Glucocorticoid Receptor Function and Hormone Therapy Resistance

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Abbreviations

A	alanine
Aa	amino acid
ACTH	adrenocorticotropic hormone
AF-1	activation function-1
AlB-1	albumin-1
AP-1	activator protein-1
ATP	adenine triphosphate
BIM	Bcl2-interacting mediator
Bolero	breast cancer trials of oral everolimus
BTM	basal transcription machinery
C-terminus	carboxyl terminal domain
CARM1	coactivator-associated arginine methyltransferase 1
CBP	p300/CREB-binding protein
CBP/p300	CREB binding protein/E1A binding protein p300 transcription complex
Cdk	cyclin E/cyclin dependent kinase
ChIP	chromatin immunoprecipitation
COPD	chronic obstructive pulmonary disease
CoRNR-box	coregulator nuclear receptor box: motive LXXXIXXXL
CREB	cAMP response element-binding protein
DAD	deacetylase activation domain
DBD	DNA binding domain
DRIP/ARC	vitamin D receptor interacting protein/activator-recruited cofactor
DRIP/TRAP	vitamin D receptor interacting protein/thyroid hormone associated protein complex
E6AP	E6-associated protein
ER	estrogen receptor
ER-α	estrogen receptor-α
ERK	extracellular signal-regulated kinase
FACT	facilitates chromatin transcription
FGD	familial glucocorticoid deficiency
FOXO3A	forkhead transcription factor 3a
Gas5	growth arrest-specific transcript 5
GCN5	general control of amino-acid-synthesis protein 5
GILZ	glucocorticoid-induced leucine zippers
GR	glucocorticoid receptor
GRB2	growth factor receptor-bound protein2
GREs	glucocorticoid responsive elements
GRIP1	glucocorticoid receptor interacting protein 1
GST	glutathionine-s-transferase
H	histone
HAT	histone acetyltransferase
HDAC	histone deacetylase
HER2	human epidermal growth factor receptor 2
hGR	human glucocorticoid receptor
HP1	heterochromatin protein 1
HPA axis	hypothalamic-pituitary-adrenal axis
HR	hormone receptor

HREs	hormone response elements
HSP	heat shock protein
iAs	inorganic arsenics
IGF	insulin-like growth factor
IGFBP1	IGF-binding protein 1
IL	interleukin
INF-ɣ/TLR-4-MyD88	interferon-ɣ/toll like receptor-4-Myeloid differentiation factor 88
iNOS	inducible nitric oxide synthase
IRF	interferon regulator factor
JNK	c-Jun N-terminal kinase
JNK/SAPKs	c-Jun N-terminal kinase and stress activated kinases
K	lysine residue
LBD	ligand binding domain
LCoR	ligand-dependent nuclear-receptor corepressor
LXXLL	leucine Xaa-Xaa-leucin leucine motif
LXXXIXXXL	leucine Xaa-Xaa-Xaa-isoleucine-Xaa-Xaa-Xaa-leucine motif
MARCoNI	microarray assay for real-time coregulator-nuclear receptor interaction
MAPK	mitogen-activated protein kinases
MCM4	mini chromosome maintenance-deficient 4 homologue
MDR1	multidrug resistance 1
Me–Lys	methylated lysine
MIF	macrophage inhibitory factor
miR	microRNA
MKP-1	mitogen-activated kinase phosphatase-1
mPUS1p	mouse PUS 1p
MRAP	melanocortin-2 (MC2) receptor accessory protein
mSIN3A	mouse Sin3 homolog A
mTOR	mammalian target of rapamycin
NO	nitric oxide
NPC	nuclear pore complex
N-terminus	amino terminal domain
NCoA1	nuclear receptor coactivator 1
NCoR	nuclear-receptor corepressor
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NFKBAI	NF-κB activation inhibitor
NRs	nuclear hormone receptors
NLSs	nuclear localization signals
NNT	nicotinamide nucleotide transhydrogenase
NURD	nucleosome remodelling and histone deacetylation complex
NRs	nuclear receptors
NR-box	LXXLL-motifs present on the C-terminus of coactivators for NR binding
NTD	N-terminal transactivation domain
ONRs	orphan nuclear receptors
P	phosphorylation
p/CAF	p300/CBP-associated protein
P-TEF	positive transcription elongation factor
P–Tyr	phosphotyrosine
PAI-1	plasminogen activator inhibitor-1
PBMCs	Peripheral Blood Mononuclear Cells

PELP1	proline-, glutamic acid- and leucine-rich protein 1
PEST	proline (P), glutamate (E), serine (S), threonine (T)
PGGH	primary generalized glucocorticoid hypersensitivity
Pi	inorganic phosphate
PI(3)K	phosphatidylinositol-3-OH kinase
PKA	protein kinase A
PLP	pyridoxal 5'-phosphate
PPARɣ	peroxisome proliferator-activated receptor ɣ
PRMT1	Protein arginine methyltransferase 1
PTM	posttranslational modifications
PUS	pseudouridine synthase
RAR	retinoic acid receptor
RIP140	receptor-interacting protein 140
RSF	remodelling and spacing factor
RSP5	reverse serine palmitoyltransferase phenotype 5
RXR	retinoid X receptors
S	serine residue
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SH2	steroid receptor coactivator -homology 2
SHC	SH2-domain-containing transforming protein
SLE	systemic lupus erythematosus
SLPI	secretory leukocyte proteinase inhibitor
SMRT	silencing mediator of retinoic acid and thyroid hormone receptors
Sp1	specificity protein 1
SRA	steroid receptor RNA activator
SRC	steroid receptor coactivator
STAR	steroidogenic acute regulatory protein
STAT	signal transducer and activator of transcription
SUG1	suppressor of gal 1
SUMO	small ubiquitin-like modifier
SWI-SNF	switching/sucrose non-fermenting complex
TAF	TATA-box-binding protein-associated factor
TBP	TATA-box-binding protein
TFIID	transcription Factor II D
TGFβ	transforming growth factor beta
Th	T-helper cells
Thr	threonine residues
TIF1	transcription intermediary factor 1
TLR	toll-like receptor
TNFα	tumor necrosis factor-α
TR	thyroid hormone receptor
UBC9	ubiquitin-conjugating enzyme 9
UIM	ubiquitin-interacting motif
V	valine
VDR	vitamin D receptor
Vps27	vacuolar protein sorting-associated protein 27

Summary in Layman's terms

Nuclear receptors (NRs) are a large family of receptors that can influence the DNA transcription of a variety of genes. Due to this DNA transcription, proteins and other factors that are required can be made inside the cells. DNA transcription is activated via signalling pathways when NRs bind their ligands. These ligands are substances that can interact with NRs and are needed for NRs to execute their function. Hereby, they can influence the transcription of specific genes needed for, amongst others, pro- or anti-inflammatory reactions or homeostasis. Nowadays, NRs are very important targets for drug development for patients that require hormone therapy. However, it is known that some patients (eventually) become resistant towards this therapy. An example is glucocorticoid (GC) hormone therapy resistance in asthmatic patients. In this phenomenon, the glucocorticoid receptor (GR), a member of the large NR family, does not function as it should be when it forms a complex with its GC ligand. As a result, the GC-bound GR does not enhance the DNA transcription of specific anti-inflammatory genes and does not inhibit pro-inflammatory genes, leading to a persisting asthma related inflammation. Hormone therapy resistance therefore harbours a large clinical burden with severe consequences and morbidity. It is still poorly understood why and how some people develop hormone therapy resistance. In order to predict and/or prevent resistance development by novel therapies, it is of significant relevance to investigate the (possible) cellular and molecular mechanisms underlying the physiology and pathology. Various mechanisms that contribute to GC hormone therapy resistance have already been described in the literature. However, the exact mechanism still remains inconclusive. Therefore, this review describes the current insights to and the currently known mechanisms behind the development of GC hormone therapy resistance in asthmatic patients. In addition, we tried to unravel novel cellular and molecular mechanisms involved. Based on the data reviewed in this thesis, it is hypothesized that the changes and alterations of posttranscriptional modifications of coregulators are involved. Posttranscriptional modifications are alterations by chemical groups of proteins that are added to amino acids after DNA transcription. In our hypothesis, these alterations are present on coregulators. Coregulators cooperate with NRs and influence the DNA transcription in a positive (coactivators) or negative (corepressors) way to regulate the gene transcription. Alterations in these coregulators can therefore lead to changes in the actions of target gene transcription by GRs and thereby eventually lead to GC hormone therapy resistance.

Abstract

Nuclear receptors (NRs) are a superfamily of transcription factors that can be activated by ligands and thereby regulate the activation of a variety of genes for, amongst others, homeostasis. Nowadays, these NRs represent very important targets for endocrine therapeutic drug development. However, it is known that some patients (eventually) become resistant towards this therapy. An example is glucocorticoid (GC) hormone therapy resistance in asthmatic patients. Hormone therapy resistance harbours a large clinical burden with severe consequences. It is still poorly understood why and how some people develop hormone therapy resistance. In order to predict and/or prevent resistance development by novel therapies, it is of significant relevance to investigate the (possible) cellular and molecular mechanisms underlying the physiology and pathology. Various mechanisms that contribute to GC hormone therapy resistance have already been described in the literature. However, the exact mechanism still remains inconclusive. Therefore, this review describes the current insights to and the currently known mechanisms behind the development of GC hormone therapy resistance in asthmatic patients. In addition, we tried to unravel novel cellular and molecular mechanisms involved. Based on the data reviewed in this thesis, it is hypothesized that the changes and alterations of posttranscriptional modifications of coregulators lead to changes in the actions of glucocorticoid receptors (members of the NR super family) and thereby eventually lead to GC hormone therapy resistance.

Introduction

Asthma is a heterogeneous disease.[1] However, many patients harbouring hormone dependent asthma are all administered endocrine therapy. Although the majority of these patient is successfully treated, some (<5%[2]) of these patients will (eventually) develop hormone therapy resistance.[3] Two types of therapy resistance exist: *de novo*/intrinsic resistance and acquired resistance. When a patient is administered hormone therapy as treatment but does not respond to it, we speak of a *de novo* resistance. When a patient initially responds to hormone therapy but eventually does not respond to it anymore, it is called acquired resistance.[4] Asthmatic patients who are resistant towards glucocorticoid (GC) hormone therapy, have a high risk of mortality and morbidity that is related to asthma. They are accounted for more than 50% of the asthma related health care costs.[2] Therefore, mechanisms involved need to be revealed and elucidated.[5]

Various factors are currently described to influence resistance. However, the physiological and pathological mechanisms behind it are complex and can involve a lot and various signal transduction pathways. These involve direct interactions with nuclear receptors (NRs) or cross-talking with other pathways.[3] Knowledge of these processes and mechanisms involved is of major importance for patients who undergo endocrine therapy. The more knowledge, the better the prognosis of the patient can be predicted and the better the patient can be treated with (novel) therapies without developing or overcoming resistance as side effect.[5,6]

To discover the mechanisms behind the development of resistance, it is important to know how healthy cells normally respond to influences from their environment. In order to insure normal development, metabolism, reproduction and hemostasis of the human body,[7] a precise regulation of transcription and gene expression is important. Therefore, coordination is needed of many regulatory mechanisms and events. To study this precise coordination in general as well as in specific mechanisms, such as GC resistance in asthmatic patients, NRs can serve as an interesting starting point.[8] NRs are involved in a lot of physiological processes in human beings, as well as in the development and progression of diseases.[9] NRs are transcription factors that can be activated by ligands and thereby regulate the activation and repression of a variety of genes for, among others, homeostasis and inflammation (**figure 1**).[10] Recent studies demonstrated a link between the precise actions and/or combinations of posttranslational modifications (PTMs) such as acetylation, phosphorylation and sumoylation of, amongst others, the NRs Estrogen Receptor α (ERα), Glucocorticoid Receptor (GR) and Peroxisome Proliferator-Activated Receptor ɣ (PPARɣ) in disease development and resistance.[7] Also various mutations, the immune system and coregulatory proteins seemed to be involved.

As reviewed in this thesis, coregulatory factors of NRs may play important roles in the development of resistance. Studies showed major importance of these coregulators in the activation or repression of gene transcription by GRs. The interactions between GRs and their coregulators depend on the presence and conformation of other steroid hormone receptors that are co-expressed, the coregulator availability and on special coregulator boxes. These boxes contain amino acid sequences which can interact with the ligand binding domain (LBD) of the GRs.[11,12,13] As mentioned in this review, it has been demonstrated that, besides mutations, PTMs of GRs and even their coregulators therefore may contribute to resistance therapy. Several PTMs, or even the same PTM at different amino acid residues, can occur within one protein. These PTMs determine the desired outcome.[7] Alterations by PTMs can thus alter the GR functioning and contribute to resistance. Therefore, PTMs are important therapeutic targets.[9]

Studies further showed that different cell types can have different transcriptional effects via GR modulation while interacting with the same promoter. In addition, in the case of an increased availability of coregulators, the gene transcription by GRs can be reversed due to other steroid receptors. Sometimes, proteasome ligand-dependent degradation of NRs and their coregulators is required for transcriptional activity. This indicates specific pathways for cellular functions. However, the exact mechanisms behind coregulator influences on GRs and their involvement in the development resistance are still not completely understood.[12] This review focused on the GRs and their GCs ligands in the development of GC hormone therapy resistance in asthmatic patients, which was originally described in 1968[5,14] by Schwartz,H.J. *et al.*.[15] Current evidence for the involvement of the immune system, mutations, coregulators and PTMs is reviewed.[12] In addition, also two interviews were conducted for this thesis. One with Dr. Rene Houtman, a NR expert at PamGene, and one with professor Dr. Herbert Michael (Bob) Pinedo, a leading oncologist. This latter interview is important since I believe we can learn a lot from discovered cellular mechanisms and therapies in cancer research as well.

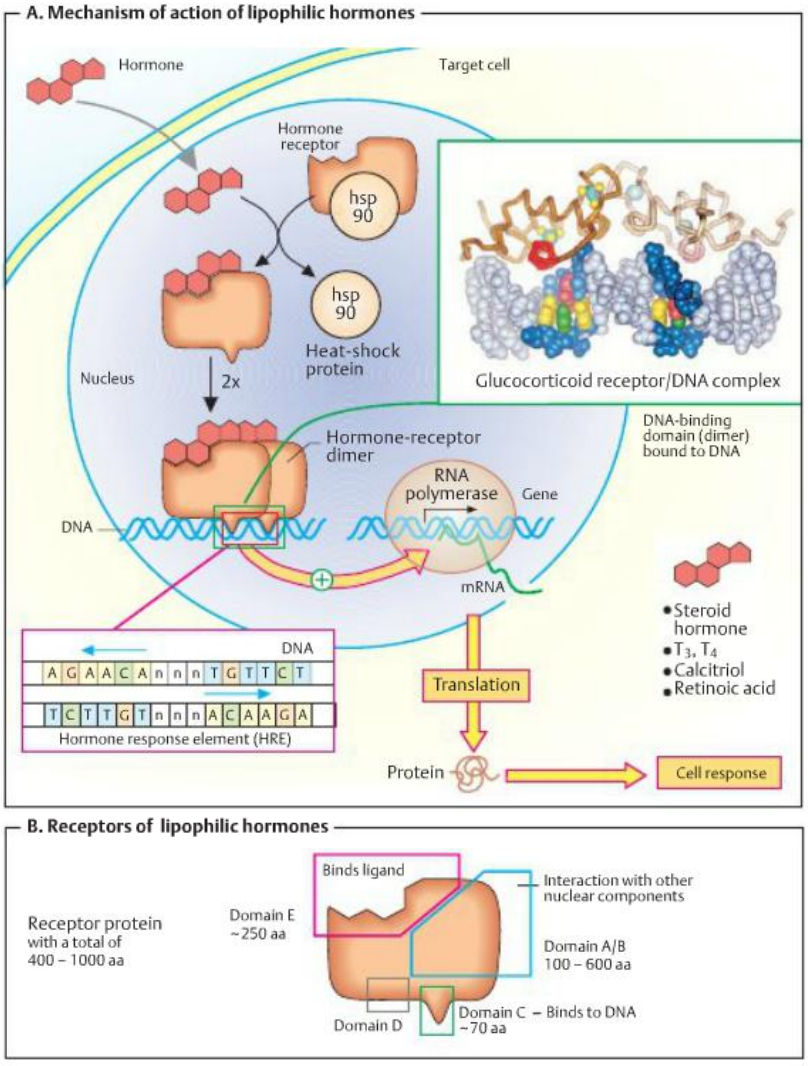


Figure 1. Mechanisms of action of lipophilic hormones (A) and their receptors (B). Figure obtained from Koolman, Jan *et al.*, 2005.[16]

Nuclear receptors

NRs form a superfamily of transcription factors. In mammals, fourthy-eight NRs are known with important roles in the regulation of homeostasis, development and growth.[8] Two different types of NR transcription factors are known. These involve nuclear hormone receptors (NHRs) and orphan nuclear receptors (ONRs). For NHRs the regulator ligands are already known, whereas for the ONRs the ligands still have to be identified[9] or were recently identified in screening strategies that discovered physiological and pharmacological ligands.[8]

Structure of nuclear receptors

All NRs have a similar structure (**figure 2**). The DNA binding domain (DBD) targets the NRs to specific DNA sequences known as hormone response elements (HREs)[17] at target genes via gene specific binding (**figure 1**).[12] The amino- (N-) and carboxyl- (C-) terminal domains of NRs are variable.[9] The N-terminal domain lies next to the DBD and contains the activation function-1 (AF-1) needed for transcriptional output.[12,18] The ligand binding domain (LBD) lies in the C-terminus of NRs and recognizes specific nonhormonal and hormonal ligands to specify the response[9] via an activation function-2 (AF-2).[12] The LBD contains three layers of antiparallel α-helixes[8] (twelve α-helixes in total) and four β-sheets.[19] Between the DBD and the LBD of NRs, hinge regions are apparent with variations in length. In addition, NRs can form homo- or heterodimers.[9]

Despite the previously described structural similarities, the function of the NR family members varies widely which makes them also interesting subjects of study. They can immediately activate or repress the gene expression due to the absence or presence of ligands,[8] by binding to their specific hormone responsive elements (HREs) in promoter or enhancer regions and by binding to other DNA activators, which are sequence specific. They can also inhibit the transcriptional activities of other classes of transcription factors[20] by inhibition of gene expression by GC-bound GRs[21] (also known as transrepression[22]).

NRs can activate or repress the gene expression in four different ways, based on their class. This classification depends on the ligand binding, DNA binding and dimerization properties according to Mangelsdorf *et al.* (**figure 2**).[17] Class I NRs include steroid hormone receptors which function as homodimers (two identical receptors). These NRs are induced by ligands and bind to HREs organized as inverted repeats (repeated DNA sequences in reverse orientations next to each other[23]). Class II NRs form heterodimers (two different types of receptors combined) with retinoid X receptors (RXR) partners and function in a ligand dependent manner. They bind to direct repeats (repeated DNA sequences in the same direction next to each other[24]) in HREs, although some bind to symmetrical repeats as well. Symmetrical repeats are repeated DNA sequences with a fixed amount of copies according to their symmetry order that can become superimposed in 180 degrees[25] and have similar chemical and physical properties.[26] Except for steroid hormones, this group includes all other known ligand-dependent receptors. Class III and IV NRs are ONRs. Class III receptors function as homodimers and bind primarily to direct HRE repeats. Class IV NR receptors function as monomers and bind to single site HREs.[17]

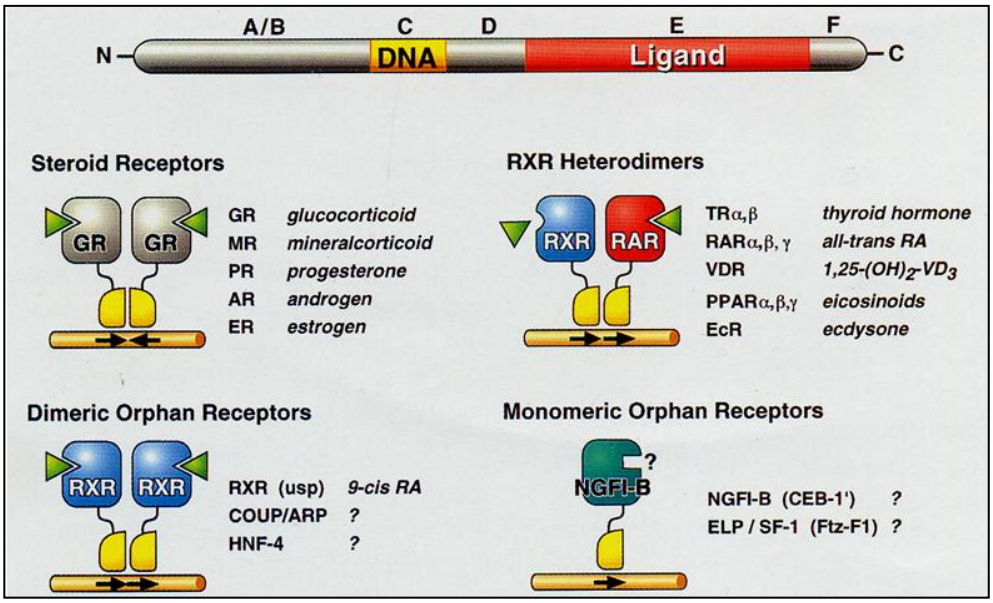


Figure 2. Overall structure of nuclear receptors. All NRs contain a variable N-terminal region (A/B), a conserved DBD (C), a variable hinge region (D), a conserved LBD (E) and a variable C-terminal region (F). NRs can be grouped in four classes: steroid receptors, RXR heterodimers, homodimeric ONRs and monomeric ONRs. Representatives for each group are shown. *DBD, DNA binding domain; LBD, ligand binding domain.* Figure obtained from Mangelshof *et al.*, 1995.[17]

Histones

For NRs to bind HREs in promoter regions and to enhance or repress the gene transcription, DNA first has to become unwind from histones and nonhistones.[27] DNA wound around histone and nonhistone proteins is called chromatin and eventually forms chromosomes. Histones are small proteins located in chromosomes. They have a positive charge by which they can bind to the negatively charged DNA. The ratio of histones and DNA in the nucleus is constant. In eukaryotes, histon 1 (H1), H2A, H2B, H3 and H4 histone proteins are associated with DNA. Eukaryotic DNA exists out of 6 x 10^9 base pairs. In absence of histones, the chromosomal DNA would therefore be over two meters for one single human cell. Thanks to histones, chromatin can be packed in such a way that it fits into the nucleus of a cell.[27]

Two of each of the H2A, H2B, H3 and H4 histone proteins are present in an 11 nm nucleosome,[27] also called an octamer.[28] Around this nucleosome, DNA of 147 base pairs is wound 1.65 times around it. This causes a six times compacter DNA which is also known as the 'beads-on-a-string' form. Subsequently, H1 can bind the DNA on the left side of the histones together with the middle DNA segment that is wrapped around the histones. This leads to a 30 nm structure of DNA, also known as the '30-nm-chromatin-fiber'. The exact packing of DNA beyond this 30 nm chromatin fiber is still inconclusive. However, it is demonstrated that DNA forms X-shaped loops by binding to a protein scaffold (also referred to as chromosome scaffold). Approximately 2,000 of these looped domains are present in one human chromosome. Nonhistone proteins from the chromosome scaffold hold these loops together at their base, where they eventually become ordered around the chromosome scaffold in a spiral like shape. In this way, the DNA becomes 10,000 times shorter and 400 times thicker (**figure 3**).[27]

Not all DNA is equally packed. There are two types of chromatin packing: via heterochromatin and via euchromatin packing. In heterochromatin packing, the chromatin remains condensed for most of the time and is in general transcriptional inactive. In euchromatin packing, the chromatin undergoes the normal cell cycle and contains DNA with repetitive sequences that can be actively transcribed.[27]

This transcription is executed by chromosome modification that alters the chromatin structure. Chromosome modification plays a major role in gene expression regulation.[29,30]

Chromosome modification can be executed in the presence or absence of covalent modification.[31] In absence of covalent modification, histon acetyltransferases (HATs) are involved that add acetyl groups[31] to lysine residues[12] at the N-terminus of all the histones.[31] Due to acetylation, the positive charge of the histone is lost, leading to a more relaxed chromatin structure in which the DNA is less wound around the histones. This makes the DNA accessible for transcription factors and can therefore enhance the gene expression.[32] This group also includes histon deacetyltransferases (HDACs) that remove acetyl groups of the N-terminus of the histones.[31] This results in a more compact chromatin structure in which the DNA is tightly wound around the histones. This makes the DNA inaccessible for transcription factors to bind their specific target genes and can therefore inhibit gene expression.[32] In absence of covalent modification, protein complexes can remodel the chromatin dependent adenine triphosphate (ATP) binding and ATP hydrolysis. This can lead to other interactions with other complexes. ATP interactions with HATs and HDACs form complexes that can lead to activation or repression of gene transcription. In humans, these complexes are known as the switching/sucrose non-fermenting (SWI/SNF-) complex, nucleosome remodelling and histone deacetylation (NURD-) complex and remodelling and spacing factor (RSF-) complex. It is also demonstrated that the vitamin D receptor interacting protein/activator-recruited cofactor (DRIP/ARC-) complex, facilitates chromatin transcription (FACT-) complex and serine palmitoyltransferase 4 and 5 (SPT4/SPT5-) complexes influence the chromatin remodelling. However, their mechanisms of action are still not completely understood.[31]

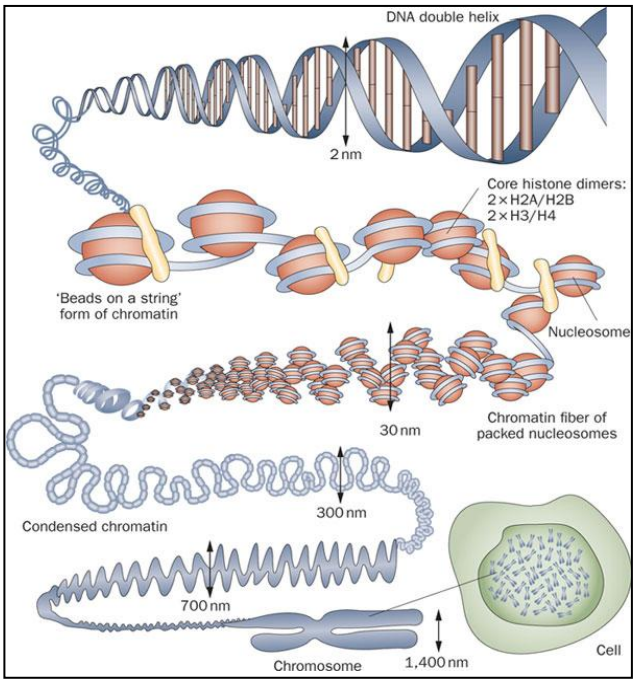


Figure 3. Schematic overview of the DNA packing by histones in the nucleus. To make DNA fit into the cell nucleus, the double helix of DNA (147 base pairs long and with a diameter of 2 nm) becomes wind around the octamer of histones and forms the 'beads-on-a-string'. When further packed, these form nucleosomes with a diameter of 30 nm and eventually finer chromatin (with a diameter of 300 nm) followed by condensed chromatin (with a diameter of 700 nm). Figure obtained from Tonna,S. et al., 2010.[28]

Coregulatory proteins involved in nuclear receptor function

NRs require a variety of coregulatory proteins to carry out their transcription functions.[8] These coregulators form the signal transduction pathway needed to activate the basal transcription machinery. They have no direct interaction with DNA, but affect transcription factors and thus the actual transcription. Coregulators can interact with one NR specific or with several NR family members. Binding of NRs with coregulators can affect the basal transcription machinery's stability, can recruit additional transcription factors and coregulators and can acetylate or deacetylate histones. These latter two mechanisms function via intrinsic HATs and intrinsic HDACs, or via recruitment of HATs and HDACs as described above.[8]

Coregulators can be subdivided into two groups according to their mechanism of action. The first group includes coregulators that covalently modify histones (for example by acetylation/deacetylation, methylation/demethylation, phosphorylation/dephosphorylation or ubiquitination/ deubiquitination). This process involves a precise and a special amino acid code for coregulators to carry out their function. The second group involves ATP-dependent chromatin remodelling coregulators that can change the promoter accessibility to transcription factors and to the basal transcriptional machinery. However, this subdivision is not very conclusive. There are coregulators, for example, that do not directly affect the chromatin structure and modification, but function in the assembly, recruitment or coregulator complexes. In addition, new coregulators are continuously being found and contain factors with unexpected functions.[8]

Furthermore, there are two types of coregulators: corepressors and coactivators. Corepressors are molecules that repress the gene transcription,[33] initiated by activators,[34] due to interactions with NRs.[34] They can prevent the activator to recruit other specific corepressors.[34] In contrast, coactivators are molecules that enhance the gene transcription by interaction with NRs.[33] They do not bind DNA directly but interact with activator and general transcription factors to participate in the activation of target gene transcription.[34] Both corepressors and coactivators have various enzymatic activities and functions. However, there are some exceptions. Some corepressors can compete with coactivators by displacing them and some coregulators can activate as well as repress the gene regulation. This indicates that coregulators are not specific for NRs and that they are also used by other DNA-binding transcription factors in a similar way. **Figure 4** depicts simplified examples of transcription units regulated by NRs together with their coactivator and corepressor complexes. In reality, gene activation and gene repression require a series of events influenced by a lot of enzymatic activities and regulatory complexes.[8]

In addition, also coactivators and corepressors themselves can be subdivided into two classes. Coactivators include proteins of the SWI/SNF family as the first class and proteins of the HAT family as the second class. They can both influence the chromatin and indirectly mediate the transcription due to interactions between histones and DNA. Coactivators can further immediately influence the basal transcriptional machinery by protein-protein interactions or via protein–protein interactions directly.[35] Examples of coregulator complexes depicted in **figure 4** are a chromatin remodelling activity complex which is ATP dependent, histon arginine methyltransferases, HATs and regulators involved in RNA processing and interactions with the RNA polymerase II machinery.[8]

Corepressors include the nuclear receptor corepressor (NCoR) and silencing mediator for retinoid and thyroid hormone receptor (SMRT)[33] as one family and the transcription intermediary factor-1 (TIF1) proteins as the second family.[35,36,37] They both recruit HDACs to the promoter regions.[35] Examples of corepressors in **figure 4** are ATP-dependent chromatin remodelling complexes, basal corepressors which take care of the recruitment of subcomplexes that often contain HDAC activity and corepressors which can recruit general corepressors on ligand induction.[8]

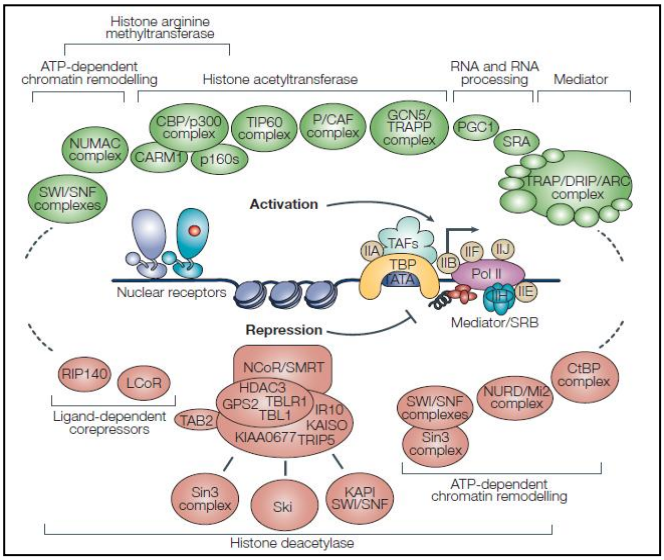


Figure 4. Simplified representation of NRs and their coactivator (green) and corepressor (red) complexes needed for the regulation of transcription. *IIA, IIB, IIE, IIF, IIH, IIJ, general transcription factors A, B, E, F, H, J; HDAC, histone deacetylase; LCoR, ligand-dependent nuclear-receptor corepressor; NCoR, nuclear-receptor corepressor; RIP140, receptor-interacting protein-140; SMRT, silencing mediator of retinoic acid and thyroid hormone receptors; TAF, TBP-associated factor; TBP, TATA-binding protein.* Figure obtained from Perissi,V. et al., 2005.[8]

Coregulatory protein interactions with nuclear receptors

When a ligand binds to a NR, this NR undergoes a conformational change allowing the occurrence of a coregulator binding site in the C-terminus of the NR.[38] For nuclear coactivators to interact with a ligand dependent NR, LXXLL- (Leucine-Xaa-Xaa-leucin-leucine[11]) motifs on the C-terminus of coactivators are acquired (**figure 5**).[39] These short α-helical[38] motifs are called NR-boxes.[11] Coactivators can possess various NR-boxes for interactions with multiple NRs with different affinities per NR-box.[12] NR-boxes contain a constant part, the 3, 3', 4, and 5 helixes, and a variable part, the AF-2 helix. In the NR LBD structures, the constant part adopts the same conformation every time. The AF-2 variable part adopts diverse conformations, depending on the ligand.[39,40] The variable part of the LXXLL-motif can recognize peptides on, and thereby interact with, the AF-2[39,40] in the E-region[41] on the LBDs of the NRs.[39,40] When this recognition takes place, especially the AF-2 helix (also known as helix 12[42]) of the LBD undergoes a significant conformational change.[43] This all contributes to the activation of the AF-2 helix leading to a charge clamp pocket.[44] This charge clamp pocket is caused by the interaction of a conserved glutamate residue with a lysine present in helix 3.[44] These two are both located in the AF-2. Between these two residues, a hydrophobic region is apparent. When a coactivator binds with its LXXLL-motif to a NR, a two-turn α –helical structure is added to the LXXLL-motif to its hydrophobic leucine side chains.[44] Subsequently, this part of the structure is transported to the hydrophobic region.[44] In this conformation, also other coregulator motifs can interact[39] to enhance the transcription regulation. Because of their large helixes, corepressors cannot be placed in the hydrophobic region of this charge clamp pocket. Hereby the NR affinity for the corepressors that contain a CoRNR-box (explained in the next alinea) is reduced, but the affinity for coactivators that contain the NR-box is increased. This mechanism therefore leads to a selection of recruited corepressors towards ligand dependent NRs.[45] Furthermore, the removal of corepressors is necessary before coactivators can be recruited.[45] Interestingly, NRs can also bind and, under certain conditions, even activate the gene transcription on a basal level. This indicates the presence of specific modifications in histones and/or factors that alter the chromatin structure needed for the activation of gene transcription.[45]

In the absence of a ligand, corepressors interact with NRs and repress the gene transcription (**figure 5**). Corepressors also contain a LXXLL-like consensus sequence domain. For N-CoR to execute its inhibitory function, a LXXXIXXXL-motif (in which I stands for isoleucine[42]) is required.[11,42,45,46] This consensus sequence is present in the N-CoR box and is called the CoRNR-box.[11] The CoRNR-box needed for interaction with NRs lies in the hinge region of corepressors between the DBD and LBD.[13] Interaction occurs when the AF-2 helix of the NR becomes displaced due to absence of a bound ligand. Hereby the CoRNR-box can interact with the same identical hydrophobic pocket as for NR-boxes to eventually repress the gene transcription.[11] The specificity of the NRs depends on the sequences that flank the CoRNR- box.[11] This box also facilitates the release of corepressors due to hormone induced changes.[35] In contrast, no LXXXIXXXL-motif is present in SMRT to execute its function.[46] However, studies demonstrated that SMRT can execute its function due to the association with mouse Sin3 homolog A (mSin3A), a corepressor, and HDAC1 *in vivo*.[47] In addition, N-CoR and SMRT both contain a deacetylase activation domain (DAD) for their interactions with HDACs to repress the transcription activity. This repression by the large molecules N-CoR and SMRT is due to their carboxyl- and amino-terminal halves.[43] Mutations of DAD can cause absence in HDAC activity leading to an increased gene transcription by GRs.[48] Repression also occurs when there is a deletion in helix 12 of the LBDs.[43]

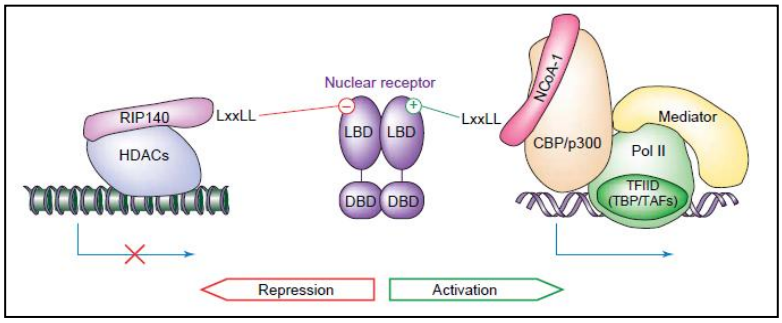


Figure 5. Mechanism of transcriptional regulation by coregulatory proteins. Regulation of transcription by NRs involves interactions mediated by LXXLL-motifs between NRs and coregulators. In the absence of a ligand (left), NRs interact with corepressor proteins and, indirectly, with HDACs. These all repress the basal transcription by producing chromatin. In the presence of a ligand (right), coactivators can bind NRs. This complex acts as an interaction platform to which multiple protein complexes can bind. These complexes have intrinsic HAT activity and can enhance the transcription by removing the chromatin repression and recruiting the RNA polymerase II (Pol II) pre-initiation complex (right). *RIP140, receptor-interacting protein-140; HDACs, histone deacetylases; LBD, ligand binding domain; DBD, DNA-binding domain; NCoA1, nuclear receptor coactivator 1; CBP/p300, CREB (cAMP-regulated-enhancer) binding protein and p300, E1A binding protein p300 transcription complex; TFIID, transcription Factor II D; TBP, TATA-box-binding protein; TAFS, TBP-associated factors.* Figure obtained from Plevin,M.J. *et al.*, 2005.[40]

The actions of NR coactivators and corepressors are due to very little differences between the NR- and CoRNR-boxes.[11] They both can interact with adaptor proteins that contain characteristics of corepressors and/or influence basal transcription machinery members. Corepressors and coactivators also require AF-2 in the NR (also known as t4) to release the corepressors and to activate the gene transcription. Furthermore, activation domains contain especially amino acids that are acidic, proline rich or glutamine rich. In contrast, corepressors do not contain similar sequences which indicate different targets. However, there are criteria which must be met. Corepressors repress the transcription when they become fused to DBDs which are heterogeneous, they interact with the transcription factor repression domain, they have a sensitive binding for inactivating mutations in the transcription factor repression domain and/or they can influence the transcription factors' repression function depending on their concentration. For example, repression of the retinoic acid receptor (RAR) and the thyroid hormone receptor (TR) occurs by N-CoR dissociation.[41]

Posttranslational modification regulation of nuclear receptors

NRs are also regulated by PTMs[7] of the receptor itself or of its coregulators (**figure 6**). PTMs are alterations by chemical groups of proteins following translation.[49] These modifications involve, amongst others, acetylation, methylation, phosphorylation, sumoylation and ubiquitination. Hereby, the PTMs can change the function of NRs and/or can control the relation between NRs, coregulators and their related target genes (as reviewed by McKenna,N.J. *et al.*).[50] These reactions are important cellular mechanisms since they can possibly influence the cellular localization, enzymatic activity (e.g. HATS and HDACs) and the recruitment of coregulators required for transcription.[8] They can do this by changing the biological activity of coregulators or receptor proteins.[51] Acetylation and ubiquitination can also change the half-life time of coregulators.[50] Phosphorylation can influence the activity of coregulators[50] and the protein turnover.[7] And finally, methylation can target histones to alter the acetyltransferase activity. All these modifications are mediated by various kinase mediated cellular signalling pathways (as reviewed by McKenna,N.J. *et al.*).[50]

PTMs are mechanisms to obtain both positive and negative cross-talks with NRs. For positive crosstalk, the first PTM serves as signal for the removal or addition of another PTM, or for the recognition by a protein for a second modification. An example is phosphorylation-dependent ubiquitination. Negative cross-talk involves competition to alter an amino acid residue. It is also possible that one PTM masks the recognition site for the second PTM. These PTM interactions are reversible processes and can alter due to changes in cellular and environmental conditions. Therefore, PTMs can be subdivided into two groups: PTMs with reversible modifications that can add or remove functional chemical groups and PTMs that modify amino acid residues due to addition of proteins or polypeptides by sumoylation and ubiquitination for example (as reviewed by Anbalagan,M. *et al.*).[7]

Overall, the coordinated regulation of gene transcription by NRs depends on the promoter properties (inverted or direct repeats, see page 8), regulatory signals (coactivators, corepressors and PTMs) and the cell-cycle stage. The latter involves repression checkpoints needed to be released in an ubiquitinated manner for each transcription cycle in order to proceed. Each of these transcriptional activation events can involve a series of changes, such as DNA demethylation and coregulator modification. These alone might not be sufficient for full activation, but together they can form the specific combinatorial code that is needed for the activation of gene transcription.[8]

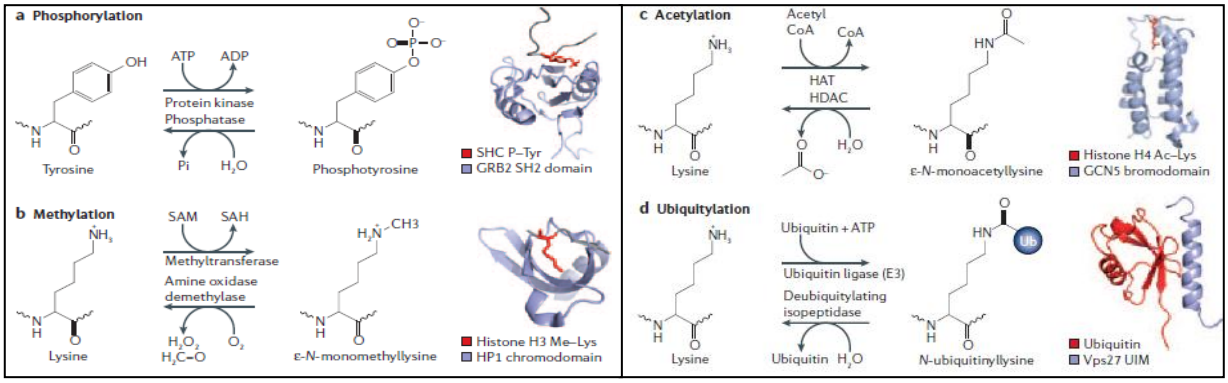


Figure 6. Examples of the post-translational modification reactions phosphorylation (a), methylation (b), acetylation (c) and ubiquitynation (d) of amino acid side chains. Enzymes on the reaction arrows displayed in this figure can add and/or remove the PTMs. Coloured structures on the right demonstrate examples of domains responsible for protein interactions in purple with their ligands in red. *GCN5, general control of amino-acid-synthesis protein-5; GRB2, growth-factor-receptor bound protein-2; HAT, histone acetyltransferase; HDAC, histone deacetylase; HP1, heterochromatin protein-1; Me–Lys, methylated lysine; Pi, inorganic phosphate; P–Tyr, phosphotyrosine; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SH2, Src-homology-2; SHC, SH2-domain-containing transforming protein; Ub, ubiquitin; UIM, ubiquitin interacting motif; Vps27, vacuolar protein sorting-associated protein 27.* Figure obtained from Seet,B.T. *et al.*, 2006.[52]

Asthma and glucocorticoid hormone therapy resistance

Asthma is one of many inflammatory diseases in which GCs are administered as endocrine therapy. GCs inhibit the expression of various genes that are involved in immune and inflammatory diseases. Examples are genes encoding adhesion molecules, chemokines, cytokines, cell-surface receptors, degradative proteinases and enzymes that produce inflammatory coactivators.[21,53] In contrast, GCs can also cause diseases such as osteoporosis and diabetes and can suppress the hypothalamic-pituitary-adrenal (HPA) axis. To what extent GCs cause anti-inflammatory actions and undesirable side effects is still not known. They can do this via similar or diverse molecular mechanisms.[53] Although GCs are used as primary treatment options for many inflammatory diseases, it is also known that patients can be irresponsive to GCs due to (developing) GC resistance (**figure 7**).[54] There are even clinical conditions associated with GC resistance, for example rheumatoid arthritis[14,55,56] and systemic lupus erythematosus (SLE).[14,22,57] Therefore, there is need to find the underlying mechanisms of GCs and the development of GC resistance.[58,59,60]

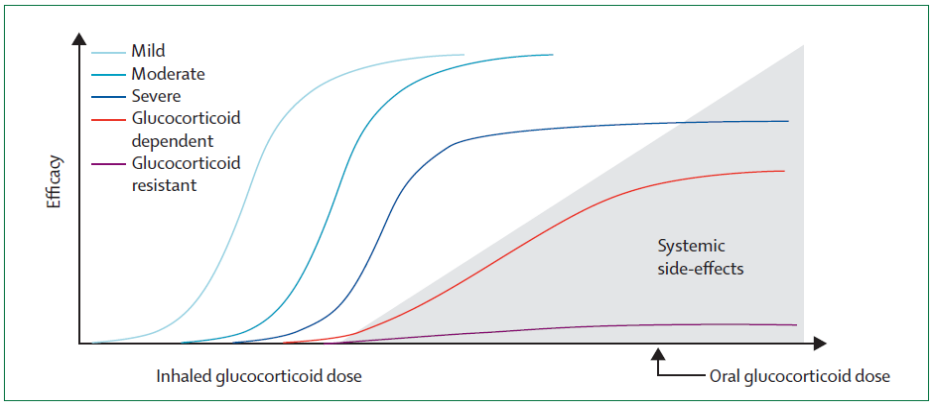


Figure 7. Responsiveness towards GCs in asthmatic patients. Patients with mild and moderate asthma can be treated with standard oral GC treatment. Patients with severe asthma need higher GC doses as therapy. Some patients are completely resistance towards GC at high doses. In addition, the side effects (grey) increase with the increasing GC doses. Figure obtained from Barnes,P.J. *et al.*, 2009.[14]

Glucocorticoid receptors and their mechanisms of action

GCs are steroid hormones[17] which are produced by the adrenal gland and their production is regulated by the HPA axis that secretes endogenous GCs.[12] They are stress related,[61] lipophilic signal molecules[62] that are synthesized from cholesterol[63] and that can directly affect the gene transcription.[64] GCs are ligands for GRs that are present at almost all cells and tissues in humans.[65] However, these cells show different responses towards GRs,[66] depending on the tissue and cell cycle.[65]

The human GR gene contains nine exons, located at chromosome 5.[67,68] At least three promoters are known to regulate the activity of the GR gene, indicating a tissue specific expression.[69] Furthermore, the promoter region of GRs (2300 base pairs) contains fifteen unique binding sites on the DNA for, amongst others, the transcription factors transcription activator protein-1[53] (AP-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), Specificity Protein 1 (Sp1) and the GR itself. Thus, the GR expression is regulated by various transcription factors that bind their response element in the GR promoter.[70]

In mice, the N-terminus of the GR is able to decrease a nonspecific DNA binding due to an acid-region and thus improves the discrimination between nonspecific and specific DNA binding sites. The

GC binding site is present in the C-terminus of the GR and represses the GR transcriptional activity in the absence of hormones. In the presence of hormones, the repression is released which allows transcription activation.[71]

Also, in human beings GRs can regulate the receptor/ligand dependent gene expression in a positive or negative manner.[21,53] GRs become activated when they bind to their GC ligands (**figure 8**).[54] This activation leads to a conformational change of GRs which then leads to its dissociation from chaperone heat shock protein (hsp) 90. The HBD of GRs determines the tight binding of GRs to chaperone hsp90[72] which is needed to maintain a GC binding site with a high affinity.[73] The conformational change also causes dissociation from other proteins[60,74,75] and dimerization of the GR.[12] Hereby the nuclear localization signals or sequences (NLSs) and phosphorylation of the GR serine residues S113, S141, S203, S211 and S226 can be activated. Phosphorylation sites are all identified in the N-terminus of the GR[76] ligand free receptor[77] and phosphorylation varies with every cell cycle.[78] It influences the half life time of GRs[79] and their transcription activity.[76] Phosphorylation takes place by the cyclin-dependent kinases[7] casein kinase II and mitogen-activated protein kinases (MAPK).[7] GRs also contain proline (P), glutamate (E), serine (S), and threonine (T) (PEST) domains with phosphorylation sites.[80] Many phosphorylation sites are consensus sequences in which a proline follows after the phosphorylated residues.[78]

Subsequently, the GC-bound and hyperphosphorylated GR translocates into the nucleus. Via conformational changes in the GR, also a coordinated recruitment of chromatin remodelling complexes and coregulators occurs.[19] Therefore, GRs can, for example, recruit members of the MAPK[81] and phosphatidylinositol-3-OH kinase PI(3)K pathway[82] signalling cascades in the cytoplasm and co-transport them into the nucleus. It is also demonstrated that AP-1 serves as a pioneer in generic cell lines to make chromatin accessible for GRs.[83] With the use of such coregulators,[12] GRs can bind as homodimer[60,74,75] to their fifteen nucleotide sequences long[12] palindromic GR responsive elements (GREs) in the promoter region of specific target genes.[21] In the promoter regions, the GRα communicates with the basal transcription machinery[60,74,75] and, together with the coregulators and complexes, they influence the RNA polymerase II activity[19,54] and the target gene expression in a positive or negative manner.[60,74,75] This depends on the GRE sequence and the promoter characteristics (**figure 8a**).[60,74] This process is also known as transactivation.[22] At this point it should also be clear that a defective GR phosphorylation will prevent GR translocation to the nucleus and can therefore be a cause of resistance.

Besides this direct protein interaction, GRs can also influence the gene expression in an indirect way. This indirect way involves protein-protein interactions of GC-bound GRs with other transcription factors,[54] such as NF-κB[21] and AP-1.[54] These two DNA-bound proteins accomplish the major immunosuppressive and anti-inflammatory effects of GCs.[19] In both cases, the activation of inflammation becomes inhibited by GC-bound GRs.[53,54] This GR inhibition of genes can occur by tethering of the GC-bound GR itself to these proteins (**figure 8b**) or by the action of these GRs in a composite way due to binding of GR to both its GRE and its transcription factors in one of the neighbouring sites (**figure 8C**).[19] In contrast, conjugation of GC-bound GRs with signal transducer and activator of transcription (STAT) family members, with or without GRE binding, can increase the GR transcription activity for certain genes (**figure 8B and 8C**). Furthermore, it is demonstrated that GCs can inhibit signalling in the mitogen-activated protein kinase (MAPK) pathways that regulate the pro-inflammatory gene expression. This inhibition is depended on *de novo* gene expression.[53]

Transrepression (inhibition of gene expression) is due to the inhibition of a variety of anti-inflammatory genes mentioned above,[21] by coregulators such as secretory leukocyte proteinase inhibitor (SLPI) and by mitogen-activated kinase phosphatase-1 (MKP-1).[7]

Inhibition can also be activated by the inhibition of the pro-inflammatory gene transcription such as interleukin-6 (IL-6)[7] and by inducing the expression of anti-inflammatory proteins.[21] GRs also repress the pro-inflammatory gene expression induced by NF-κB (**figure 8**).[7] Because GRs can inhibit cell processes by binding to their GC ligand, GCs are, among others, used as immunomodulatory and anti-inflammatory agents. Therefore they are prescribed as endocrine therapy for allergies, chronic inflammatory diseases[7] and auto-immune diseases.[21] GCs can do this by controlling transcriptional regulation of GR target genes.[21]

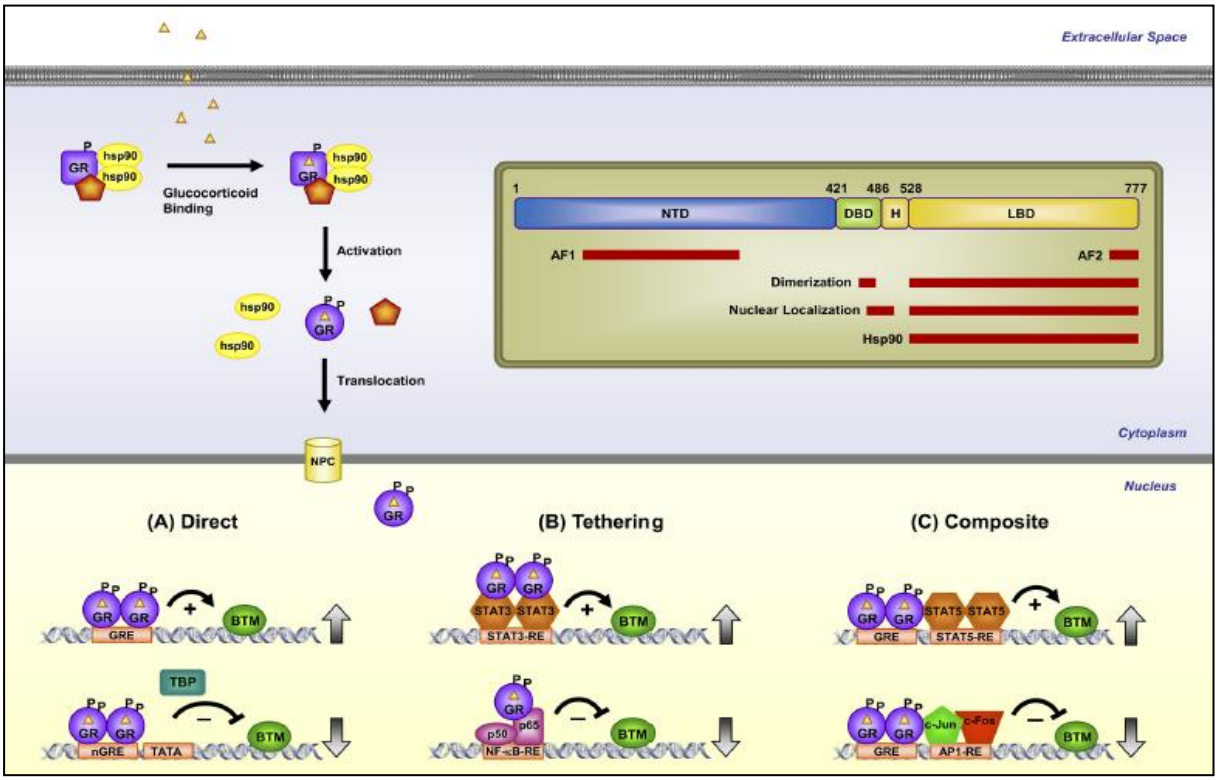


Figure 8. Schematic depiction of the mechanisms of action of glucocorticoid receptors. Target gene expression by GRs can be executed via a direct manner by binding of GR to GRE (**A**), by tethering of the GR itself towards other transcription factors with the exception of DNA binding (**B**) or via binding of the GR to both its GRE and transcription factors to the adjacent sites (**C**). In the grey box, it is demonstrated that the GR consist of an N-terminal transactivation domain (NTD), a DNA binding domain (DBD), a hinge region (H) and a ligand binding domain (LBD). Furthermore, regions important for the activation of gene transcription (AF-1 and AF-2), dimerization, nuclear localization and hsp90 conjugation for human GRs are demonstrated. *NPC, nuclear pore complex; BTM, basal transcription machinery, GR, glucocorticoid receptor; GREs, glucocorticoid responsive elements; HSP, Heat Shock Proteins; TBP, TATA-binding protein; nGRE, negative GRE; RE, response element.* Figure obtained from Oakley,R.H. *et al.*, 2011.[19]

Coregulatory proteins involved in glucocorticoid receptor functions

Corepressors and coactivators are required for a proper gene expression by GRs.[84] Coactivators (**figure 9**) can recruit the basal transcription machinery, remodel chromatin and are, via the functional protein domain AF-1 of the GRs (see page 7), attracted to the promoter regions of GR target genes. Coactivators for GRs include, amongst others, the vitamin D receptor interacting protein/thyroid hormone associated protein (DRIP/TRAP-) complex, SWI/SNF-complex, p160 coactivators, glucocorticoid receptor interacting protein 1 (GRIP1), steroid receptor coactivator 1 (SRC1), p300/CBP-associated protein (p/CAF), p300/CREB-binding protein (CBP) cointegrators, HATs (as reviewed by Charmandari,E. *et al.*),[60] E6-associated protein (E6AP), reverse Spt phenotype 5 (RSP5) and ubiquitin-conjugating enzyme 9 (UBC9).[79,85,86]

Upon ligand binding, corepressors dissociate and SWI/SNF-complex chromatin remodeling enzymes appear to modify the chromatin domains. The interaction between SRCs and CBPs results in a disruption of the local structure of the nucleus and in HAT activity. Signaling pathways mediated by kinases can directly communicate with GR target promoters. Phosphorylation of AF-1 further strengthens ligand-dependent interactions between GRs and SRCs in order to recruit SRCs immediately to the target promoter in the absence of ligand. The TRAP/DRIP-complex interacts with the basal transcription machinery for transcriptional initiation. Certain TATA-box-binding protein-associated factors (TAFs) have additional input in the GR transcription specific for promoters. However, to what extent overlap is present in the binding of specific complexes to the target promoter is still unclear. It is possible that the requirement of certain local coactivators may vary.[50]

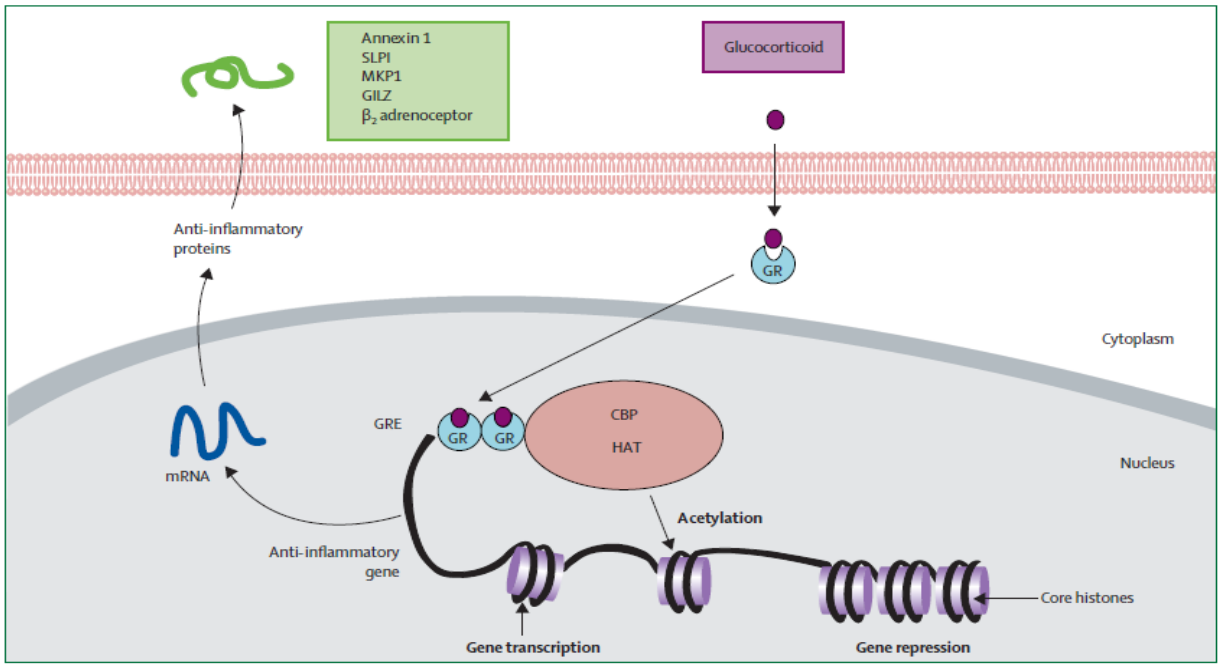


Figure 9. Scheme of the activation of anti-inflammatory gene expression. Upon ligand binding, GRs translocate to the nucleus and bind their GRE in the promoter region of responsive genes and to coactivator molecules. These coactivators can acetylate histones because they possess intrinsic HAT activity. This leads to anti-inflammatory gene activation. *CBP, cyclic AMP response element binding protein; GR, glucocorticoid receptor; GILZ, glucocorticoid-induced leucine zipper protein; MKP1, mitogen-activated protein kinase phosphatase 1; SLPI, secretory leucoprotease inhibitor.* Figure obtained from Barnes,P.J. *et al.*, 2009.[14]

Corepressors (**figure 10**) contain HDAC activity which enables DNA-binding proteins to interact with N-CoR and SMRT. Hereby, a repression of the transcription of target genes occurs. N-CoR and SMRT are targets for the signalling pathways of cells which influence their subcellular localization, expression levels and their interaction with other proteins.[87] A lot of corepressors are associated with redox pathways.[87] Corepressors of GRs include, amongst others, SMRT,[46] N-CoR,[46] HDACs,[88] ligand dependent nuclear corepressor (LCoR),[89] receptor interaction protein 140 (RIP140),[90-93] growth arrest-specific transcript 5 (gas5)[94] and NURD complexes.[87] These corepressors can work at the same time or in succession in order to enhance or repress the GR gene transcription.[87]

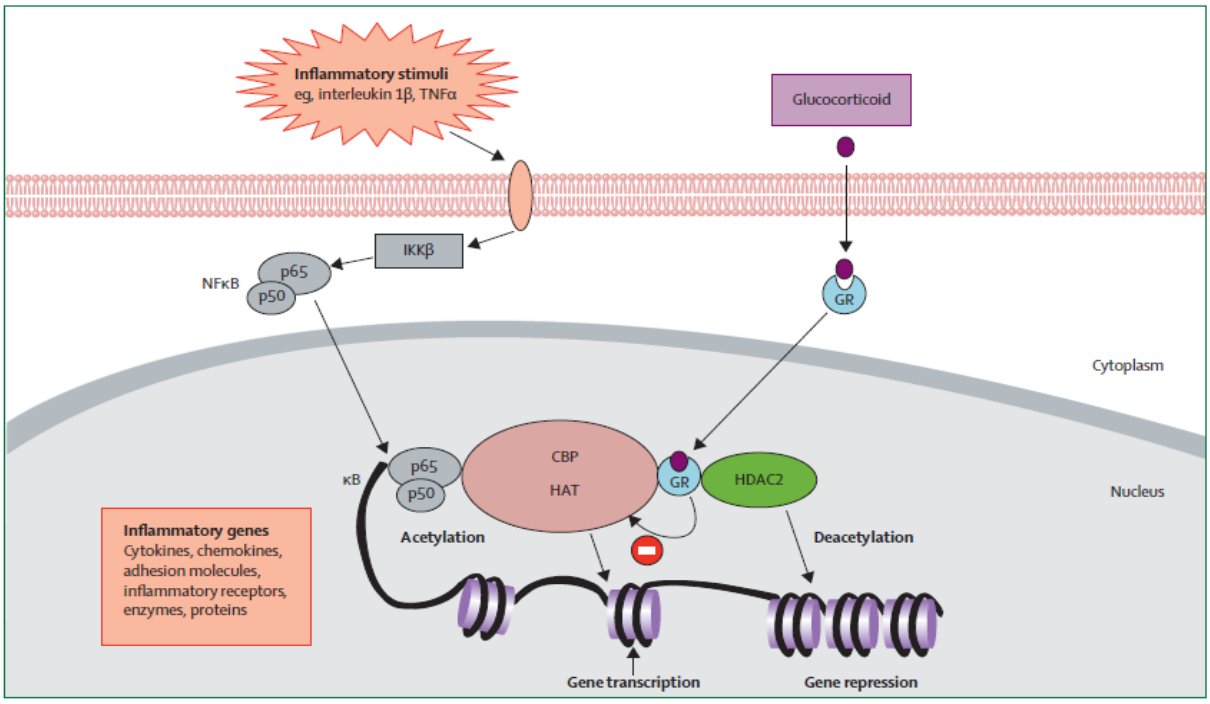


Figure 10. Scheme of the suppression of inflammatory gene expression. Due to inflammatory stimuli, the NF-κB kinase inhibitor (IKKβ) becomes activated. This leads to activation of NF-κB. Subsequently, a complex of p50 and p65 NF-κB proteins becomes translocated to the nucleus. Here they bind with their κB recognition sites to inflammatory gene promoters and also to coactivators, for example CBP. These coactivators acetylate core histones and thereby activate the inflammatory gene expression. In addition, GRs that are activated also bind to coactivators in the nucleus and inhibit their HAT activity. Because of the recruitment of HDAC2 by GRs, activation of inflammatory genes becomes suppressed. *CBP, cyclic AMP response element binding protein; GR, glucocorticoid receptor; TNFα, tumour necrosis factor α.* Figure obtained from Barnes,P.J. *et al.*, 2009.[14]

Interestingly, it is possible that PTMs can influence the coregulators in such a way, that they can change their role. Instead of a corepressor, they can become a coactivator.[92-96] This is for example seen with the GR corepressor RIP140.[92,93] By arginine phosphorylation and methylation, RIP140 becomes conjugated to Pyridoxal 5'-phosphate (PLP), the active form of vitamin B6. PTMs alter the function of RIP140 via coactivator competition with receptor agonists. This way, RIP140 can modify the transcription activity of these agonists in a reverse way.[92,93] The same phenomenon is seen in mice studies with pseudouridine synthase (PUS), which demonstrates that mouse PUS 1p (mPUS1P) can bind with NRs. In this complex, mPUS1p acts as a coregulator by modifying positions in the steroid receptor RNA activator (SRA) through pseudouridylation. This psuedouridylation is a PTM and can control the switch between a coactivator and corepressor function of the SRAs. This has major effects on the NR signalling.[97]

Mechanisms behind glucocorticoid hormone therapy resistance in asthmatic patients

The type, local concentration and pattern of the exposure of ligands can influence the activity of GRs.[12] Additional regulation can also influence their activity. These can include GR expression levels, GR dimerization, GR interactions with chaperones located in the cytoplasm, PTMs of GRs and/or their coregulators, GR translocation to the nucleus, GR binding with DNA and GR interactions with transcription and coregulators.[12] These mechanisms may all influence GC resistance. Various mechanisms for acquired resistance are known and thirteen of them are described in this chapter. On the GR level, for example, resistance can be caused by quantitative and qualitative defects, at the GC levels due to an increased metabolism or an increased elimination of GCs, or by the intervention of components downstream the death signalling pathway induced by GCs.[54] Three of these thirteen mechanisms involved in GC resistance are schematically depicted below (**figure 11**). All mechanisms are described in the following text.

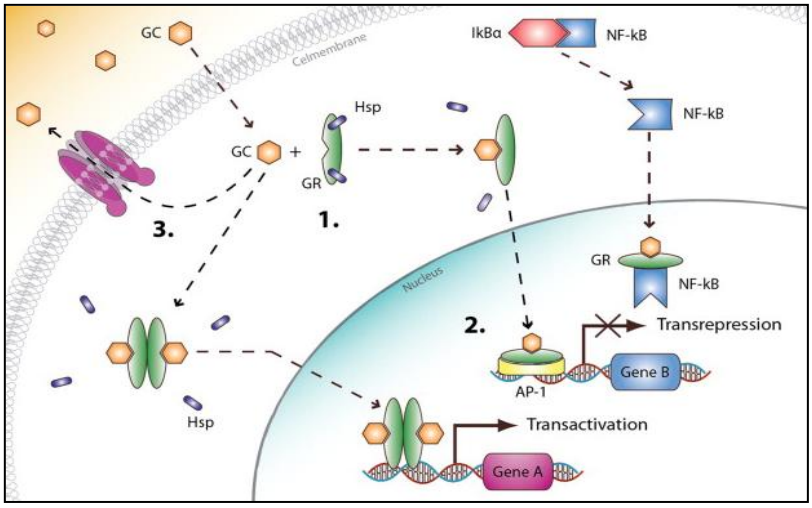


Figure 11. Three mechanisms involved in the development of glucocorticoid resistance. GRs can act via transactivation and transrepression. GC transactivation leads to metabolic side effects whereas GC transrepression leads to anti-inflammatory effects. This figure depicts the three main mechanisms that are currently known to develop GC resistance. These include the inhibition of GC action via the inability of GRβ to bind with GCs (**1**), the increased amount of inflammatory transcription factors (AP-1 and NFκB) that compete with the actions of GCs (**2**) and the activity of the P-glycoprotein transporter that can export GCs out of the cell cytoplasm (**3**).[22] In addition, JNK activated AP-1 and increased binding of GRβ to GREs can prevent the GR binding with its GRE and inhibit its binding with NF-κB.[14] *GC, glucocorticoid; GR, glucocorticoid receptor; hsp, heat shock proteins; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; AP-1, activator protein-1.* Figure obtained from Luijten,R.K. *et al.*, 2013.[22]

I. GC hormone therapy resistance due to DNA methylation

In vitro it is shown that hypomethylation[98] and GC demethylation of DNA around the GRE[99] can lead to activation of gene transcription of initially unexpressed genes.[98] In contrast, DNA *de novo* methylation that occurs spontaneously can lead to GC insensitivity by arresting the gene expression and can therefore achieve GC resistance.[98] Interestingly, it is demonstrated in animal studies that prenatally administration of synthetic GCs can lead to DNA methylation. Hereby, it can alter the gene expression of several genes and thereby influence the epigenetic state[100] and GC sensitivity of the cell. It is even described that DNA methylation can cause loss of GR expression.[101] Hereby, the ligand GC cannot bind its GR leading to GC insensitivity of cells.

II. GC hormone therapy resistance due to multiple mRNAs

Two homologous mRNA transcripts of the human GR (hGR) gene exist due to alternative splicing, hGRα and hGRβ, which differ only in exon 9.[60,102,103] They are both expressed in almost all cells and tissues in humans, although hGRβ usually in lower concentrations. hGRβ is primarily located in the cell nucleus, binds no GCs, is inactive in the transcription and inhibits transactivation of target genes mediated by hGRα. These characteristics of hGRβ can mediate the regulation of the sensitivity of cells and tissues towards GCs. Research indicated an increased hGRβ expression in patients with GC resistance. As a result, hGRα is less able to bind to GREs. This imbalance between the expression of hGRα and hGRβ induced by inflammatory cytokines[22,58] can be an underlying mechanism of the pathogenesis of conditions associated with GC resistance[22,58,60,104] In addition, it is currently described that IL-17 and IL-23 can enhance the production of hGRβ and thus enhance GC resistance.[105]

III. GC hormone therapy resistance due to multiple microRNAs

MicroRNAs (miRs) are small, noncoding RNAs that can cause GC hormone therapy resistance when they post transcriptionally bind to their specific target mRNAs via base pairing.[106] This way, they can inhibit or induce these mRNAs leading to an imbalance between pro- and anti-inflammatory genes. Lymphocytic leukemia cells that contain mutations in miR-128b have a reduced miR-128b expression. This miR normally restores the GC sensitivity by down-regulating proteins which contribute to GC insensitivity. A reduction in miR-128b will therefore result in GC insensitivity. However, the exact mechanism is not understood.[107] In the presence of over-expression of miR-130b in multiple myeloma, a decrease of the endogenous GR expression and GC response of the ubiquitous expressed GC-induced leucine zipper (GILZ) Tsc22d3-2 occurs. This GILZ is a downstream target of the GR[108] and identified as a GC-regulated gene with six GREs.[109] GILZ up-regulation may have a role in controlling the growth and death of immune cells and can block the actions of NF-κB and AP-1.[109] A decrease in GILZ expression may therefore lead to inhibition of apoptosis, induction of inflammation and irresponsiveness of cells for GCs and thereby causes hormone therapy resistance to GCs.[108] GC resistant cells in lymphoblastic malignancies appear to have an increased miRNA-182 expression. miR-182 targets forkhead transcription factor 3a (FOXO3A)[110] and when overexpressed, FOXO3A becomes reduced.[111] This reduction leads to a reduction in GC-induced apoptosis and a reduced Bcl2-interacting mediator (BIM) expression, a target of FOXO3A.[111] A reduced BIM expression leads to a reduction in the acetylation of H3 and therefore to a chromatin conformation that is not able to become transcribed.[112] So, GC-bound GR is not able to transcribe its target genes anymore, leading to GCs resistance of cells. In brain, miR-18 and miR-124a can reduce events mediated by GRs and thus induce GC hormone therapy resistance.[113] Furthermore, *in vitro* it is demonstrated that miR15b ~ 16 cluster overexpression causes an increase in GC sensitivity. In contrast, silencing of miR15b ~ 16 cluster causes a decreased GC sensitivity. This is probably due to changes in miRs and their related mitron expression. Mitrons are 22 nucleotide long intronic miRs which can control biological functions like apoptosis.[106] The same mechanism is most likely responsible for an increased GC-induced miR-223 expression in acute lymphoblastic leukemia patients.[106] Since miRs can target numerous genes inside one signalling pathway, it is also possible that these previously described miRs can contribute to GC resistance by targeting other factors.[108]

IV. GC hormone therapy resistance due to mutations in the GR gene

Mutations in the GR gene can cause GC resistance[54] by altering the sensitivity of tissues towards GCs.[60] In mice, for example, a deletion in the C-terminus is described that can even activate gene transcription without the presence of a ligand.[71] A similar point mutation in the C-terminus of the LBD of the human GR is recently found to cause generalized glucocorticoid resistance.[114] So far, 19 mutations have already been discovered which are associated with GC resistance (**figure 12**).

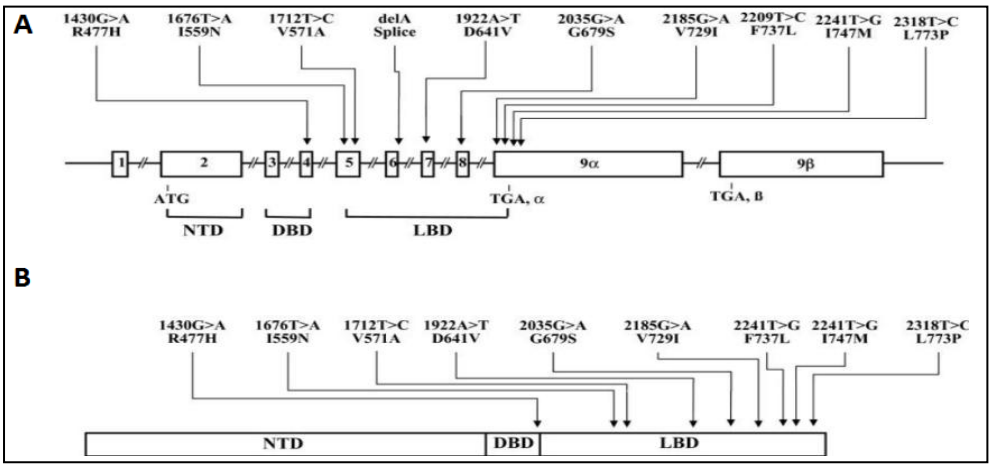


Figure 12. Diagram of the 19 known DNA mutations and polymorphisms in the human GR gene (A) and mutations in the protein (B) leading to GC resistance. *NTD, N-terminal transactivation domain; DBD, DNA binding domain; LBD, ligand binding domain.* Figure obtained from Charmandari,E. *et al.*, 2008.[60]

Mutations

Besides the mutations depicted in figure 12, several other mutations are already discovered. Mutations at the phosphorylated GR serine residue (S) S203,[77] S211,[77] S122, S150, S212, S220, S234, S315, S412 and threonine 159 to alanine in the GR, cause a reduction in the ligand dependent GR activity and thus also in its transcription activity.[7,115,116] Depending on the target gene, this can lead to pro- or anti-inflammatory responses by GRs[7] and can thus contribute to GC insensitivity. Furthermore, research performed on acute lymphoblastic leukemic cells resistant to GCs demonstrated a gene mutation that can occur in the HLH1 gene, which takes care of the mismatch repair (MMR) pathway after replication. This mutation causes an increased chance of developing other mutations, since the errors of DNA polymerase do not become corrected anymore. This can result in inactive cells[54] and thus in cells with insensitivity to GCs. It is also demonstrated that a partial or total deletion of the human GR gene can cause GR insensitivity.[117] In addition, mutations of chromosome 5 also play a role. Deletions or even a loss of the long chromosome arm 5q can cause GR loss as well as the replacement of a part of chromosome 5 with chromosome 15.[117]

Heritable mutations

Heritable mutations for the development of GC resistance also exist. Familial glucocorticoid deficiency (FGD) or Chrousos syndrome is a rare genetic condition leading to HPA hyperactivation and a partial but generalized tissue insensitivity for GC.[118] FGD is caused by a splice site deletion in the gene of the human GRs. It occurs only in one of the alleles encoding for the GR gene and leads to a 50% decrease in GR protein expression.[119] This deletion can be caused by mutations in the receptor for adrenocorticotroop hormone (ACTH) or Melanocortin Receptor Associated Protein (MRAP), mutations in the steroidogenic acute regulatory protein (STAR), mutations in the mini chromosome maintenance-deficient 4 homologue (MCM4), which is involved in DNA replication, and nicotinamide nucleotide transhydrogenase (NNT), which is involved in anti-oxidant defence. This can cause pathologies in organs. It can also be that the adrenal gland is hypersensitive to oxidative stresses.[120] In contrast, primary generalized glucocorticoid hypersensitivity (PGGH) shows target tissue hypersensitivity and HPA axis hypoactivation. Both FGD and PGGH are caused by mutations in the hGR gene by which the tissue sensitivity for GC is altered.[118,12]

GC hormone therapy resistance due to mutations in the DNA binding domain

Recently, a new point mutation has been discovered in the human DBD that causes GC hormone therapy resistance. Due to this V423A point mutation, in which valine (V) is substituted to alanine (A)

at position 423, the GR affinity for its GRE in target genes was decreased as well as the translocation of ligand dependent GRs to the nucleus. Since this point mutation affects various step in the cascade needed for GR action, it causes generalized GC resistance.[122]

GC hormone therapy resistance due to mutations in the hormone binding domain

The majority of mutations in the HBD of GRs greatly reduce or eliminate the steroid binding. The HBD is located in the C-terminus of the NR which forms a hydrophobic charge clamp pocket surrounding the ligand. Sequences responsible for chaperone hsp interaction, receptor dimerization and transcription activity are also apparent in the HBD. The HBD is thus involved in reducing the transcriptional activity of GRs, binding of GRs to hormones and determining the stimulation of GRs regulated by hormones.[72] Mutations and alterations in this HBD can therefore lead to weaker target gene activation and thus to GC resistance.[123,124] A HBD mutation at V641 causes an impaired binding between the GR and its ligand, leading to GC resistance.[123] This is also seen for a HBD substitution mutation of valine to amino acid 729[125] and for deletions in this HBD.[124] In contrast, also point mutations in the HBD are described that can increase the affinity of the GCs to GRs leading to increased target gene activation.[126,127]

Polymorphisms

Finally, also various polymorphisms are known to influence the hormone therapy resistance for GC. These polymorphisms include gene deletions, mutations in the 5' flanking region of the promoter, a four base pair deletion at exon/intron 6 splice site, mutations in the 3' untranslated region of GRβ and mutation in nucleotide 1221 in N363S.[70]

V. GC hormone therapy resistance due to orphan nuclear receptors

Homologous down-regulation is known to cause GC resistance. Hereby, GCs cause reduced GRs levels via a ligand-dependent receptor-mediated mechanism and thereby limit the responsiveness of cells to GCs.[132] Besides homologous down-regulation, one *in vivo* study demonstrated that also ONRs can contribute to GC insensitivity. In this study, ER-related 1 and 2 could repress the GR gene transcription. In the absence of GR, no promoter activity was notable of the ONRs.[133] This indicates that this ONR is receptor and also cell specific[133] and can lead to GC insensitivity of cells.

VI. GC hormone therapy resistance due to proteasome activity

When there is need for ATP in a degradation process, proteasome activity is increased.[79] Since GRs become degradated in an ATP-dependent fashion,[134] it is suggested that ubiquitin-dependent pathways of proteolysis are involved in degradation of GRs.[79,134] This degradation reduces the transcription signalling by GCs and thus may lead to GC resistance.[79] Studies already demonstrated blocking of induced degradation by inhibition of proteasomes.[79,135-137] Inhibition of proteasomes increases the GC hormone responsiveness and will therefore lead to an increased transcription activity of GRs.[79] It is further demonstrated that when proteasomes become inhibited, GRs with a higher molecular weight are present. This indicates ubiquitinated GRs.[79] A mutation of lysine 426 (K426) in a PEST domain is also demonstrated to block the ligand dependent GR down-regulation and enhances the GR transcription activity.[79,138] Therefore, K426 can be used as a proteasomal inhibitor which leads to a recurrent activation of GRs. Overall, the literature implicates the existence of a mechanism that uses proteasome pathway induced protein degradation to down-regulate the ligand-dependent GR signalling pathway.[79] This mechanism can be used to limit or increase the GC responses and is most likely involved in the turnover of the GRs.[139]

VII. GC hormone therapy resistance due to PTMs of glucocorticoid receptors

For GRs to function in the correct manner, PTMs are important as described earlier. However, when these PTMs function inappropriately, they can influence the target gene transcription activity of GRs. This will lead to no or a different response of cells towards GCs and thus GC resistance. PTMs of GR are described in table 1.

Table 1. Currently described PTMs of GRs known to influence GC hormone therapy resistance

PTM	***Mechanisms***
Acetylation [7,140,141]	Via the two ligand-dependent acetylation sites, lysine 494 and 495 GRs can be acetylated. Acetylation of lysine 494 and 495 of GRs causes loss of affinity of GRs towards NF-κB and GRs cannot repress the NF-κB induced inflammatory gene transcription which increases GC insensitivity.[7] In contrast, absence of acetylation of ligand- dependent GRs can repress the NF-κB inflammatory gene transcription by binding to NF-κB.[7]
	Administration of HDAC2 leads to a reversible affinity between GRs and NF-κB. However, HDAC2 can also be silenced by RNA interference which results in a reduced GC sensitivity. In contrast, overexpression of HDAC2 in GC resistant alveolar macrophages from chronic obstructive pulmonary disease (COPD) patients caused an increased in GC sensitivity.[140,142] However, in the absence of HDAC2, GR hyperacetylation may lead to GC insensitivity.[7]
Phosphorylation [143,79]	In asthmatic patients who are resistance to GCs, the p38 MAPK is expressed and active in alveolar macrophages.[7,144] P38 MAPK can phosphorylate a serine residue in the GR leading to a reduction in the response to GCs.[145] However, the phosphorylated serine residue still has to be discovered. Contenders are the serine residues (S) S211[77,146] or S226 (as reviewed by Anbalagan,M. *et al.*).[7,77] Administer of p38 MAPK inhibitors together with GCs increased the anti-inflammatory and beneficial effects of GC in these asthmatic patients.[147]
	A lot of asthmatic patients with GC resistance are a not able to fully translocate the GR to the nucleus to bind GREs in peripheral blood mononuclear cells (PBMCs) after GC administration. This might be due to changes in the phosphorylation of the GR.[7,148,149]
S-nitrosylation[14]	GR tyrosine residues can be S-nitrosylated by nitric oxide (NO)[14] as well as cysteine residues and -SH groups on GRs. S-nitrosylation of GRs reduces the GR affinity for GCs and thus also reduces the responsiveness of cells towards GCs.[150] Since inflammatory diseases often have an increased inducible nitric oxide synthase (iNOS) expression, this can lead to large NO amounts and thus to GC resistance.[14] Furthermore, the S-nitrosylation of GR cysteine residues appears to be (almost) irreversible since the removal of *S*-nitrosothiol does not reverse the binding between GRs and their GC ligands.[150]
Sumoylation[151]	Sumoylation of GRs by the small ubiquitin-related modifier-1 (SUMO-1) peptide can take place at two possible sumoylation sites (KxE, in which X can be any amino acid[152]) in the N-terminus[151,153] and at one in the LBD domain of the GRs.[153] The function of SUMO-1 depends on the promoter context,[154] whereas the amount of SUMO-1 conjugation depends on environmental factors. This way, SUMO-1 can also regulate a subgroup of genes responsive to GCs.[151] Several proteins, such as UBc9[151,153,155] and coactivator protein inhibitor of activated STAT (PIAS),[156,157] can catalyze GR symoylation[152] and can bind with SUMO-1. However, the exact sumoylation mechanisms, where these occur and how these contribute to the GC response is still needs to be clarified.[151]
	Overexpression of SUMO-1 in the presence of ligand-bound GRs leads to an enhanced transcription activity of GRs. However, GRs will become instable and a dramatic reduction of GRs occurs. This hyperactivation requires several GREs in the target gene promoter region and synergy of various GR molecules. The action of SUMO-1 increases when the number of DNA binding sites increases in the promoter regions[151] leading to resistance.
Ubiquitination[79]	Ubiquitination of GRs leads to GR proteasome degradation.[14] Phosphorylation plays a major role in GR ubiquitination. Phosphorylation sites in PEST domains are often surrounded by a lysine residue that can be ubiquitinated. Studies performed on these residues suggest that K426 is important in the hormone dependent GR regulation and responsiveness.[79,158] However, a mutation in this residue still leads to ubiquitination and a more powerful GR. This suggests the presence of other (unidentified) ubiquitination sites on GRs or a difference in the overall ubiquitination levels for GRs.[79,158]

VIII. GC hormone therapy resistance due to the immune system

T-helper 2 cells (Th2) play an important role in the inflammatory processes seen in asthma.[1,159] Other cytokines involved in this pathway are IL-4 and IL-13 that induce IgE, IL-5 that induces tissue damage and eosinophilic inflammation and IL-13 that causes Th2 activation.[1] In addition, also Th17 and IL-17 are found to be involved in this asthma induced inflammation since they activate transcription factors and induce neutrophilic inflammation.[1,159] GCs are administered to down-regulate mRNA of these cytokines and to up-regulate inflammatory suppressor cytokines. An increased IL-10 expression for example inhibits the T-cell response towards GCs. However, asthmatic patients fail to synthesize a sufficient amount of IL-10, leading to an increased expression of pro-inflammatory T-cells.[1,159] Furthermore, in asthmatic patients an increased expression of IL-2, IL-4 and IL-13 is found in inflammatory cells leading to a reduced GR sensitivity and thus function.[1,149,160,161] The underlying mechanisms behind IL-2 and IL-4 overexpression might be phosphorylation by p38 MAPK kinases.[162] MAPKs represent a family of signalling proteins[21] which are activated by IL-2, IL-4, IL-13 or macrophage inhibitory factor (MIF), by pro-inflammatory cytokine activated c-Jun N-terminal kinase (JNK) or by extracellular signal-regulated kinase (ERK) that is activated by microbial super antigens.[14] MAPKs can activate intracellular transduction pathways due to extracellular influences. Activation occurs by the phosphorylation of a variety of substrates by which apoptosis, proliferation and development can be regulated. When this activation does not occur in a proper way, this can contribute to the expression of the pro-inflammatory cascade. The anti-inflammatory response occurs due to a negative intervention of the GCs with the MAPK pathways.[21] In addition, tumour necrosis factor α (TNFα) can induce the IL-13 expression.

Besides Th2, also Th1 is involved in resistance via Interferon-ɣ/Toll like receptor-4-Myeloid differentiation factor 88 (INF-ɣ/TLR-4-MyD88) dependent mechanisms when the innate immune system is primed. Furthermore, the ratio Th2 and Th1 is involved.[1,159,160,163] Resistance occurs when the production of the pro-inflammatory cytokines cannot be repressed and the anti-inflammatory cytokines cannot be induced.[163] Also AP-1, NF-κB and kinase pathways upstream the gene transcription are increased activated in patients with GC hormone therapy resistance.[1,163,164] They enhance the activation of pro-inflammatory genes and thereby contribute to resistance.[1,163,164]

IX. GC hormone therapy resistance due to excessive AP-1 activation

AP-1 can be activated in the presence of pro-inflammatory cytokines, for example by TNFα, via the JNK pathway. In a situation of excessive AP-1 activation, GC resistance can occur. AP-1 can bind the GR and thereby prevent the interactions of this receptor with its GREs and its other transcription factors. Studies found increased JNK activation in PBMCs and lung biopsy samples from GC resistant asthmatic patients. After administration of GCs, no decrease in the JNK activity was found. This might be an explanation of why an increased inflammation in inflammatory diseases can result in secondary GC resistance. Furthermore, increased expression of c-Jun depolymerises the cytoskeleton and may therefore reduce the GR transactivation activity.[14]

X. GC hormone therapy resistance due to non-GR molecules and coregulatory proteins

IL-10

Regulatory T-cells secrete IL-10 in response to GCs[14] and to protect for development of inflammatory diseases such as asthma.[165] One study demonstrated that some asthmatic patients resistant for GC, have T-helper cells that are not capable of IL-10 secretion *in vitro*. However, administration of vitamin D3, resulted again in increased IL-10 levels to levels seen in GC sensitive patients. After treating asthmatic patients with vitamin D, the response of T-cells towards IL-10 restored again leading to inhibition of pro-inflammatory cells as described above. This indicates that vitamin D may serve as a possible therapy and that low levels of vitamin D, due to intake or lack of sunlight, may contribute to GC irresponsiveness in inflammatory diseases. [14,166]

Macrophage migration inhibitory factor

Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine with anti-GC effects that can lead to GC resistance when its over-expressed.[167] It is induced by GCs and can inhibit the anti-inflammatory effects via inhibition of mitogen-activated protein kinase phosphatase-1 (MKP1) induction.[168-170] Increased MIF expression is found in several GC resistant patients with different diseases such as rheumatoid arthritis and asthma.[167] Therefore, anti-MIF therapy may serve as a potential treatment in various disease related to GC resistance.[14]

P-glycoprotein

The efflux pump P-glycoprotein 170 is an ATP-binding cassette transporter member. It transports, amongst others, GCs out of cells. If this pump does not function sufficiently due to polymorphisms of the multidrug resistance 1 (MDR1) gene, it can contribute to GC resistance in inflammatory diseases.[14,128-130] It can excessively pump GCs out of cell, and thus administer of GCs does not make sense. In contrast, professor Dr. Bob Pinedo stated that the effect of the P- glycoprotein is negligible and that other approaches leading to resistance have a higher priority (personal communication with professor Dr. Bob Pinedo).[131]

Vitamin B6

Studies implied that GC hormone action can be modulated by the physiological role of PLP.[93,171] Vitamin B can, in high concentrations, decrease the transcription activation of GRs. This is a results of GR isoelectric point alterations, changes in the binding capacity of GCs and GREs and changes in properties of the GC-dependent gene transcription activation. Deficiency of vitamin B can, in contrast, enhance the GC responsiveness and thus increase transcription activation.[172] Furthermore, these modulator effects of vitamin B are not restricted nor limited to specific cell types and the human form of GRs.[171]

Calreticulin

Calreticulin is a ubiquitously expressed multifunctional protein. It is a calcium-binding (storage) protein primarily expressed in the endoplasmic reticulum. Since it is also apparent in the nucleus, it may influence the transcription regulation. Calreticulin can bind the KXGFFKR peptide (in which X can be either G, A or V).[173] This synthetic peptide is almost indistinguishable with a certain amino acid sequence located in the DBD of the NR superfamily. The N-terminus of calreticulin can therefore also interact with the GR DBD and thereby prevent the GC-bound GR to bind with its GRE.[174] Calreticulin therefore inhibits the target gene transcription by GC-bound GRs,[173,175] and thus may lead to GC resistance of the cell.

HATs and HDACs

HATs and HDACs play a major role in gene expression induced by ligand dependent GRs. In asthmatic patients, an increase in HAT and a decrease in HDAC are apparent.[142,176] This causes enhancement of pro-inflammatory gene transcription. Interestingly, in the absence of HDAC6, GRs are not able to mature in a proper way[177,178] whereby GCs are not able to bind. These processes consequently lead to GC insensitivity.

XI. GC hormone therapy resistance due to glucocorticoid receptor transrepression by coactivator competition

Transrepression of the inflammatory responses can contributes to GR resistance. NF-κB plays a major role in this mechanism. Whether or not cofactor interferon regulatory factor 3 (IRF3) is used for NF-κB dependent activation, depends on special sequences in κB sites.[179] P65 in NF-κB is needed as coactivator for IRF3 genes upon toll-like receptor 3 (TLR3) activation, not upon TLR4 activation. IRF3 and p65 can interact with each other, but when the GR binds to p65 this interaction is blocked.[45,180] Thus, GRs repress NF-κB genes in which IRF3 is present as cofactor and repress IRF3 genes activated by TLR4 in which p65 is present as cofactor. However, GC resistance for IRF3 genes occurs when TLR3 becomes activated because of the absence of coactivator p65 for IRF3.[45,181]

Also IL-8 is involved in transrepression. It is known that NF-κB activation inhibitor (NFKBAI) and IL-8 transcription are induced by TNFα while GR can only repress IL-8. Therefore, NF-κB requires positive transcription elongation factor (P-TEF) as coactivator for the induction of IL-8. When the interaction between NF-κB and P-TEFb is disrupted on the IL-8 promoter, the activation of TNFα is blocked.[182] Interestingly, in this case P-TEFb phosphorylates the RNA polymerase II carboxyl terminal domain and thus acts as a PTM.[45] Hereby it can influence and/or alter the functions of GC bound GRs and may induce insensitivity towards GCs.

Another GR-coactivator interaction that leads to transrepression is GRIP1. GRIP1 is a member of the p160/SRC family and can act both as corepressor and as coactivator. When GRs become activated, they can interact with the AP-1 element leading to recruitment of GRIP1. GRIP1 functions in this cascade as corepressor for GRs.[45,183] This characteristic depends on an repression domain that is specific for GRIP1. Other members of the p160/SRC family do not contain this intrinsic domain. Due to this domain, GRIP1 can enhance GR transrepression of gene expression that is NF-κB dependent. In addition, GRIP1 and SRC-1 can also induce the GR repression of plasminogen activator inhibitor-1 (PAI-1) expression which is induced by transforming growth factor beta (TGFβ).[45]

Also an enzyme required for the vitamin D production, 25(OH)D3 1_-hydroxylase gene (1_(OH)ase), can cause transrepression. This key enzyme can undergo transrepression via the vitamin D receptor (VDR). For the protein kinase A (PKA) dependent phosphorylation of VDRs, coactivator complexes of p300 are recruited. These have acetylation functions on histones and can set a repression function for the promoter. When active vitamin D binds to VDR, NCoR/SMRT/HDAC-complexes appear to the promoter of 1_(OH)ase and cause deacetylation of nucleosomes surrounding the promoter. This causes gene repression.[45]

XII. GC hormone therapy resistance due to histone modification by coregulators

Gcn5 is a histone lysine acetyltransferase that catalyses coactivator HAT complexes important for the activation of several genes. When Gcn5 acetylates histones, a positive interaction occurs in which the SWI/SNF-complex remains. However, the Snf2 unit itself can also be acetylated by Gcn5. When this occurs, the SWI/SNF-complex becomes dissociated from acetylated histones which cause a reduction in its connection with promoters. This leads to a reduction in gene expression and thus causes hormone therapy resistance.[184]

Protein arginine methyltransferase 1 (PRMT1) is a H4 specific histone methyltransferase that can methylate arginine 3 of H4 both *in vitro* and *in vivo*. Due to this methylation, acetylation by p300 can occur of the H4 tails.[185-187] In contrast, this acetylation of H4 leads to inhibition PRMT1 methylation of H4 by competing with methylation and thus a repressed gene transcription.[185,187] In addition, when a mutation appears in the PRMT1 binding site S-adenosyl-L-methionine, no methylation can take place and thus the GR coactivator activity becomes repressed. This indicates that methylation of arginine 3 by PRMT1 is important in the gene transcription regulation.[185] It can contribute to GC hormone therapy resistance in that it can repress gene transcription in absence or presence of ligand bound GRs.

GRIP1 is a coactivator that belongs to the p160-family and allows CARM1 to interact with promoters.[188] S736 in p160 is needed for induction of the transcriptional activation of GRP1.[189] CARM1 can induce methylation of histon H3R17 and H3R26 and acetylation of H3R17 and H3K18 due to its activation by these GR-regulated promoters. Binding of CARM1 towards promoters is due to the three P-160 coactivators SRC1 (a HAT that can recruit coregulators like CBP/P-300[12]), SRC2/GRIP1/TIF2 and SRC3/pCIP/AIB1/ACTR/RAC3. Subsequently, these bind to the DNA bound GRs and this mechanism influences the GR gene transcription activation. *In vitro*, inorganic arsenics (iAs) represses chromatin remodelling mediated by GRs and represses transcription initiation as well as both histone methylation and acetylation. It is found that CARM1 and GRIP1 are targets of iAs. Interestingly, CARM1 appears to be absent whereas GRIP1 appears to be present in the promoter after iAs treatment. Furthermore, CARM1 over-expression can restore the gene transcription that was inhibited by iAs.[188] iAs can further enhance or inhibit signalling pathways such as p38 MAPK (by which p160 can be phosphorylated[189]), ERK1/2, c-Jun terminal and stress activated kinases (JNK/SAPKs) and MAPKS. All of these can modify CARM1 or GRIP1 post transcriptional.[188] iAs can therefore also have an indirect effect on CARM1 and GRIP1 in that can repress the gene transcription activation due to these inappropriate PTMs. It is suggested that TIF1a/Trim24 can restore the roles of CARM1 and GRIP1 by stabilizing their interaction, making it a candidate target for iAs.[188]

XIII. GC hormone therapy resistance due to PTM of coregulatory proteins

Coregulators of GRs are important for a proper gene expression. It is demonstrated that coregulators can be derived from amino acids via PTMs. Tyrosine residues play an import part in this process.[190] Studies conducted also showed that PTMs of coregulators themselves can influence the function and transcriptional effects of GRs and other coregulators due to changes in the conformation[191-196] and may lead to hormone therapy resistance. PTMs of coactivators can induce the activities of coregulator enzymes by acetylation,[197] induce the recruitment of other coregulators[198] or inhibit complexes of coregulators.[199] Coactivators and corepressors therefore might serve as controllers for a proper cell response.[50] PTMs of several coregulators are currently known to influence resistance and are described in table 2.

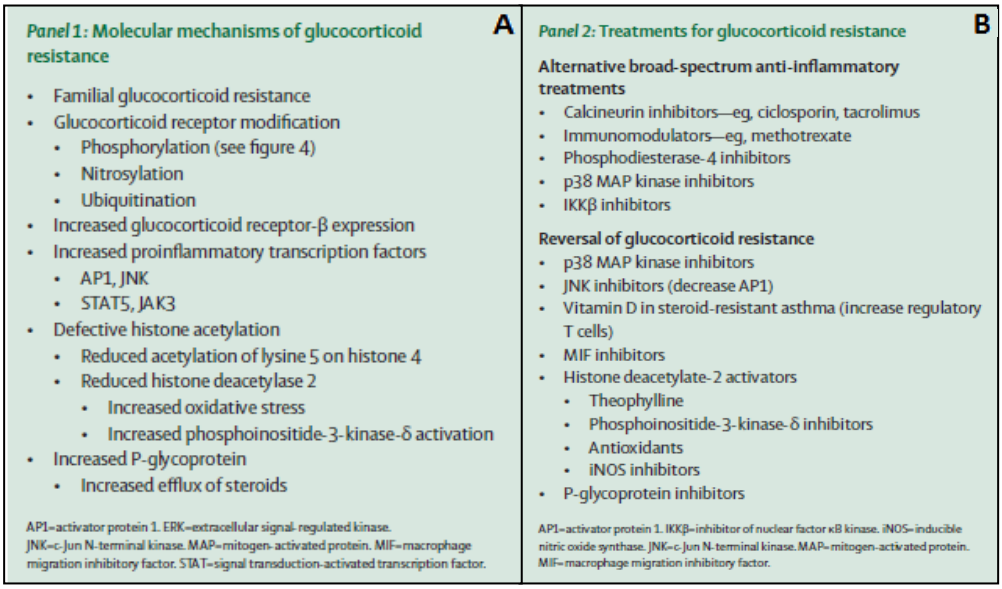
Table 2. Currently described PTMs of coregulators known to influence GC hormone therapy resistance

Coregulator	***PTM***	***Mechanism***
HDAC 1 and 2	Phosphorylation [200,201]	HDAC 1 and 2 interactions with corepressors will become disrupted,[202-205] leading to an increase in inflammatory gene expression and thus GC hormone therapy resistance.
HDAC 2	Phosphorylation, Ubiquitination	Phosphorylation and ubiquitination by cigarette smoke in asthma and COPD patients causes a degradation of HDAC 2.[164,206] leading to a decrease in inflammatory gene expression and thus GC hormone therapy resistance.
HDAC 4	Phosphorylation	Phosphorylation by extracellular signal-regulated kinase 1 (ERK1) and ERK2 causes an accumulation of HDAC 4 in the nucleus leading to a decrease in inflammatory gene expression and thus GC hormone therapy resistance.[204]
HDAC 4 and 5	Phosphorylation [200,201]	Calmodulin-dependent kinase (CDK) phosphorylation leads to differences in the composition of these proteins[205,207] causing a decrease in the expression of their target genes,[200,201,205,208] leading to a decrease in inflammatory gene expression and thus GC hormone therapy resistance.
RIIP140 (corepressor)	Phosphorylation	Phosphorylation at tyrosine residues Thr202 and Thr207 of the RIP140, represses RIP140 and leads to a strong interaction between RIP140 and HDAC.[92] This complex is resistant to the MAPK inhibitor,[92] leading to an increase of inflammatory signals and thus resistance of GCs.
		When PLP conjugates with the RIP140 modification site to K613, transcriptional corepression occurs. This might be due to an increased interaction between RIP140 and HDAC, leading to a nuclear decrease in coactivator RIP140[93] and thus GC resistance.
SMRT (corepressor)	Phosphorylation [209,210]	Absence of phosphorylation by IKKα causes chromatin bound SMRT. Therefore, NF-κB will not be transcribed and recruited to target gene promoters. This will lead to transcription blocking and cells will be sensitized to go in apoptosis.[211] SMRT phosphorylation is further associated with the inhibition of corepressor function[212] and thus enhancement of gene transcription, leading to resistance.
HSP 90 (chaperone protein)	Acetylation [213,214]	Acetylation (regulated by HDAC6 and SIRT2 (a NAD-dependent deacetylase[215])) leads to inhibition of the hsp90 interaction with GRs.[177,216] Hereby hsp90 cannot bind GRs leading to a reduction in the transcription activity of GRs.[217]
	Ubiquitination	GR levels decline when the interaction between GRs and hsps is disrupted. ATP is acquired for this degradation of the GR by hsp which suggest that ubiquitin-dependent pathways of proteolysis are involved.[79,135,218]
Snf2 bromodomain (subunit of the SWI/SNF-complex) [219,220]	Acetylation	Due to Gcn5 acetylation, the SWI/SNF remodelling complex becomes dissociated from acetylated histones which cause a reduction in its connection with promoters. This leads to a reduction in gene expression and thus causes hormone therapy resistance.[184]
Brahma (factor of the SWI/SNF complex, target gene and coregulator of GRs that remodel chromatin) [221]	Acetylation [222]	Acetylation is needed to coordinately increase the gene transcription by RNA polymerase II. In presence of acetylation, brahma is inhibited. Without acetylation and in presence of HDACs, the brahma transcriptional activity and growth inhibitory function increase.[222] These mechanisms may lead to GC hormone therapy resistance since the cell response towards GCs is not sufficient anymore.

NF-κB (transcriptional activator that is redox-sensitive)	Acetylation	P300/CBP (a HAT[12,223]) can acetylate the p53 subunit of NF-κB at K221 leading to a bigger affinity of NF-κB to DNA and thus an increased gene expression.[224,225] Deacetylation at NF-κB K221 leads to a decreased gene expression.[226]
		Acetylation of K310[227] and mutations or alterations in acetylation sites K315 and K315 of NF-κB enhances its transcriptional activity, leading to enhanced expression of inflammatory genes.[228]
	S-nitrosylation	Inhibition of NF-κB can be induced by S-nitrosylation of an important thiol in its p50 subunit that can interact with DNA. The level of S-nitrosylated of p50 is modulated by TNFα. Cytokine-activated nitric oxide synthase (NOS) is also involved in NF-κB inhibition. Due to these inhibitions, NF-κB cannot bind DNA anymore leading to a reduction of the expression of pro-inflammatory genes. These mechanisms can also be reversed by denitrosylating agents, such as dithiothreitol.[229]
	Phosphorylation	Phosphorylation of NF-κB and p65-p65 and p50-p65 by PKA.[230] leads to enhanced expression of inflammatory genes.[228]
P300/CBP (HAT)	Methylation	Methylation occurs by coactivator phosphorylation and coactivator-associated arginine methyltransferase 1 (CARM1).[231] Due to an arginine in the CARM1 binding region steroid receptor coactivator 3 (SRC-3), SRC-3 is regulated by dependent methylation. The mechanisms are not clearly understood.[232]
C/EBP (transcription factor)	Acetylation	Acetylation can be mediated by CBP/p300[233] and by GCN5. This can repress the interaction between HDAC1 and C/EBPβ and can decrease the C/EBPβ affinity for mSin3a, a corepressor.[233,234] Due to dysfunctioning of C/EBPs in GC presence and thus unintended enhanced or repressed gene transcription this can lead to resistance.
	Phosphorylation	Phosphorylation increases the C/EBP function leading to enhances transcription. Studies found that C/EBP-delta and C/EBP-beta are induced by inflammatory signals.[235]
	Sumoylation	Sumoylation represses the C/EBP function. For example, the inhibition of the interaction between C/EBPα and SWI/SNF by lysine sumoylation leading to a decrease in gene expression by GRs.[233]

Discussion

Literature demonstrates that long term treatment of asthmatic patients with GCs can lead to a down-regulation of GRs and thus a resistance against GCs.[79,236] However, there is still a lot that needs be clarified about the cellular and molecular mechanisms involved in the development of GC hormone therapy resistance. This is important for the prognosis and prediction of GC resistance in patients and for better treatment options preventing the development of resistance.[6] This review described various factors and agents that are currently known to be responsible for the development of hormone therapy resistance of GCs. As a consequence, these factors influence the GC-regulated target gene expressions. Investigation of new mechanisms involved in resistance is still ongoing. The data of currently known mechanisms and treatments is summarized in **figure 13** and based on *in vitro* cell line models and *in vivo* and clinical studies. Mechanisms involved in GC hormone therapy resistance include multiple mRNAs and miRNAs, mutations in the GR gene itself, its DNA binding domain and ligand binding domains, the immune system, mechanisms involved in coregulator functioning such as PTMs and transrepression and PTMs of GRs themselves. Especially the PTMs like acetylation, phosphorylation, S-nitrosylation, sumoylation and ubiquitination play a role. With these PTMs, also different pathways, transcription factors and proteins (such as kinases) are involved that control the signal transduction and transcription in a positive or negative manner. The relation between all these described mechanisms is important to elucidate the mechanisms behind the development of GC hormone therapy resistance. This is complicated, since GRs function via at least three different mechanisms that can contain different targets.[12] The first mechanism is non-genomic signalling via classical receptors on the cell membrane or in the cytoplasm. The second transcriptional signalling of GRs involves interaction with other transcription factors. Thirdly, it is known that anti-inflammatory actions of GRs partly depend on the binding of a GR with its GRE in presence of coregulators.[12] So far, the questions how resistance towards GC hormone therapy occurs and the interplay of the mechanisms involved, still remain unsolved with the currently available data. Therefore, a new hypothesis is conceived based on the possibility that PTMs of coregulators contribute to the development of GC hormone therapy resistance in asthmatic patients.



A

Panel 1: **Molecular mechanisms of glucocorticoid resistance**

- Familial glucocorticoid resistance
- Glucocorticoid receptor modification
  - Phosphorylation (see figure 4)
  - Nitrosylation
  - Ubiquitination
- Increased glucocorticoid receptor-β expression
- Increased proinflammatory transcription factors
  - AP1, JNK
  - STAT5, JAK3
- Defective histone acetylation
  - Reduced acetylation of lysine 5 on histone 4
  - Reduced histone deacetylase 2
    - Increased oxidative stress
    - Increased phosphoinositide-3-kinase-δ activation
- Increased P-glycoprotein
  - Increased efflux of steroids

AP1=activator protein 1. ERK=extracellular signal-regulated kinase. JNK=c-Jun N-terminal kinase. MAP=mitogen-activated protein. MIF=macrophage migration inhibitory factor. STAT=signal transduction-activated transcription factor.

B

Panel 2: **Treatments for glucocorticoid resistance**

Alternative broad-spectrum anti-inflammatory treatments

- Calcineurin inhibitors—eg, ciclosporin, tacrolimus
- Immunomodulators—eg, methotrexate
- Phosphodiesterase-4 inhibitors
- p38 MAP kinase inhibitors
- IKKβ inhibitors

Reversal of glucocorticoid resistance

- p38 MAP kinase inhibitors
- JNK inhibitors (decrease AP1)
- Vitamin D in steroid-resistant asthma (increase regulatory T cells)
- MIF inhibitors
- Histone deacetylate-2 activators
  - Theophylline
  - Phosphoinositide-3-kinase-δ inhibitors
  - Antioxidants
  - iNOS inhibitors
- P-glycoprotein inhibitors

AP1=activator protein 1. IKKβ=inhibitor of nuclear factor κB kinase. iNOS=inducible nitric oxide synthase. JNK=c-Jun N-terminal kinase. MAP=mitogen-activated protein. MIF=macrophage migration inhibitory factor.

Figure 13. Molecular mechanisms (A) and treatments (B) of patients harbouring glucocorticoid resistance. Figure obtained from Barnes,P.J. *et al.*, 2009.[14]

Posttranslational modifications of coregulators: hypothesis for glucocorticoid hormone therapy resistance

Studies demonstrated that both PTMs of coregulators and PTMs of GRs themselves influence the GR-controlled gene transcription.[146,191-196] PTMs largely influence the function of coregulators, leading to changes in the GR gene transcription. Coregulators have a major influence on the cell responsiveness and thus sensitivity towards GCs. Alterations or mutations of these coregulators can therefore cause severe problems in patients,[54,92,93,184,212,217] such as GC hormone therapy resistance and its associated conditions such as SLE.[14,22,57] Coregulators influence the gene transcription of pro- and anti-inflammatory cells by interaction of their NCoRNR- or NR-boxes with the LBD of the GR.[111,213] It is described that coregulators are widely post translational modified to perform their actions.[92,191-196] These PTMs include acetylation, methylation, phosphorylation, s-nitrosylation, sumoylation and ubiquitination. Furthermore, also addition or removal of PTMs on amino acids in the NCoRNR- or NR-boxes of coregulators can occur, for example due to mutations or environmental factors, that can alter their initial functions as described in this thesis. Coregulators can be repressed or enhanced by PTMs, leading to variations in the levels of coregulator expression. It can be hypothesised that this may be the missing link of developing GC hormone therapy resistance. Because the coregulator functions lie in the amplification of gene expression, even minor differences can have major effects in the development of organ systems,[50] hormone therapy resistance and so on. In addition, the ratio between coactivators and corepressors expressed in cells, and the influence by PTMs, might also be an interesting topic of research towards understanding GC hormone therapy resistance in asthmatic patients. Differences in expression or activity of these coregulators can alter the activity of the basal transcription machinery towards drugs.[4] In addition, it is known that asthma follows a circadian rhythm, in that it is worst during the night and early morning.[237] Also circulating steroids levels have fluctuations during day and night time due to a 24 hour circadian rhythm (personal communication with professor Dr. Bob Pinedo). It has even been demonstrated that GCs can interact with circadian rhythms.[238] PTMs of coregulators and proteins are known to influence the circadian rhythms[239,240,241] of steroids levels in blood. Hereby, they can lead to differences in gene expression of, for example, pro- and anti-inflammatory genes.[239] This can eventually contribute and lead to resistance and even morbidity of human beings.[239,242]

Translational research: cellular and molecular mechanisms involved in glucocorticoid hormone therapy resistance

This exploratory review indicates that PTMs of GRs and especially PTMs of coregulators are important mediators for the development of GC hormone therapy resistance.[191-196] These mechanisms on cellular and molecular level are expected to be of clinical importance. The knowledge so far may lead to a combination therapy of GCs together with an agent to prevent insensitivity and thus resistance towards GCs. The BOLERO (breast cancer trials of oral everolimus) trial in breast cancer is an example of a study which already demonstrated that combination therapy results in resensitization for hormone therapy.[243,244] The mammalian target of rapamycin (mTOR) pathway plays an important role in the development of hormone resistance in patients with ER-positive and human epidermal growth factor receptor 2 (HER2) negative breast cancer by phosphorylating the ER, which is correlated to resistance.[243] The hypothesis is that preventing this phosphorylation, by inhibiting the mTOR pathway, also prevents the development of resistance. In the BOLERO trial, a group of breast cancer patients was divided in two groups. One group was administered everolimus,[244] an inhibitor of the mTOR pathway[244] that acts by dissociating the Raptor coregulator from mTOR,[245] together with exemestane, an inhibitor of steroidal aromatase such as anastrozole. The other group was administered a placebo together with exemstane. Treatment with exemestane plus everolimus resulted in a better quality of life and a decrease in deterioration.[244]

In addition, it is recently demonstrated that GRs and androgen receptors can bind the same responsive elements on target genes. As a consequence, patients with prostate cancer treated with androgens can develop androgen resistance due to GR-upregulation.[246] Therefore, androgen resistance could also be treated with GC-therapy. These findings suggests that a combination therapy can be used as novel therapy and could be an important approach to develop novel treatments for GC hormone therapy resistant patients. In this case, according to my hypothesis, this combination therapy would consist of NRs together with the blocking or enhancement of PTMs of coregulators.

Directions for future research

Despite the provided evidence of mechanisms involved in the development of GC hormone therapy resistance, still a lot more knowledge must be gained about the mechanism involved. The BOLERO trial and several other breast cancer studies already demonstrated the importance of coregulators (such as ERα coregulators,[247] proline-, glutamic acid-, and leucine-rich protein 1 (PELP1),[248] albumin-1 (AlB-1),[249,250] NCOR1, Human Epidermal growth factor Receptor 2 (ERBB2/HER2), SRC-1 and suppressor of gal 1 (SUG1) (as reviewed by Girault,I. *et al*., 2006[250]) in endocrine therapy. Studies further demonstrated that viruses (the papillomavirus and adenovirus) capture coregulators when infecting cells,[197,251] by mimicking phosphorylation and acetyltransferase activity.[197] This gives them the control over basal transcription machinery components of the cell.[50] In addition, also genetic diseases are known which can influence the actions of coregulators.[252] They can do this via, amongst others, influencing the histone acetyltransferase activity[253] and thereby contributing to the development of hormone therapy resistance. It is therefore interesting to examine the influences on and presence of PTMs on GR specific coregulators in relation to GC hormone therapy resistance development.

So far, *in vivo* cell line models and a few mouse models have been used to investigate this phenomenon. It is also demonstrated that patients with complete GC resistance can be identified by administration of oral GC or via one single injection of triamcinolone acetonide (a depot GC).[14] In addition, chromatin immunoprecipitation (ChIP) is often used. ChIP can be used to investigate NRs and coregulator recruitment to promoters in their target genes. When cross-linking DNA and proteins using formaldehyde, chromatin undergoes enzymatic digestion or sonication to finally become precipitated with antibodies. After reversing the cross-links, DNA can be amplified by using primers that are targeted to a specific locus. If an interaction between the target protein and this locus occurs, DNA becomes amplified and the amount can be quantified.[254] However, besides these described models, no well defined methods are currently known to quantify the GC responsiveness.[14]

In addition, new insights are expected to be gained with PamChip technology from PamGene. This research will provide valuable information by using patient-derived materials instead of cell lines and other models, which have limitations (see appendix I). Even medicines can be tested directly on patient-derived tissue lysates using this PamChip technology (personal communication with Dr. René Houtman). Such chips are being developed specific for NRs, also referred to as the MARCoNI assay: Microarray Assay for Real-time Coregulator-Nuclear Receptor Interaction. Development of new PamChips that can detect influences of coregulator PTMs may provide evidence that these PTMs are involved in the development of GC hormone therapy resistance. Ultimately, this could lead to a new (combinatorial) therapy for GC hormone resistant patients.

The MARCoNI assay is based on a micro-array in which the conditions of the interaction between NRs and ligands or coregulators can be varied per array. Every array is made of a metal oxide carrier that is porous. Peptides are spotted to this metal using piezo spotting technology. In one array, 48 or 53 peptide motifs are present for detection. These motifs are from known coregulators (L*XX*LL-motifs

in coactivators as well as L*XXX*I*XXX*L-motifs in corepressors) and possess a common binding motif. The diameter for every spot is 100 µm and due to the multiple porous metal, the surface area is approximately 500 times larger than a flat surface. One single spot contains multiple pores with a length of 60 µm and a diameter of 0.2 µm (**figure 14A**). These pores are interconnected and branched. To detect interactions between NRs and their ligands or coregulatory factors, first a blocking buffer is applied onto the chip. Subsequently, the NR ligand or coregulator is added and incubated (**figure 14B**). During this incubation a fluorescent anti-glutathionine-stransferase (GST) antibody GST-NR-LBD is present in the reaction mixture and pumped through the porous metal oxide membrane that contains the peptides (**figure 14C**). This is done for 80 cycles with a rate of 2 cycles per minute. Binding of the GST-NR-LBD with the peptides are detected with a fluorescence detecting camera.[42] Using Bionavigator software (PamGene International B.V.), image analysis such as quantification and binding velocities can be calculated (**figure 14D**).[42]

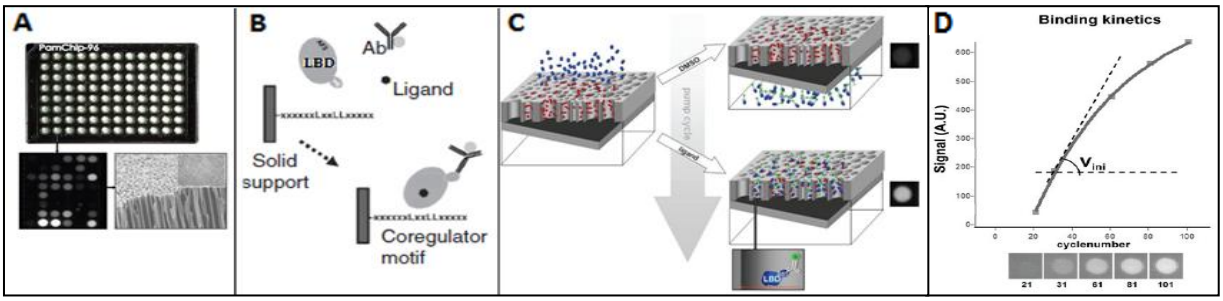


Figure 14. Demonstration of the MARCoNI PamChip (A), the mechanisms behind the PamChip (B and C) and an example of a graph from a time dependent binding of NRs (D). Figure A, C and D are adapted from Koppen,A. *et al.*, 2009.[42] Figure B is obtained from Houtman,R. *et al.*, 2012.[255]

To determine whether PTMs of coregulators cause GC hormone therapy resistance, we have to immobilize posttranscriptional modificated coregulators on such a MARCoNI PamChip. Subsequently, we can execute a similar protocol as described above. In the analysis, in the absence of bright spots of a coactivator and in the presence of bright spots of corepressors, GRs gene expression is repressed or reduced. This means that the posttranscriptional modificated coregulator contributes to GC hormone therapy resistance. In the absence of bright spots of a corepressor and in the presence of bright spots of a coactivator, GRs are not inhibited in their function and the posttranscriptional modificated coregulator leads to GC hypersensitivity. Looking at these results, novel therapies can be developed. For example, combination therapy as in a GC and inhibitor or activator of a PTM at a certain coregulator.

Conclusions

Already important effort has been made to unravel the mechanisms involved in the development of GC hormone therapy resistance are described. However, the exact mechanisms behind it still need to be clarified. By reviewing the currently known possible mechanism involved, there seems to be growing evidence that PTMs of coregulators can play an important role in the irresponsiveness of cells towards GCs. This theory is based on the fact that PTMs can modify the binding sites of GR specific coregulators, leading to alterations in the GR functions. This can eventually lead to impairment of the GC signal transduction and therefore insensitivity towards GCs. PamChip technology might clarify the importance of PTM influences on coregulators in GC responsiveness and may lead to the development of combination therapies to treat asthmatic patients with GC hormone therapy resistance.

Appendix

Limitations of cell line models, animal models, human studies and PamChip technology

Several mechanisms behind the development of GC hormone therapy resistance have been described, such as mutations and PTMs. These can therefore offer important animal and *in vitro* cell line models for clinical and genetic studies[66] to study the mechanisms behind the development of GC hormone therapy resistance in asthmatic patients. They can also be used to test new therapeutics to block the development of resistance. However, studying these mechanisms in animal and *in vitro* cell line models is not without limitations.[4]

Cell line models to study the variety of mechanisms behind resistance are limited available and often hard to culture. When available, they are most of the time simplified models of the true alterations and mechanisms that cause resistance. Therefore, they do not show all mechanisms involved which leads to wrong conclusions about the understanding and development of resistance. In addition, drugs that show potency in cell line models may not show potency or even cause toxicity in animal models. Therefore, such drugs are not usable in the clinic and animals studies are of great value to also study the entire system and environment of organisms in response to therapies.[4,54] However, when using animal models, there is still need to consider whether the effects seen in such studies are also of relevance for human beings. Different species can show different outcomes and thus appropriate models are required to study human pathology and physiology.[256]

Studying the mechanisms behind GC hormone therapy resistance in human beings themselves, is of course the best solution to unravel the mechanisms behind diseases and resistance. However, this is also not without limitations. As well as cell line and animal models, human material used for studies can undergo some change. The quality of material and labile modifications, in particular phosphorylation, can be considerably affected by pre-analytic circumstances. These can involve hypoxia, fixation time, temperature and exposure to other drugs. During sample collection, ischemia and hypoxia can change endogenous phosphatase, kinase, protease and sumo ligase activity. Hereby, phosphorylation and sumoylation of proteins can alter in dissected tissues and cell lines. Accordingly, there is need for guidelines to prevent the influences of other factors on the various PTMs.[7]

The MARCoNI PamChip might offer a solution. This assay works from recombinant LBD to patient and can therefore be directly translated to the patient. The activity within one patient can be demonstrated with just a small volume and results can be quickly obtained. This is due to the pumping capacity of the machine and because this assay does not work with diffusion (personal communication with Dr. René Houtman)**.** However, this approach also has a few drawbacks. This assay is not able to distinguish between agonists and antagonist of the GR and animals are probably needed to obtain the posttranscriptionally modified coregulators.[257]

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