a signaling inhibits synoviocyte activation (222). Wnt5a has also been detected in the sera of patients with severe sepsis (173). These reports suggest a

role for Wnt signaling pathways in the pathology of various inflammatory disease conditions and thus, might be candidate molecular targets for treatment by anti-inflammatory drugs e.g. GC therapy.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The MAPK signaling pathways, the canonical and non-canonical NF-κB pathways, and the canonical and non-canonical Wnt pathways have all been implicated in inflammatory diseases. GCs, potent anti-inflammatory hormones, inhibit these signaling pathways in part by stimulating expression of their archetypical inhibitors (e.g. IκBα, MKP-1 and DKK-1) and in the case for canonical NF-κB signaling, also through several transrepression mechanisms involving an inhibitory GR interaction with activated p65/p50. This thesis covers aspects of molecular cross-talk between GR and these signaling pathways.

The relevance of GCs capability to transactivate anti-inflammatory genes for their overall anti-inflammatory effects of GCs is still debated (see above). Animal knock-out models of GC target genes (e.g. IL-$10^{-/-}$ and MKP-$1^{-/-}$) exist to study this purpose. However, the mechanisms by which GCs induce expression of some of these genes remain either elusive or unaddressed. In **paper I**, we demonstrate that the transcriptional activation mechanism of MAPK-inhibitor MKP-1 by GCs is independent of a direct DNA-binding by GR, but instead involves a positive tethering between a promoter-bound C/EBP and the GR around an evolutionary conserved region -1.4kb upstream of the transcription start site. Non-classical transcriptional activation mechanisms as the one described in **Paper I**, would explain why some dissociated GCs and GR mutants that are transcriptionally inactive at GRE-containing genes still can induce a subset of GC target genes such as MKP-1. In addition to our study, other studies have expanded on the mechanisms underlying GR-regulation of the MKP-1 and have provided interesting new characteristics for GC-induction of this gene. Therefore, GC-regulation of genes that are suggested to be transactivated by GR through non-classical mechanisms, e.g. MKP-1, IL-10 and IκBα (223) merits more studies and could provide important clues about the mechanisms behind transcriptional regulation by GCs. Ultimately, it would be interesting to utilize genome-wide profiling methods to further investigate transcriptional regulation of GC target genes by dissociated GR agonists and GR mutants, such as GR_{LS7}. This could provide important information about the proportion and relationship of GC-target genes that are regulated through direct and indirect mechanisms.

In **papers I-III** GR mutants are used as investigative tools. Our results in using GR_{R488Q} showed that it was unable to repress NF-κB activity following stimulation by the phorbol ester TPA, but did so following activation by TNFα, while wild-type GR repressed NF-κB in both cases (**paper II**). This seemed to relate to the differential ability to activate of ERK1/2 by TPA and TNFα in the HEK293 cells. Blocking ERK1/2 enhanced the ability of a GR_{R488Q} to inhibit TPA-stimulated NF-κB activity. These results stress that the nature of the inducer of NF-κB activity may have profound effects on the mechanism by which the GR represses NF-κB. This is in line with previous observations (54) and provides an additional layer of complexity to cross-talk between NF-κB and GR that is worth to further investigate**.** Notably, GR activity may be impaired by both p38 MAPK (165, 224, 225) and JNK (162, 226, 227) and GR mutants, due to their mutations, might be more susceptible to post-translational modifications. More in depth studies investigating if GR monomers are more sensitive to post-translational modifications could be valuable to investigate, with the use of GR dimerization mutants and clinically aspiring monomer inducing GC agonists.

The study of GC-crosstalk with NF-κB has mainly involved the canonical or "classical" NF-κB pathway due to its involvement in the inflammatory response and its deregulation in inflammatory disorders. In **paper III** we demonstrate that GCs extend their inhibitory actions to the non-canonical NF-κB pathway. This is to the author's best knowledge the first time a cross-talk between non-canonical NF-κB pathway and GC signaling has been described. Thus, a broad range of future studies could provide more knowledge into this area of research. For instance, if a good model gene for the non-canonical pathway could be found that is repressed by GCs, as is the case for COX-2 and IL-8 for the canonical pathway, GR transrepression mechanisms at non-canonical genes could be studied in more detail. Furthermore, the implications of cross-talk between GC-signaling and non-canonical NF-κB activity in inflammatory disease and hematopoietic malignancies remain to be investigated. This should be further explored in e.g. B-cells where the cytokine BAFF signals purely through the non-canonical NF-κB pathway. Furthermore, the effect of GCs on a deregulated non-canonical NF-κB pathway and excessive BAFF production in animal models of inflammatory disease could provide important insights into the anti-inflammatory actions of GCs. Interestingly, two recent studies has demonstrated that dexamethasone inhibits BAFF expression in synoviocytes from patients with rheumatoid arthritis (228) and in patients with immune thrombocytopenia (229), without explicitly connecting this to an ability by GCs to inhibit non-canonical RelB/p52 activity. Furthermore,

elevated BAFF levels have been shown to counter the pro-apoptotic effects of GCs and protect myeloma cells from apoptosis (230). Moreover, it would be interesting to compare the effects of particular dissociated GCs on the ability of GC-crosstalk with the non-canonical NF-κB signaling pathway, in addition to the canonical pathway. The effects of these dissociated GCs on both canonical and non-canonical NF-κB target genes could be analyzed on a genome wide scale using high-throughput gene array platforms, which could in turn provide clues about the possible *in vivo* effectiveness of future compounds and give a powerful reflection of their dissociative nature with regards to transcriptional regulation.

Because of the ubiquitous tissue distribution of GR and the wide variety of genes under GC-control, prolonged GC therapy of inflammatory disorders is often associated with unwanted side effects. For instance, GCs affect the development, survival and death of neurons and excessive levels GCs have been linked to neurodegeneration of the hippocampus. Also, GC therapy is often predictably accompanied by GC-induced destruction of bone tissue. Interestingly, both these adverse effects has been suggested to be mediated by GC-inhibition of canonical Wnt signaling in NPCs and osteoblasts respectively, through the transcriptional activation of the soluble Wnt signaling inhibitor DKK-1 by GCs (109, 218). In **paper IV** we demonstrate that GCs induce expression of DKK-1, by a direct binding of GR to the GRE in the DKK-1 promoter. This inhibits Wnt-dependent transactivation of Cyclin D1 and ID2 and halts proliferation and differentiation of NPCs. Thus, antibodies targeting DKK-1 could be valuable in the management of GC-induced osteoporosis and manifestations of depression and cognitive disorders caused by HPA-axis hyperactivity or exogenous GCs. Importantly, since GC-activation of DKK-1 expression requires a direct DNA-binding by GR to a functional GRE in the DKK-1 promoter, the use of dissociated GCs that do not activate GRE-containing genes might be warranted to limit GC effects on the Wnt signaling pathway. On the other hand, both the canonical and non-canonical Wnt pathways have recently been implicated in inflammatory disease. Here, the role of GC-crosstalk with Wnt signaling and the possible effects on inflammation still remains an unexplored area of research. The significance of GC up-regulation of DKK-1 and IL-10 for a deregulated Wnt signaling in inflammatory disease should be examined. GCs have been shown to inhibit TAK1 in other contexts (231) and may serve as a way by which GCs can inhibit both TLR-stimulated Wnt5a expression and Wnt5a signaling. Although no reports have studied a direct transcriptional repression of Wnt5a-activated

NFAT by GR, GR can inhibit NFAT DNA-binding activity in thymocytes by disrupting AP-1 and NFAT transcriptional partnership (232). Moreover, pro-inflammatory target genes of Wnt5a include IL-1, IL-6 and COX2, which are negatively regulated by GCs when they are activated by other signaling pathways, including NF-κB and MAPK signaling pathways (87 and **paper II**).

Ultimately, the use of available high-trough put based systems in GC-target tissues to evaluate the dissociated qualities of selective GC agonist and to predict therapeutic index of these compounds is bound to be more fruitful than the use of simple GRE reporter based system and a selection of biomarker genes in limited number of cell types. However, a complete separation of transrepression and transactivation of GR may be difficult to achieve due the multiple mechanisms described for transcriptional regulation by GR (e.g. DKK-1, which is up-regulated by GR through direct GRE-binding and MKP-1, which is up-regulated by GR through positive tethering) and could possibly be undesirable because of a potentially critical interdependency between these two regulatory processes for GC-suppression of inflammatory responses (e.g. MKP-1 stimulation and NF-κB-repression) (Fig. 3). Nevertheless, dissociated GR agonists hold significant promise as anti-inflammatory drugs and could potentially provide a safer and more effective treatment option for some inflammatory disorders that today require treatment with non-dissociated GC equivalents, although ultimately, a single dissociated GR ligand might not be useful for all inflammatory disorders considering that gene-specific mechanisms and a variety in cross-talk mechanisms may be involved in inflammatory pathologies and side effects in different tissues.

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