Glucocorticoid receptor function:
New insights from genetic and chemical biology approaches

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medical and Human Sciences

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List of publications arising from this thesis

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- Oral presentation at the Society for Endocrinology BES 2010 in Manchester, UK. *Familial GC resistance: a novel, naturally occurring mutation which has dominant negative effects on ligand-dependent and –independent GR action.*

List of abbreviations

ACTH.....Adrenocorticotrophic Hormone
AF1 .....Activation Function-1
AF2 .....Activation Function-2
AP1 .....Activator protein-1
AR.....Androgen receptor
AVP.....Arginine Vasopressin
BMI.....Body mass index
BSA.....Bovine serum albumin
CBG .....Corticosteroid Binding Globulin
CBP.....Creb Binding Protein
CDK.....Cyclin-Dependant Kinases
CF.....Cystic fibrosis
CRH .....Corticotrophin Releasing Hormone
ChIP-Seq.....Chromatin immune-precipitation sequencing
CVZ.....Cortivazol
COPD.....Chronic obstructive pulmonary disease
DBD .....DNA Binding Domain
Dex.....Dexamethasone
DHEAS .....Dehydroepiandrosterone sulfate
DMEM.....Dulbecco's modified Eagle's medium
DTH .....Delayed type hypersensitivity
DUSP1 .....Dual specificity protein phosphatase-1
EJC.....Exon junction complex
eNOS.....endothelial Nitric Oxide Synthase
ER .....Estrogen receptor
FAI.....Free androgen index
FBS .....heat inactivated fetal bovine serum
FRAP.....Fluorescence recovery after photobleaching
FP .....Fluticasone propionate
GA.....Geldanamycin
Gc .....Glucocorticoids
GILZ .....Glucocorticoid induced leucine zipper

GFP ..... Green fluorescent protein
GR ..... Glucocorticoid Receptor
GRE ..... Glucocorticoid Response Element
GRE1/2 ..... Half GREs
GRIP1 ..... GR-Interacting Protein 1
GRUs ..... Gc Responsive Units
GPCRs..... G-protein coupled receptors
HATs ..... Histone Acetyl Transferases
HC..... Hydrocortisone
HDACs ..... Histone Deacetylases
HEK 293 ..... Human embryonic kidney 293 cell
hGR..... Human glucocorticoid receptor
Hop ..... Hsp70-Hsp90 organising protein
HPA ..... Hypothalmic-Pituitary-Adrenal
HSD ..... 11-β-Hydroxysteroid Dehydrogenase
Hsp ..... Heat shock protein
IκB..... Inhibitor of κB
IL..... Interleukin
iNOS ..... Inducible nitric oxide synthase
JNK ..... Jun N-Terminal Kinase
LBD..... Ligand Binding Domain
MAPK ..... Mitogen-Activated Kinase
MHC ..... Major histocompatibility complex
MKP-1..... MAPK phosphatase-1
MMTV ..... Mouse Mammary Tumour Virus
MR ..... Mineralocorticoid receptor
NCoR ..... Nuclear receptor corepressor
NEAA ..... Non essential amino acid
NF1 ..... Nuclear Factor 1
NFκB..... Nuclear Factor-κB
nGRE ..... Negative GREs
NLS ..... Nuclear Localisation Signal
NMD ..... Nonsense mediated messenger RNA decay
NO ..... Nitric Oxide

NOS ..... Nitric Oxide Synthase
NREs ..... NFκB Response Elements
NSGs ..... Non-steroidal glucocorticoids
NTD ..... N Terminal Domain
qPCR ..... Quantitative polymerase chain reaction
Pred ..... Prednisolone
PBMCs ..... Peripheral Blood Mononuclear Cells
PBS ..... Phosphate buffered saline
PGGH ..... Primary generalised glucocorticoid hypersensitivity
PGGR ..... Primary generalised glucocorticoid resistance
P-GR ..... Phosphorylated glucocorticoid receptor
PKB ..... Protein kinase B
PMT ..... Photomultiplier tube
POMC ..... Pituitary Proopiomelanocortin
PP5 ..... Protein Phosphatase 5
PPI ..... Peptidylprolyl isomerase
PTC ..... Premature translation-termination codon
PTM ..... Post-translational Modifications
PR ..... Progesterone receptor
PPARγ ..... Peroxisome proliferator-activated receptor γ
RLU ..... Relative light unit
SEGRAs ..... Selective glucocorticoid receptor agonists
sFCS ..... charcoal dextran stripped fetal calf serum
SHBG ..... Sex hormone binding globulin
SUMO-1 ..... Small Ubiquitin-related Modifier-1
SRC ..... Steroid receptor coactivator
SP ..... Spironolactone
TAT3-Luc ..... Tyrosine aminotransferase 3 luciferase
TNF-α ..... Tumour necrosis factor-α
TBP ..... TATA Binding Protein
TFIID ..... Transcription factor II D
TGF-β ..... Transforming growth factor-β
TPR ..... Tetratricopeptide repeat
TRIP6 ..... Thyroid receptor-interacting protein 6

UTR..Untranslated region

UPF ....Up-frameshift proteins

Declaration

I declare that no portion of the work in this thesis has been submitted in an application for another degree or qualification of this or any other university or other institute of learning.

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Alternative format thesis

This thesis is presented in the alternative format in accordance with the University of Manchester guidelines. Chapters two and three present my work from two published manuscripts formatted in a style suitable for this thesis. My contributions to the manuscripts are as follows.

Chapter 2: Familial glucocorticoid resistance caused by a novel frameshift glucocorticoid receptor mutation

Published December 2010 in The Journal of Clinical Endocrinology and Metabolism.

I performed all experiments for this paper. My supervisors Dr. Laura Matthews and Professor David Ray provided advice and guidance on all experimental work. Dr. John Blaikley carried out statistical analysis on the results. As first author on this paper, I was also fully responsible for writing the text of the manuscript. The first draft was produced by me; which my co-authors then reviewed and provided comments, specifically Dr. Laura Matthews and Professor David Ray. These comments were then compiled by me into the final version in print.

Chapter 3: A ligand-specific kinetic switch regulates glucocorticoid receptor trafficking and function.

Published online May 2013 in The Journal of Cell Science.

I carried out the vast majority of experiments for this paper. Dr. Laura Matthews carried out the MTS proliferation assay in A549 and Hela cells. Bill Leavens carried out mass spec analysis on samples I prepared and James Woolven advised with the crystal structure work during my 3 month placement at GlaxoSmithKline. My supervisors Dr. Laura Matthews, Professor Stuart Farrow and Professor David Ray provided advice and guidance on all experimental work. As first author on this paper, I was fully responsible for writing the text of the manuscript. The first draft was produced by me; which my co-authors then reviewed and provided comments, specifically Dr. Laura Matthews, Professor Stuart Farrow and Professor David Ray. These comments were then compiled by me into the final version submitted.

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Abstract

Glucocorticoids (Gc) are vital for development, maintenance of glucose homeostasis and the resolution of inflammation. As potent modulators of the immune response Gc are routinely prescribed in the management of a variety of inflammatory diseases including asthma and rheumatoid arthritis. However clinical use of Gc is limited by variation in patient sensitivity to Gc treatment and development of a wide range of side effects. In this thesis I present two studies that have advanced our understanding of Gc action *in vivo*. The first defines and characterises the cause of familial Gc resistance, and the second describes the action of two potent non-steroidal Gc in a cell line model.

Familial Gc Resistance: Cases of primary generalised Gc resistance are very rare and typically present as mineralocorticoid and androgen excess leading to hypertension, hypokalemia and hirsutism. Gc resistance is attributed to loss of function mutations within the glucocorticoid receptor (GR). Here I identify a family with a novel mutation in GR exon 6 that gives rise to a very mild phenotype. Analysis of transformed patient peripheral blood lymphocytes revealed a 50% reduction in full length GR but no expression of a mutant form. As this did not rule out expression *in vivo*, the mutant receptor (Δ612GR) was characterised in a cell line. Investigation using reporter genes revealed that Δ612GR lacked any activity, but had dominant negative action when co-expressed with full length GR. In response to Gc Δ612GR was not phosphorylated or targeted for degradation. Fluorophore tagged Δ612GR was unable to translocate to the nucleus in response to Gc, but delayed the translocation of full length GR when co-expressed. Together this indicates that Δ612GR is unable to bind ligand but has dominant negative action upon full length GR most likely due to heterodimerisation. Therefore I describe a novel GR mutation that results in Gc resistance but presents with a mild very phenotype.

Novel Non-steroidal Gc: Non-steroidal Gc can be used as tools to determine how ligand structure directs GR function. Here I describe two highly potent non steroidal Gc ligands, GSK47867A and GSK47869A which alter the kinetics of receptor activity. Treatment with either ligand induces slow GR nuclear translocation, promotes GR nuclear retention and prolongs transcriptional activity following ligand withdrawal. Crystal structure analysis revealed that GSK47867A and GSK47869A specifically alter the surface charge of the GR at a site important for Hsp90 binding. GR bound to GSK47867A and GSK47869A shows prolonged activity in the presence of Hsp90 inhibitor geldanamycin. Therefore this work identifies a new chemical series that could prolong GR activity due to altered pharmacodynamics rather than altered pharmacokinetics.

In summary this work uses a combination of genetic and chemical biology approaches to broaden our understanding of GR function. Characterisation of naturally occurring GR mutations gives insight into the complex function of the GR, and non-steroidal Gc act as useful tools that will aid in the design of improved therapeutics.

Chapter 1: Introduction

1.1 Glucocorticoid function

Glucocorticoids (Gc) are required for development and are essential for life. They act upon a wide range of cells and tissues including muscle, adipose tissue, liver, bone and cells of the immune system. Gc perform a dual role acting to control glucose homeostasis and regulating the immune response. Therefore the action of these steroid hormones is tightly regulated on multiple levels. Therapeutically Gc are the most potent anti-inflammatory agents known and are routinely prescribed to treat a wide variety of autoimmune and inflammatory diseases.

1.1.1 Glucocorticoid metabolic action

Gc were identified through studies in adrenalectomised animals that developed hypoglycaemia. This observation led to the discovery that Gc maintain glucose homeostasis and for this role they were named (COHN *et al.*, 1952). Gc are produced in response to low blood glucose to prevent glucose uptake in peripheral muscle and adipose tissue and promote catabolism of protein and lipid reserves (Figure 1.1) (Vinson, 2009). Gc also drive gluconeogenesis in hepatocytes, thereby providing a dual mechanism to elevate blood glucose (Figure 1.1) (Hanson and Reshef, 1997). Initial studies in knockout mice demonstrated that the receptor for Gc, the glucocorticoid receptor (GR), is critical for life as mice die at birth due to failure of lung maturation. Other complications of GR loss include high levels of endogenous Gc in the blood and enlarged adrenals (Cole *et al.*, 1995). Subsequent gene targeting studies using the Cre/LoxP system allowed for generation of mice that survive to adulthood (Reichardt *et al.*, 1998). These studies revealed the importance of GR in inflammation, nervous system control and stress-induced erythropoiesis (Reichardt *et al.*, 2000).

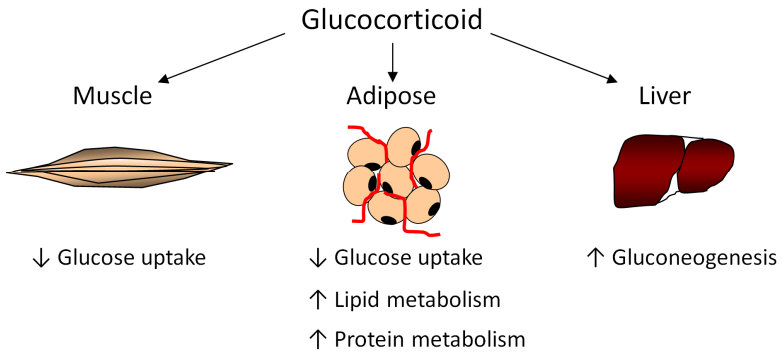

Figure 1.1: Metabolic action of glucocorticoids. In response to low blood glucose Gc is produced and acts upon muscle, adipose and liver tissues facilitating restoration of normal blood glucose level.

1.1.2 Glucocorticoid immune action

Gc are released in response to stress. This enables mobilisation of energy reserves required to combat potential immune threats, whilst concomitantly limiting the immune reaction itself, preventing damage to host tissue (Munck, 2005). Gc are therefore potent modulators of the immune response (Liberman *et al.*, 2009). The importance of Gc directed immunosuppression is evident from the poor survival rate of adrenalectomised rodents challenged with the bacterial coat protein, lipopolysaccharide (Yeager *et al.*, 2004). Gc activate a broad range of inhibitory mechanisms in both primary and secondary immune cells (Figure 1.2). Gc act on primary immune cell number by decreasing myelopoiesis, inhibiting proliferation and activating apoptosis of monocytes, macrophages, T lymphocytes (Amsterdam and Sasson, 2002;Buttgereit *et al.*, 2005;Abe *et al.*, 2011). Additionally, Gc reduces the proliferation of endothelial cells and fibroblasts (Akkoyun *et al.*, 2007;Nehme and Edelman, 2008;He *et al.*, 2011). Gc also down-regulate expression of MHC class II proteins and Fc receptors in macrophages, effectively decreasing sensitivity to inflammatory stimuli (Buttgereit *et al.*, 2005). Gc potently inhibit the production and activity of rapidly synthesised pro-inflammatory molecules. Gc inhibit production of arachidonic acid, an important inflammatory mediator, by preventing expression of the enzymes required for its synthesis (Newton *et al.*, 1997). Gc also restrain the activity of cytokine secreting T cells and inhibit macrophage activity by preventing synthesis of interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α) and prostaglandins (Abe *et al.*, 2011). Gc also prevent fibroblast production of fibronectin and prostaglandins and inhibit endothelial cell production of IL-1, and prostaglandins (Nehme *et al.*, 2008;He *et al.*, 2011). Inflammation triggers production of inducible nitric oxide synthase (iNOS) which drives vasodilation, increasing blood vessel permeability and promoting accumulation of leukocytes at sites of inflammation (Farsky *et al.*, 1995). Gc decrease iNOS gene transcription through inhibiting the pro-inflammatory transcription factor nuclear factor-κB (NF-κB), and also upregulating the production of IκB, an NF-κB inhibitor (De Vera *et al.*, 1997;Matsumura *et al.*, 2001). Targeting of immune cells to sites of inflammation is disrupted by Gc. Chemotaxis is inhibited by decreasing chemokine production and down-regulating adhesion molecule synthesis. In addition to the inhibitory action, Gc also upregulates production of anti-inflammatory molecules. Gc induce expression of cytokine receptor variants, that are unable to transmit the inflammatory signal, sequestering

cytokine molecules. In addition to quenching the cytokine signal, Gc also increases production of anti-inflammatory cytokines including transforming growth factor-β (TGF-β) and IL-10 along with their corresponding receptors TGF-βR and IL-10R (Almawi *et al.*, 1996).

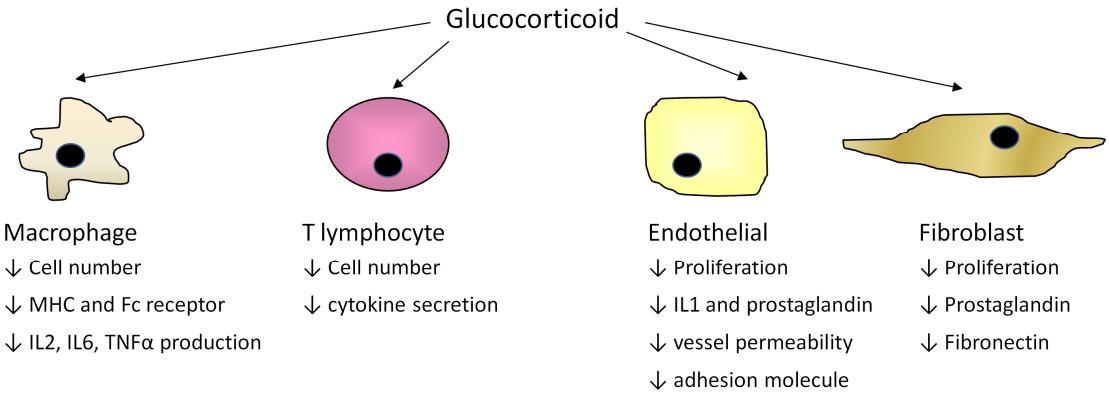

Figure 1.2: Immune action of glucocorticoids. In response to inflammatory challenge Gc is produced and acts upon cells of the immune system to restrain the immune response to prevent damage to host cells and tissues.

1.1.3 Glucocorticoid production and bioavailability

Given the functional importance of Gc for glucose homeostasis and control of the immune response, the synthesis and bioavailability of endogenous Gc is tightly regulated. Gc production is governed by the hypothalamic-pituitary-adrenal (HPA) axis, an interlinked feedback mechanism that orchestrates synthesis from the molecular precursor cholesterol (Payne and Hales, 2004). Cortisol, the main Gc in humans (corticosterone in rodents), is synthesised in cells of *zona fasciculata* in the adrenal cortex and released in a highly regulated manner (Enyeart, 2005). If the glucose homeostatic balance is perturbed, chemical and neurological signals are interpreted by the hypothalamus, which triggers the production of corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) (Figure 1.3) (Papadimitriou and Priftis, 2009). These chemical mediators act synergistically upon cells in the pituitary driving transcription of pituitary proopiomelanocortin (POMC) which in turn is processed to adrenocorticotrophic hormone (ACTH) (Raffin-Sanson *et al.*, 2003). ACTH stimulates synthesis of cortisol in the adrenal cortex which is released into the bloodstream enabling transport throughout the body (Papadimitriou *et al.*, 2009). Rapid feedback occurs when cells of the pituitary sense elevated

circulating Gc and consequently down-regulate production of ACTH. Long term feedback occurs through Gc interaction with the hypothalamus that blocks production of CRH and AVP (Buckingham, 2006;Papadimitriou *et al.*, 2009). This limits prolonged exposure of the body to elevated Gc, allowing smooth release of nutrients whilst permitting rapid release of glucose in response to stress. The HPA axis drives circadian rhythms allowing for energy availability to coordinate with activity, thereby pre-empting metabolic demand (Dickmeis, 2009). Cortisol release is pulsatile and diurnal in pattern, with a peak in the morning followed by a steady decrease until a second lesser peak in the afternoon, with levels falling to their lowest around midnight (Linkowski *et al.*, 1993;Young *et al.*, 2004).

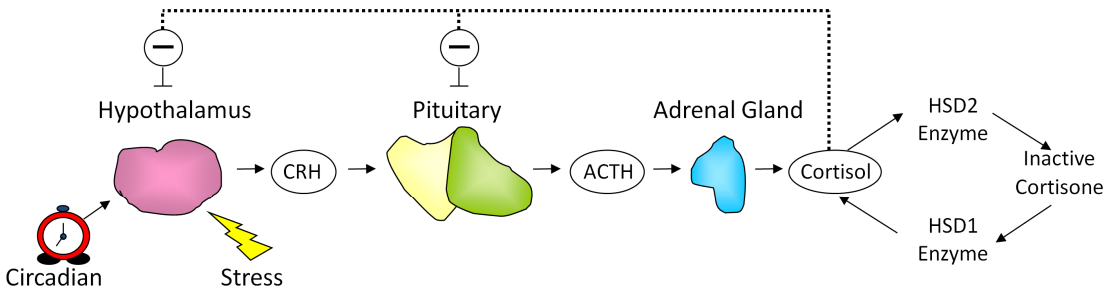

Figure 1.3: Schematic of the HPA axis. Environmental signals such as stress or time of day are interpreted by the hypothalamus that results in production of CRH, which subsequently acts upon the pituitary. ACTH is produced from the pituitary in response to CRH and acts upon the adrenal glands triggering synthesis of cortisol. Tissue specific expression of HSD enzymes adds further layer of Gc control. CRH: corticotrophin releasing hormone; ACTH adrenocorticotrophic hormone; HSD1/2: 11-β-Hydroxysteroid Dehydrogenase 1/2.

Upon entry into the circulatory system the vast majority of Gc is bound by corticosteroid binding globulin (CBG) or serum albumin which acts a reservoir to limit bioavailability. Only 4-5% of circulating Gc is unbound and able to act on target cells and tissues directly (Lin *et al.*, 2009). At the cellular level, tissue-specific expression of 11-β-Hydroxysteroid Dehydrogenase (HSD) enzymes introduces a further level of cortisol regulation. HSD1 is a reductase and converts inactive cortisone into active cortisol, whereas HSD2 catalyses the opposite reaction, producing inactive cortisone. Tissue specific expression of both enzymes in different ratios therefore permits fine-tuning of cortisol bioavailability (Figure 1.3) (Draper and Stewart, 2005). HSD1 expression is highest in adipose tissue, brain, gonads and liver which are classic targets for Gc action (Moisan *et al.*, 1990;Tannin *et al.*,

1991;Bujalska *et al.*, 1997;Ricketts *et al.*, 1998). HSD2 is predominantly expressed in the tissues of the kidney and colon which are primary targets for the action of mineralocorticoids, a class of steroid hormone that control salt and water homeostasis (Agarwal *et al.*, 1994;Whorwood *et al.*, 1994). The mineralocorticoid receptor has a high affinity for Gc, and so the expression of HSD2 in these mineralocorticoid target tissues protects it from non-specific Gc activation (Krozowski, 1999).

1.1.4 Therapeutic use of glucocorticoids

The potent immunomodulatory activity of Gc was quickly recognised to be of potential therapeutic benefit, which led to development of synthetic Gc such as dexamethasone (Dex) and prednisolone (Pred). As such Gc are amongst the most frequently prescribed drugs in the treatment and management of chronic inflammatory diseases such as rheumatoid arthritis and asthma (Schett *et al.*, 2008;Krishnan *et al.*, 2009). Additionally, Gc are also used as an adjunct treatment in chemotherapy as they prevent chemotherapy induced nausea and reduce tumour swelling (Sionov *et al.*, 2008). The synthetic steroidal Gc are adaptations of the molecular structure of cortisol, differing through the addition or removal of various functional groups (Figure 1.4) (Bledsoe *et al.*, 2004). Fluticasone propionate (FP), a highly potent synthetic Gc, is used in inhalers for the treatment of asthma (Cerasoli, Jr., 2006). More recently, non-steroidal Gc (NSG) have provided an alternate route for drug design, yet much is still to be understood about how these drugs work. Non-steroidal arylpyrazole compounds have been tested in different cells lines (A549 and 3T3-L1 cells) and activate distinct Gc target gene profiles (Wang *et al.*, 2006). For example, the NSG 2,5-dihydro-9-hydroxy-10-methoxy-2,2,4-trimethyl-5-(1-methylcyclohexen-3-y1)-1H-[1]benzopyrano[3,4-f]quinoline (A276575) and its four enantiomers each regulate Gc target genes differently, revealing that the structure of the Gc directly influences function (Figure 1.4) (Lin *et al.*, 2002).

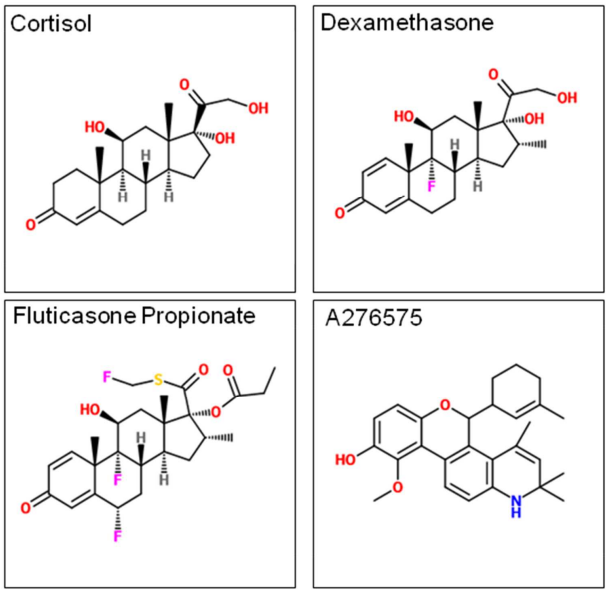

Figure 1.4: Chemical structure of natural and synthetic glucocorticoids. Cortisol is the endogenous glucorticiod in humans. Dexamethasone is a classic synthetic agonist. Fluticasone Propionate used in asthma treatment. A276575 is a non steroidal Gc.

1.1.5 Glucocorticoid side effects

Design of synthetic Gc initially focused on identifying compounds that had greater potency than the naturally occurring Gc cortisol. This search yielded numerous highly potent Gc. Unfortunately, prolonged treatment with these highly potent synthetic compounds, that cannot be inactivated by HSD2, results in a number of unwanted effects. This is a reflection of the complex multifaceted role of Gc, and so the side effect profile of Gc treatment is large, including osteoporosis, metabolic syndrome and cardiovascular disease (Canalis *et al.*, 2002;McMaster and Ray, 2007;McMaster and Ray, 2008b). For instance in humans Gc treatment increases the action of osteoclasts, responsible for bone resorption, and also decreases the action of osteoblasts which are responsible for bone formation. As such this results in dose dependant bone loss, compounded by decreased calcium uptake from the intestine and kidneys leading to osteoporosis (Compston, 2011). Long term Gc treatment also results in dyslipidemia, insulin resistance, glucose intolerance and hypertension which are all risk factors for cardiovascular disease. In addition, Gc act directly on cells and tissues of the cardiovascular system promoting atherogenesis and influencing the remodelling of vasculature following insult (Walker, 2007). The immunosuppressive action of Gc also makes patients receiving treatment more susceptible to secondary

bacterial or viral infections (Cutolo *et al.*, 2008). There is also a greater incident of skin, bladder and prostate cancer in patients on long term Gc therapy (Karagas *et al.*, 2001;Dietrich *et al.*, 2009;Severi *et al.*, 2010;Seguro *et al.*, 2012).

1.2 The glucocorticoid receptor

Gc actions are mediated by the ubiquitously expressed glucocorticoid receptor (GR) a member of the nuclear hormone receptor superfamily. GR is the product of a single gene locus 80 kb in length found on chromosome 5 q31-q33 (Hollenberg *et al.*, 1985;Encio and Detera-Wadleigh, 1991). Upstream of the GR coding sequence is a promoter region that contains binding sites for various transcription factors such as AP1 and NF-κB (Nobukuni *et al.*, 1995). GR is a ligand activated transcription factor. It is controlled by a variety of other transcription factors and is able to regulate its own production (Breslin *et al.*, 2001).

1.2.1 Glucocorticoid receptor domain organisation

The GR gene comprises 9 exons, with alternative terminal exons 9α and 9β. Only exons 2 to 9 contribute to the translated protein. There are several isoforms of the GR that will be discussed later but GRα is the most well characterised isoform. In humans GRα is a single chain polypeptide consisting of 777 amino acids (aa) (Hollenberg *et al.*, 1985). As a member of the nuclear hormone superfamily the GR shares an arrangement of domains homologous to other nuclear receptors (Giguere *et al.*, 1986;Thornton, 2001). At the amino end of the GR is the N terminal domain (NTD) that flanks a central DNA binding domain (DBD)(Luisi *et al.*, 1991;Lu and Cidlowski, 2005). The ligand binding domain (LBD) lies at the C-terminus (Weinberger *et al.*, 1985;Mittelstadt and Ashwell, 2003;Bledsoe *et al.*, 2004). Exon 2 encodes the majority of the NTD whilst exons 3 and 4 contribute to the DBD and the final exons 5 through to 9α or 9β encode the LBD (Figure 1.5). Although crystal structures of the LBD and DBD have been resolved, due to the fluid nature of the NTD the entire crystal structure for the GR is yet to be solved (Bledsoe *et al.*, 2004).

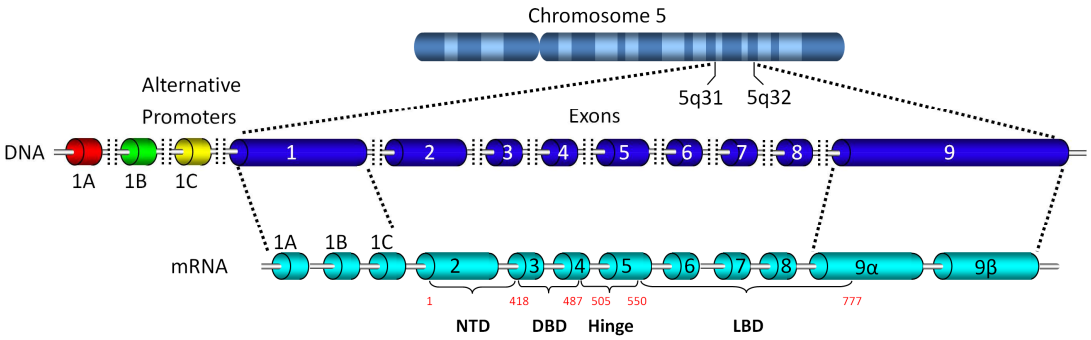

Figure 1.5: Organisation of the human GR gene. The GR is located on chromosome 5 and consists of 9 exons.

1.2.2 The N terminal domain

Residues 1 to 417 form the NTD of the GR, and contain the transcriptional activation function-1 (AF1) domain responsible for transactivation and interaction with other transcription factors (Hittelman *et al.*, 1999). Also known as the immunogenic region of the GR, this is a major site of post-translational modification as it contains several serine residues that can be phosphorylated in both ligand-dependent and -independent mechanisms (Ismaili and Garabedian, 2004). The AF1 interacts with key proteins of the transcriptional machinery such as the TATA box binding protein (TBP) and is therefore required for effective transactivation (Kumar *et al.*, 2004b). Upon interaction with DNA a conformational change within the AF1 selectively promotes the recruitment of additional proteins. The selection of binding partners for the AF1 subsequently determines the level of transcriptional activity (Kumar and Thompson, 2005). The removal of the AF1 in transgenic mice has no affect on their viability however, suggesting a modulatory role of the NTD. The majority of the functional activity of the receptor is mediated by the DBD and LBD region of the GR (Miesfeld *et al.*, 1987;Mittelstadt *et al.*, 2003).

1.2.3 The DNA binding domain

Residues 418 to 487 form the central DBD. This has a high degree of sequence homology across species and contains two conserved zinc finger motifs (Luisi *et al.*, 1991). The conformation of this domain allows for highly specific interaction with Gc response elements (GREs) located upstream of target genes (La and Yamamoto, 1994). Variation within the copy number and sequence of the response elements is thought to confer allosteric alteration to the GR which enables differential recruitment

of cofactors generating further specificity (Meijsing *et al.*, 2009). Recent genome wide chromatin immune-precipitation sequencing (ChIP-Seq) has revealed that GR predominantly binds to its consensus GRE (Voss *et al.*, 2011). The first zinc finger motif in the DBD contains a 'P-Box' that contains residues responsible for direct interaction with the GRE in the major groove of the DNA. The distal zinc finger of the DBD recognises bases within the minor groove of the DNA through its α helices (Hard *et al.*, 1990b). The DBD also contains a 'D-box' required for dimerisation of the GR upon interaction with a GRE. The GRE binds monomeric GR with a subsequent alteration in the receptor conformation that favours binding of a second GR to the adjacent major groove in the correct orientation (Luisi *et al.*, 1991). The hinge region of GR (residues 505 to 550) is required for receptor dimerisation prior to translocation to the nucleus (Savory *et al.*, 2001).

1.2.4 The ligand binding domain

The ligand binding domain spans residues 527 to 777 and adopts a complex globular tertiary structure composed of eleven α helices and four short β sheets (Bledsoe *et al.*, 2002). The helices and sheets pack together forming a hydrophobic pocket of residues that serves to bind lipophilic ligand with high affinity (Bledsoe *et al.*, 2004). In addition to this, a side pocket enables the GR to bind ligands with a large side group at C17α allowing binding to both conventional steroidal ligands, alternative non-steroidal ligands and non classical ligands such as rosiglitazone (a full PPARγ antagonist and partial GR agonist) or RU486 (a full progesterone receptor and partial GR agonist) (Kauppi *et al.*, 2003;Matthews *et al.*, 2009). The ligand binding domain also contains a transcriptional activation function-2 (AF2) spanning residues 526-556 (Giguere *et al.*, 1986). The AF2 has been implicated with recruitment of transcriptional machinery and cofactors following binding to Gc (Bledsoe *et al.*, 2002).

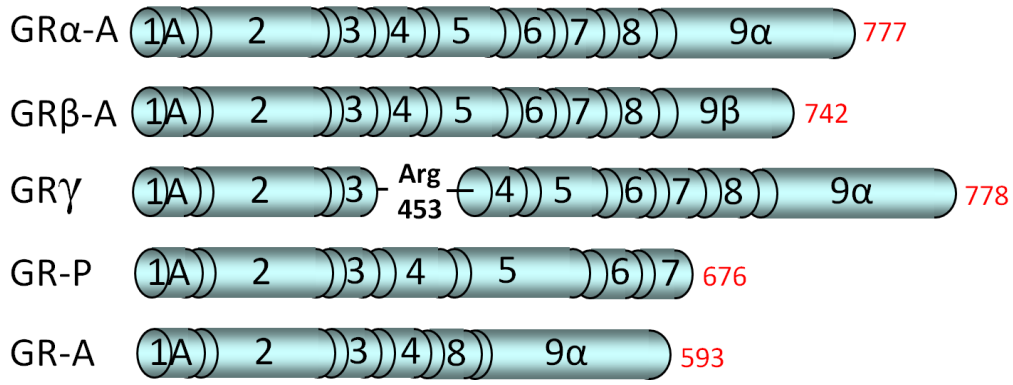

Figure 1.6: GR gene protein products. Alternate splicing of the GR mRNA gives rises to a variety of protein products. GRα-A is most the abundant.

1.2.5 Alternative promoter and splice variants usage in GR expression

Exon one contains several transcription initiation sites that allow for generation of a variety of mRNA transcripts. The alternative 5' untranslated exon 1 transcripts (1A, 1B, 1C, 1D, 1E, 1F, 1H, 1I and 1J) all join to exon 2 and are known to be differentially expressed in different cell types, suggesting that there could be a functional difference for each of these mRNA transcripts (Zhang *et al.*, 2004;Turner and Muller, 2005). Additional variation has been observed through splice variants of the GR with exons 9α and 9β forming GRα and GRβ respectively (Figure1.6) (Hollenberg *et al.*, 1985).

1.2.6 GR β

GRβ is 742 aa, as exon 9β encodes for a distinct set of 15 aa at the carboxy terminal of the GR (Oakley *et al.*, 1996). Due to the truncation conferred by inclusion of exon 9β, initially GRβ was mistaken as a cloning artefact as it lacks conventional LBD and is unable bind ligand (Yudt and Cidlowski, 2002;Yudt *et al.*, 2003). However it has since been recognised to be present at low levels in most cells and tissues (Oakley *et al.*, 1996). Due to conflicting reports in the literature there is a degree of uncertainty surrounding the role of GRβ. It constitutively resides in the nucleus and is thought to play an inhibitory role by forming heterodimers with GRα and thus has dominant negative activity (Oakley *et al.*, 1999). It appears to be preferentially upregulated in response to IL8 and TNFα supporting a role for GRβ in inflammation (Webster *et al.*, 2001;Strickland *et al.*, 2001). However, since GRβ appears to have no negative effect

upon the ability of GRα to transrepress target genes, the function of GRβ remains controversial (Hecht *et al.*, 1997).

1.2.7 GR translational variation

As well as the diverse number of mRNA transcripts for GR, leaky ribosomal scanning adds an additional level of translational variation (Lu *et al.*, 2005). The predominant GR product is formed from the first AUG start codon and is known as GRα-A. An additional methionine (met-27) in exon two leads to a 751aa GRα product (GRα-B) and a 716aa GRβ product (GRβ-B). This allows an increase in physiological flexibility since GRα-B has twice the transactivation activity of the GRα-A *in-vitro* (Yudt and Cidlowski, 2001). The ratio of these isoforms therefore potentially dictates the sensitivity of a cell to Gc.

1.2.8 Alternative GR isoforms

GRγ is similar to full length GRα but includes three nucleotides from the intronic region between exons 3 and 4 which results in the insertion of an additional arginine residue R453 (Rivers *et al.*, 1999). GRγ therefore comprises 778aa. The additional arginine residue is importantly located between two zinc fingers that form the DBD. This single residue addition profoundly reduces the activity of GRγ on some templates but microarray analysis suggests that GRγ may preferentially target a subset of genes (Meijsing *et al.*, 2009). Other splice variants, GR-P (also known as GR δ) and GR-A are elevated in disease conditions such as myeloma and leukaemia. Both these isoforms have large truncations, GR-A lacks exons 5 to 7 and GR-P lacks exons 8 and 9 which result in non functional forms that are linked to Gc resistance (Moalli *et al.*, 1993;Krett *et al.*, 1995;De Lange *et al.*, 2001).

1.3 Glucocorticoid receptor function

In the absence of ligand GR is predominantly cytoplasmic sequestered as part of a heteromeric multiprotein complex. Derived from cholesterol, Gc are lipophilic and are therefore able to passively diffuse across the plasma membrane of target cells (Siddiqui *et al.*, 1989). Upon encountering and binding to Gc, GR exerts effect via two pathways. As a transcription factor the classical route for activation requires translocation to the nucleus with subsequent alteration in gene expression, termed

‘genomic signals’. More recently it has also been observed that there are rapid ‘non-genomic’ actions mediated through the activation of cytoplasmic kinase cascades.

1.3.1 GR folding

To enable rapid binding to Gc the newly translated GR protein undergoes a highly regulated folding process. Heat shock protein (Hsp) 70 aids folding of the GR through a dynamic ATP-dependant cyclic process of association and dissociation, following presentation of the nascent GR polypeptide by Hsp40 (Figure 1.7) (Laufen *et al.*, 1999). Subsequent binding of Hop (Hsp70-Hsp90 organising protein) via its tetratricopeptide repeat (TPR) domains enables passage of the low affinity conformation GR from the Hsp70 to the Hsp90 (Hutchison *et al.*, 1994;Chen and Smith, 1998). Binding of dimerised Hsp90 induces a conformational change in the GR LBD that exposes the hydrophilic steroid binding pocket (Nemoto *et al.*, 1990;Grenert *et al.*, 1999). Association of Hsp90 not only facilitates ligand binding but also acts to anchor GR in the cytoplasm through masking of a nuclear localisation signal 1 (NLS1). Removal of the LBD abolishes Hsp90 interaction resulting in a GR that is constitutively nuclear and transcriptionally active (Godowski *et al.*, 1987;Savory *et al.*, 1999). The use of geldanamycin to inhibit ATP binding to the Hsp90 active site reveals a further role in maintaining GR protein stability (Whitesell and Cook, 1996). Addition of P23 results in a stabilisation of the GR complex in an active state facilitating ligand binding (Grad *et al.*, 2006). Regulation of this system is exerted through interaction with co-chaperone proteins such as Bag-1 that serve to inhibit the folding of GR and can target it for degradation by the proteasome (Figure 1.7) (Kanelakis *et al.*, 2000). As such the components of the mature GR heteromeric multiprotein complex include Hsp90, Hsp70, Hsp40, immunophilins, and P23 (Grad and Picard, 2007).

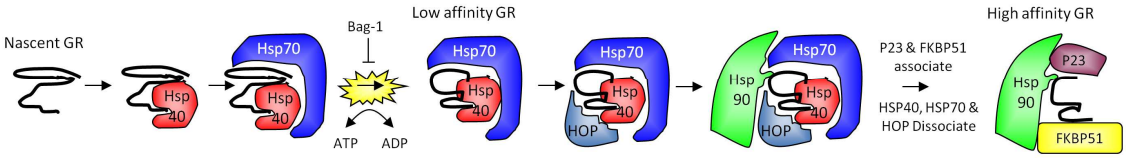

Figure 1.7: GR folding. Nascent GR associates with Hsp40 and Hsp70 and folds into a low affinity conformation in an ATP dependant manner. Recruitment of HOP facilitates passage of GR to Hsp90 which enables a high affinity conformation ready to bind free steroid.

Cytoplasmic GR heterocomplexes contain one immunophilin such as the structurally related FK506-binding proteins (FKBP) 51 or 52, cyclophilin-40 (Cyp40) or protein phosphate 5 (PP5) a immunophilin homologue (Silverstein *et al.*, 1997;Silverstein *et al.*, 1999;Galigniana *et al.*, 2001;Galigniana *et al.*, 2002;Hinds, Jr. and Sanchez, 2008). Each immunophilin possesses peptidyl-prolyl isomerase (PPIase) activity and they all share a common tetratricopeptide (TPR) domain. As mentioned previously Hop binds to the Hsp90 dimer through its TPR acceptor site which consists of a MEEVD pentapeptide motif located at the end of the Hsp90 C-terminal (Scheufler *et al.*, 2000;Brinker *et al.*, 2002). Dissociation of Hop allows the immunophilins to bind the unoccupied TPR site in a mutually exclusive manner, however the immunophilins can also interact directly with GR influencing ligand binding (Silverstein *et al.*, 1999;Riggs *et al.*, 2003). The GR heterocomplex undergoes constant cycles of assembly and disassembly and the composition of TPR binding partners is directed by ligand binding.

1.3.2 Non-genomic GR activity.

After Gc binding, GR mediates non genomic effects (Figure1.8). By definition, these cellular effects occur rapidly, and do not require new transcription. In cell lines treated with Gc short term (5-30 minutes) observations revealed that phosphorylation of kinases occurs (Croxtall *et al.*, 2000;Croxtall *et al.*, 2002;Liu *et al.*, 2005). In the same way high doses of Gc used to treat myocardial infarction activate phosphatidylinositol 3-kinase and protein kinase Akt signalling. This results in the rapid activation of endothelial nitric oxide synthase which facilitates vasorelaxation (Hafezi-Moghadam *et al.*, 2002). It has also been found that Gc treatment suppresses stimulated insulin release from β-cells in the pancreas via a rapid non-genomic mechanism (Sutter-Dub, 2002). Gc promote adipocyte production by acting to inhibit histone deacetylase complex in preadipocytes which triggers differentiation (Wiper-Bergeron *et al.*, 2003). Furthermore the rapid regulation of brain function and regulation of the HPA occurring within minutes of Gc exposure is due to non-genomic GR activity (Tasker *et al.*, 2006). The non genomic action of Gc is hypothesised to function through membrane bound GR associated with G-protein coupled receptors (GPCRs). In support of this, rapid Gc mediated suppression of ACTH is reversed by the classic GPCR inhibitor pertussis toxin (Tasker *et al.*, 2006). GR is targeted to the membrane through processing in the Golgi which most likely

results in post-translational modifications that enable association with the membrane (Strehl *et al.*, 2011). Membrane GR is found as part of lipid raft microdomains, which are small rigid regions of the plasma membrane that have a high concentration of sphingolipids and cholesterol, disruption of which inhibits GR transcriptional activity. Additionally Dex treatment has been demonstrated to increase the recruitment of GR to these lipid raft microdomains (Jain *et al.*, 2005). More recently GR has been shown in complex with caveolin coated lipid rafts called caveolae. Studies using a mice knock model out for caveolin-1 demonstrated a loss of Gc anti-proliferative action, with no effect on GR transactivation (Matthews *et al.*, 2008). The non genomic action of membrane GR can be selected for by using BSA conjugated Dex which is unable to cross the plasma membrane. Proteomic studies with BSA-Dex confirmed the association of GR and caveolin-1 via the AF-1 domain. Knock down of caveolin-1 using siRNA resulted in a 70% loss in GR-caveolin dimers with a subsequent loss in total membrane associated GR (Vernocchi *et al.*, 2013).

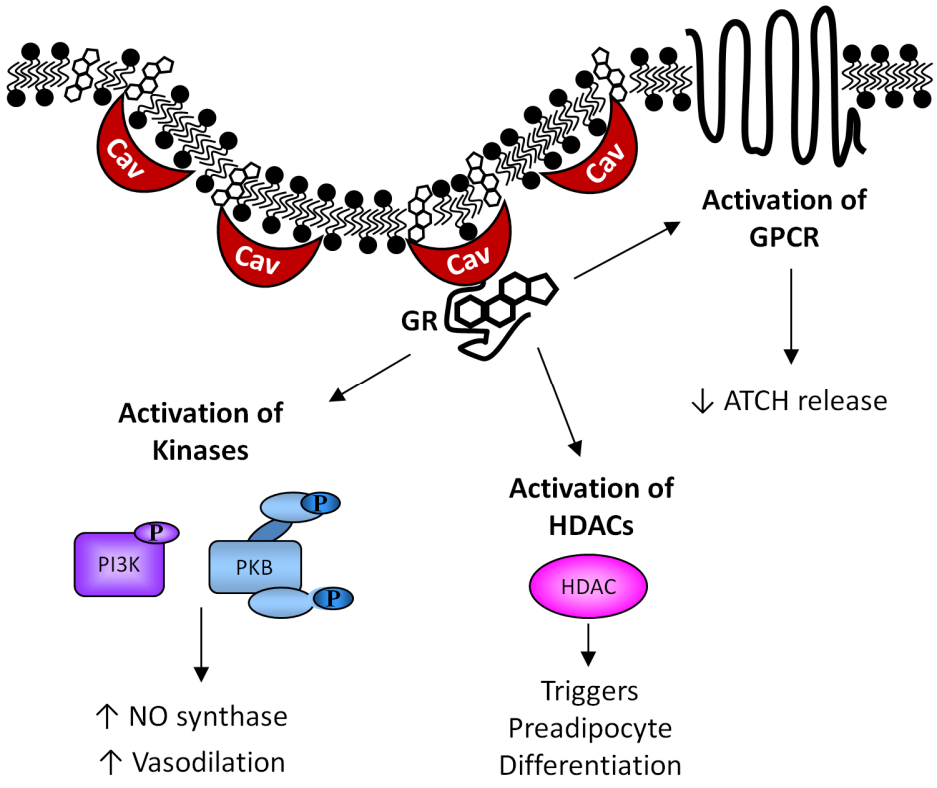

Figure 1.8: GR non genomic signalling. Cytoplasmic high affinity GR binds Gc rapidly activates cytoplasmic kinase signalling cascades, G protein coupled receptors (GPCRs) and histone deacetylases (HDACs).

1.3.3 Ligand induced GR nuclear translocation

After Gc binding, GR translocates to the nucleus, with the majority of cellular GR being nuclear 30 minutes following treatment with highly potent synthetic Gc Dex (100nM). The conformational change of GR induced upon binding ligand results in exposure of NLS1 and NLS2 enabling nuclear import (Savory *et al.*, 1999). FKBP51 is predominantly associated with the inactive Hsp90 GR complex and is replaced by FKBP52 following Gc binding (Davies *et al.*, 2002). FKBP52 docks with the molecular machine dynein via dynamitin through its PPIase domain, facilitating retrograde transport along the microtubules (Figure 1.9) (Czar *et al.*, 1994;Silverstein *et al.*, 1999;Galigniana *et al.*, 2002;Harrell *et al.*, 2004). In order for GR to cross the nuclear membrane it must pass through the nuclear pore, which is a large protein complex (125MDa). Importin α binds to NLS1 and importin β which facilitate the passage of GR through the nuclear pore (Freedman and Yamamoto, 2004). Other components of the GR heterocomplex also interact with importin β and the nuclear pore glycoprotein Nup62, indicating that the entire heterocomplex could translocate across the nuclear pore (Echeverria *et al.*, 2009). Remarkably, prior to translocation of the GR heterocomplex, Gc treatment results in rapid restructuring of the nuclear envelope resulting in clustering and dilation of the nuclear pores (Shahin *et al.*, 2005a;Shahin *et al.*, 2005b).

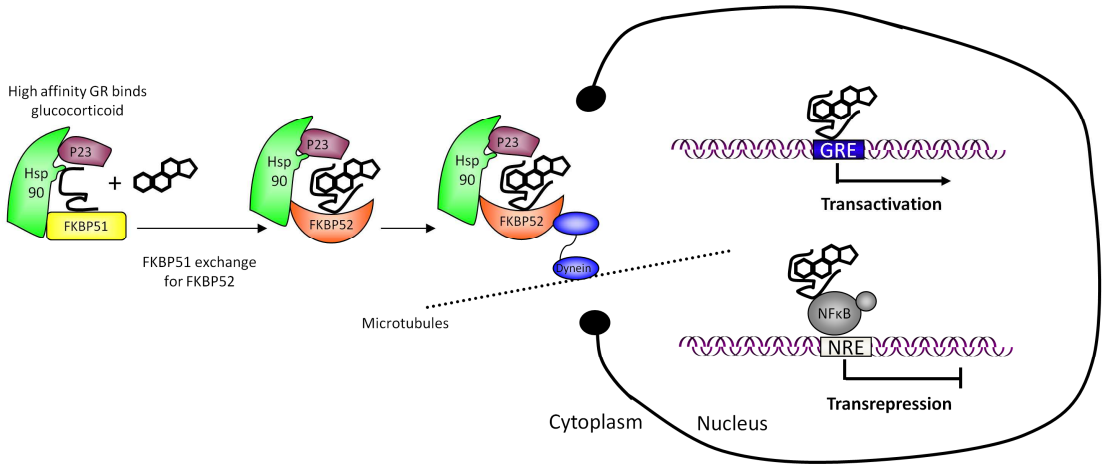

Figure 1.9: GR nuclear translocation. Cytoplasmic high affinity GR binds Gc and translocates to the nucleus via the microtubule network to modulate gene expression.

1.3.4 GR regulation of gene expression

Ligand-bound nuclear GR modulates expression of Gc target genes. This is achieved through two mechanisms, monomeric GR can interact with other DNA-bound transcription factors (tethering) or dimeric GR can directly bind specific DNA sequences. In general (although with exceptions) repression of target genes (transrepression) occurs through tethering of monomeric GR, whereas activation of target genes (transactivation) occurs through direct DNA binding of GR dimers (Uhlenhaut *et al.*, 2013).

1.3.5 GR transactivation

Liganded GR binds DNA directly through recognition of sequences referred to as glucocorticoid response elements (GRE) (Figure 1.10). Simple GREs are defined as an imperfect palindrome with a consensus sequence of 5' GGT ACA nnn TGT TCT 3' (Nordeen *et al.*, 1990). Monomeric GR first binds to the more conserved 3' site which induces an allosteric alteration to the GR that is favourable for the binding of a second GR monomer to the 5' site (Dahlman-Wright *et al.*, 1990;Hard *et al.*, 1990a;Dahlman-Wright *et al.*, 1991;La *et al.*, 1994). Binding to this type of GRE typically results in a relatively small induction of the target gene transcription (between 2- and 4-fold) (Schoneveld *et al.*, 2004b). The presence of a GRE alone in a gene promoter region does not necessarily denote sensitivity to Gc. When located amongst a cluster of response elements for other transcription factors this can promote acute regulation by Gc (23-fold induction for the carbamoyl-phosphate synthase gene) (Schoneveld *et al.*, 2004a). These clusters are referred to as Gc responsive units (GRUs). Mechanistically this allows for tissue specific gene regulation where the expression and activity of other transcription factors can directly influence Gc sensitivity (Schoneveld *et al.*, 2004b). Due to the interactions between the bound transcription factors the order of the response elements within a GRU is paramount for activation (Stafford *et al.*, 2001;Schoneveld *et al.*, 2004a). A GRE within a GRU can be in close proximity to or even overlap other response elements which means that GR occupancy can block binding of other transcription factors (Stromstedt *et al.*, 1991). In this way GRUs can be both activators and repressors of transcription. GR also binds to half GREs (GRE1/2) as a monomer with relative low affinity, but much like GRUs alteration in gene transcription is only achieved through interaction with accessory proteins (Segard-Maurel *et al.*, 1996;Schoneveld *et al.*, 2004b). The mouse

mammary tumour virus (MMTV) enhancer contains a GRE1/2, for example, that requires interaction with nuclear factor-1 (NF-1) (Hebbar and Archer, 2003). In this case, cooperation with the accessory protein NF-1 stabilises the weak affinity interaction with the GRE1/2. Not all GRE1/2 work in this way. Multiple GRE1/2 repeats do not require additional accessory proteins. The hCYP3A gene contains two adjacent GRE1/2 sites which drive expression by a bound GR dimer (Schuetz *et al.*, 1996). It is thought that the ligand binding domain rather than the D-box in the DBD enables GR dimerisation on neighbouring GRE1/2s (Aumais *et al.*, 1996).

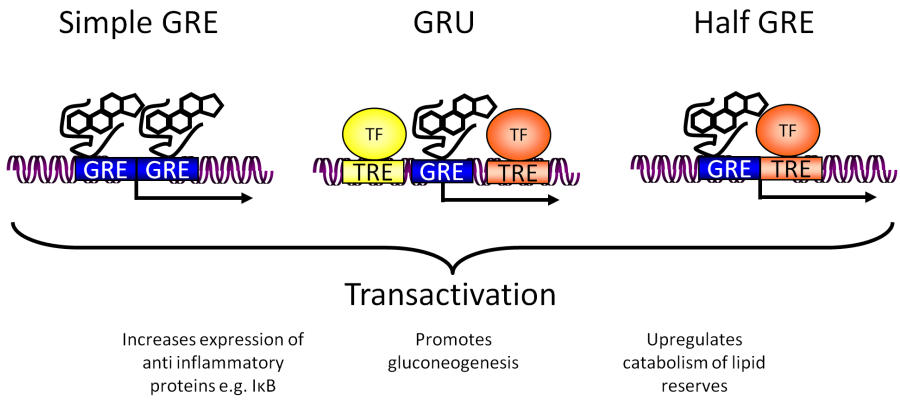

Figure 1.10: Mechanisms of GR transactivation. TF: Transcription factor; TRE: Transcription factor response element; GRE: glucocorticoid response element.

1.3.6 GR transrepression

Transrepression of Gc target genes predominantly occurs through GR interaction with other transcription factors through a process known as tethering. Many of the anti-inflammatory effects of Gc are through the inhibition of NFκB and AP1 which themselves drive expression of pro-inflammatory proteins (Figure 1.11). Several pathways triggered by environmental and inflammatory stimuli converge on NFκB and as such it is a key regulator of the immune response (Bonizzi and Karin, 2004). NFκB acts as a dimer comprising any combination of the five NFκB family members, forming either hetero or homodimers (Kumar *et al.*, 2004a). Of most importance to immune function is the heterodimers of p65 and p50 subunits (Phelps *et al.*, 2000). In unstimulated cells IκBα and IκBβ sequester the NFκB heterodimer in the cytoplasm preventing its activation (Baeuerle and Baltimore, 1988). Upon TNFα pathway activation for example, phosphorylation of IκB occurs leading to ubiquitination and

degradation by the proteasome, which permits nuclear translocation of NFκB where it binds to NFκB response elements (NREs) (Henkel *et al.*, 1993;Brown *et al.*, 1995;Chen *et al.*, 2002). NREs are located in promoter regions of genes responsible for leukocyte activation, cytokine production and adhesion molecule synthesis (Barnes *et al.*, 1998;Tian *et al.*, 2005). As part of a negative feedback mechanism there is also an NRE upstream of the gene for the IκB allowing for replenishment of degraded inhibitory molecules (de Martin R. *et al.*, 1993;Tam *et al.*, 2001).

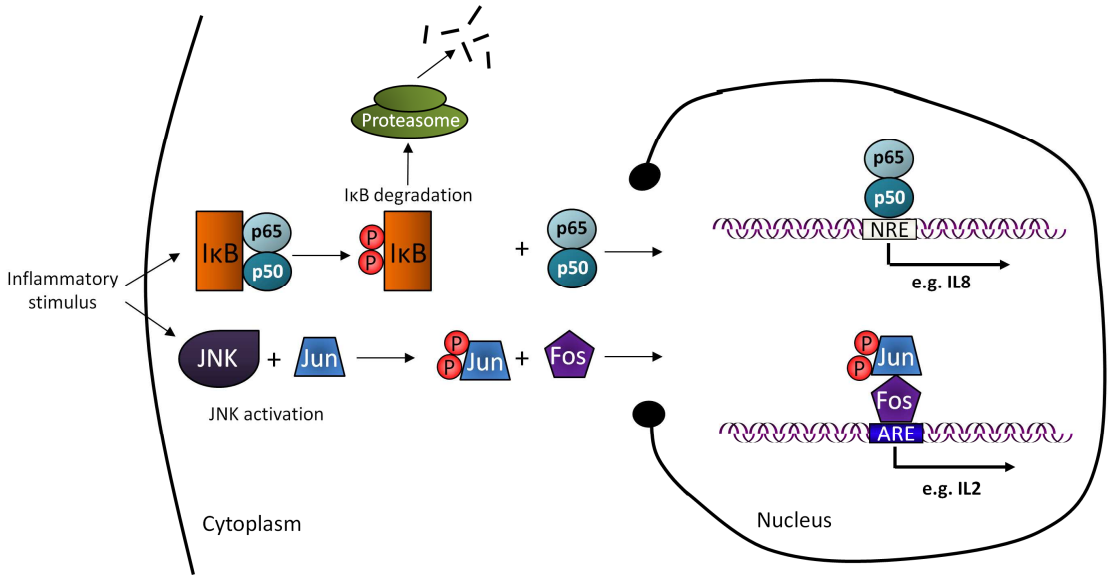

Figure 1.11: NFκB and AP1 activation. NFκB as a dimer of p50 and p65 is held in the cytoplasm by IκB which is degraded in response to proinflammatory stimulus. Free NFκB translocates to the nucleus where it binds NFκB response elements (NRE) activating production of proinflammatory genes such as IL8. Inflammatory stimulus also activates Jun N-terminal kinase (JNK) resulting in phosphorylation and dimerisation of Fos and Jun forming AP1. Following dimerisation AP1 translocates to the nucleus to bind AP1 response elements (ARE) to transactivate proinflammatory genes such as IL2.

The transcription factor activator protein-1 (AP1) influences the expression of proteins involved in a diverse range of processes, most notably, those involved in inflammation. Target genes include those that drive proliferation and the differentiation of immune cells (Zenz *et al.*, 2008). Analogous to NFκB, AP1 acts as a dimer consisting of proteins from the Fos, Jun, ATF and MAF families of proteins (Hess *et al.*, 2004). AP1 transcription factors all dimerise through a leucine-zipper motif with the most common form of AP1 comprising a heterodimer of members of the Fos and Jun families. Transcription of Fos and Jun is up-regulated by a variety of

environmental stressors. Activated Jun N-terminal kinase (JNK) phosphorylates Jun promoting dimerisation with Fos and binding to AP1 response elements (Beck *et al.*, 2009). As each dimer has a slightly different function, the variety of binding partners that form AP1 allow a complex level of transcriptional regulation (Hess *et al.*, 2004;Beck *et al.*, 2009).

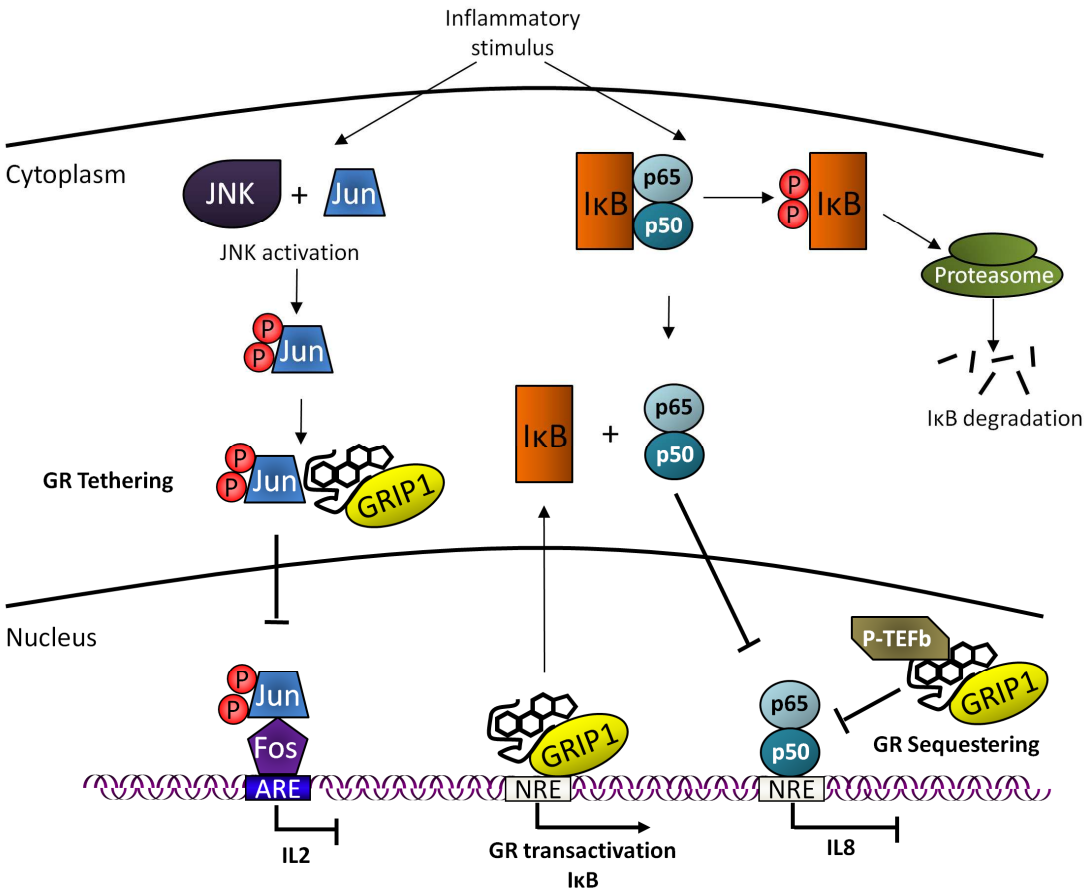

Figure 1.12: GR anti-inflammatory action. Ligand bound GR represses AP1 action through tethering to phosphorylated Jun. GR inhibits NFκB by sequestering cofactors required for transactivation and by upregulating production of IκB.

As an exception to the rule, activated GR can antagonise NFκB without binding it directly (De *et al.*, 2003). Phosphorylated NFκB at the IL8 promoter binds to P-TEFb, a factor which is required for transcription (Figure 1.12). Ligand-bound GR antagonises this interaction through sequestration of free P-TEFb thereby reducing IL8 expression through competitive inhibition (Mukaida *et al.*, 1994;Luecke and Yamamoto, 2005). As an additional exception, GR does not inhibit the NFκB feedback loop at the IκB site, but instead potentiates NFκB to increase IκB expression

and in turn terminate NFκB signalling (Auphan *et al.*, 1995;Scheinman *et al.*, 1995;Deroo and Archer, 2001). Concomitant with binding and inhibiting NFκB, GR also binds phosphorylated Jun preventing it partnering with Fos. In this way GR prevents expression of AP1 target genes such as IL-2 (Paliogianni *et al.*, 1993). Importantly, binding also prevents GR from interacting with GREs and so serves to modulate GR activity. The GR DBD is crucial for binding to AP1 as disruption of it abolishes GR mediated repression of AP1 target genes (Heck *et al.*, 1994). Repression of both NFκB and AP1 are mediated through GR interaction with corepressor molecules such as GR-interacting protein 1 (GRIP1) and thyroid receptor-interacting protein 6 (TRIP6). Knockdown of either of these corepressor proteins impairs GR transrepression (Rogatsky *et al.*, 2002;Kassel *et al.*, 2004;Diefenbacher *et al.*, 2008). Interaction of the GR with corepressors therefore not only facilitates transrepression, but also fine tunes GR function.

1.3.7 GR transactivation of anti inflammatory genes

Although most anti-inflammatory GR actions are mediated by monomeric receptor acting through tethering mechanisms, there are several examples of anti-inflammatory genes that are induced by dimeric GR binding to DNA. Studies using the dimerisation deficient $GR^{dim/dim}$ mouse have shown that Gc treatment is ineffective in mouse models of allergic contact dermatitis, antigen-induced arthritis, glucose-6-phosphate isomerase-induced arthritis, or LPS and TNFα induced septic shock (Tuckermann *et al.*, 2007;Baschant *et al.*, 2011;Kleiman *et al.*, 2012;Vandevyver *et al.*, 2012). In this context, Gc induced genes that play a role in regulating inflammation include NFκB inhibitors (IκB), MAPK phosphatase1 (MKP-1/DUSP1), Gc induced leucine zipper (GILZ) and Annexin 1.

1.3.8 MAPK phosphatase1 (MKP-1)/Dual specificity protein phosphatase 1 (DUSP1)

MAPK phosphatase1 (MPK-1), also known as Dual specificity protein phosphatase 1 (DUSP1), is a highly potent anti-inflammatory protein expressed in a variety of cells in response to Gc treatment (Abraham *et al.*, 2006;Shipp *et al.*, 2010). Mitogen activated protein kinases (MAPKs) such as ERKs, JNKs and p38 MAPKs respond to inflammatory challenge by triggering signalling cascades and the production of proinflammatory proteins (Raingeaud *et al.*, 1995). MPK-1 attenuates MAPK signalling by catalysing the removal of phosphate groups from threonine and tyrosine

residues conferring deactivation, with preference for p38 and JNK (Alessi *et al.*, 1993;Franklin and Kraft, 1997). The importance of MPK-1 is highlighted through studies in MPK-1 knockout mice where animals are more vulnerable to endotoxic or induced inflammatory shock (Wang *et al.*, 2008;Vandevyver *et al.*, 2012). GR homodimers bind to a GRE in the promoter region of MPK-1 driving the induction of this anti-inflammatory protein (Abraham *et al.*, 2006;Frijters *et al.*, 2010;Vandevyver *et al.*, 2012).

1.3.9 Gc induced leucine zipper (GILZ)

Gc induced leucine zipper (GILZ) is a well established Gc target gene frequently used to measure response to Gc treatment. Expression of GILZ is induced following stimulation with Gc in various cells, however this is lost in $GR^{dim/dim}$ mice (D'Adamio *et al.*, 1997;Rauch *et al.*, 2010). GILZ binds to p65 of NFκB and both fos and jun of AP1, interfering with proinflammatory signalling (Ayroldi *et al.*, 2001;Mittelstadt and Ashwell, 2001). In addition GILZ also inhibits activation of Ras and Raf-1 preventing the initiation of kinase signalling cascades that activate ERK (Ayroldi *et al.*, 2002). Furthermore mouse models of inflammatory bowel disease, rheumatoid arthritis and multiple sclerosis clearly demonstrated the immuno protective action of GILZ confirming its role in Gc mediated regulation of inflammation (Cannarile *et al.*, 2009;Beaulieu *et al.*, 2010;Srinivasan and Janardhanam, 2011).

1.3.10 Annexin 1

Treatment with Gc leads to production of the 37 kDa protein annexin 1, which in turn binds to phospholipids in a calcium dependent manner (Blackwell *et al.*, 1980;Errasfa and Russo-Marie, 1989). Annexin 1 directly interacts with phospholipase A2 preventing the production of proinflammatory mediators such as leukotrienes and prostaglandins by inhibiting production of the precursor molecule arachidonic acid (Kim *et al.*, 1994;Croxtall *et al.*, 1995). Nuclear annexin 1 also sequesters NFκB by binding to the p65 subunit and preventing interaction with DNA and COX-2 (Zhang *et al.*, 2010;Wang *et al.*, 2011). Expression of annexin in leukocytes prevents migration to, and adhesion at sites of inflammation (Lim *et al.*, 1998;D'Amico *et al.*, 2000). In animal models of arthritis, knock out of annexin 1 leads to a heightened inflammatory response unresponsive to Gc treatment (Yang *et al.*, 2004).

Collectively, this suggests that Gc induced production of annexin 1 is essential for resolution of inflammation.

1.3.11 Negative GRE

In addition to the transactivation of anti-inflammatory proteins GR also binds negative GREs (nGRE), resulting in transrepression of target genes. A consensus binding site for nGREs has been derived from comparison of known nGREs such as ATYACnnTnTGATCn which is less well conserved than the GRE consensus sequence (Dostert and Heinzel, 2004;Schoneveld *et al.*, 2004b). An example of a gene regulated by an nGRE is POMC which allows Gc to regulate their own production (Drouin *et al.*, 1989). nGREs account for more than 1000 genes, enabling finely tuned repression in response to fluctuating Gc levels, a result of circadian and stress signals (Surjit *et al.*, 2011).

1.4 GR coregulators

A range of comodulatory GR partners have been identified and their tissue specific expression dictates the cellular response to Gc. These comodulators fall into two broad categories: partners that confer increased transactivation (coactivators) and those that facilitate transrepression (corepressors) (Horwitz *et al.*, 1996). GR binds to comodulatory proteins through its AF1 and AF2 domains located in the NTD and LBD respectively (Warnmark *et al.*, 2000;Kumar *et al.*, 2001;Bledsoe *et al.*, 2002). Most comodulators bind to the GR through interaction with the AF2 domain, located in the LBD, via L-X-X-L-L motifs (where L=leucine and X=any aa) known as the nuclear receptor box (Heery *et al.*, 1997). Comodulator recruitment is highly dependent upon the structure of the ligand that is bound as this alters the conformation of the LBD. Investigation of GR crystal structures bound to agonist or antagonist ligands reveals an alteration in the position of helix 12 in the LBD, providing a mechanism to account for differential comodulator binding (Kauppi *et al.*, 2003). GR interacting proteins alter transcription by either promoting or inhibiting the formation of the transcriptional machinery, through modifying the constituents of the transcriptional machinery themselves or by directly altering DNA structure (Ford *et al.*, 1997;Johnson *et al.*, 2008).

1.4.1 Modifiers of transcriptional machinery

Once in the nucleus, ligand bound GR rapidly associates and dissociates from target DNA, resulting in recruitment of transcriptional machinery in a 'hit and run' manner (Nagaich *et al.*, 2004). The components of the GR heterocomplex facilitate the dynamic exchange of GR with its DNA template and enable reacquisition of the ligand signal (Stavreva *et al.*, 2004;Conway-Campbell *et al.*, 2011). DNA bound GR associates with comodulators with rapid nuclear mobility, enabling dynamic modulation of GR activity in response to changing environmental stimuli. The interaction of GR with comodulatory proteins is necessary for efficient recruitment of the transcriptional machinery. The steroid receptor coactivator (SRC/p160) family of cofactors are well defined GR coactivators that bind to ligand-bound GR via three L-X-X-L-L motifs on the surface of the protein (Heery *et al.*, 1997). The three members of this family, SRC1, SRC2 (GRIP1) and SRC3 are vital for recruitment of other coactivators such as CREB binding protein (CBP) which cooperatively bind to GR through SRC binding at the AF1 and AF2 domains. CBP recruits basal transcription machinery such as TBP and RNA pol II (McInerney *et al.*, 1998).

1.4.2 Modifiers of DNA organisation

In addition to promoting effective recruitment of transcriptional machinery, comodulatory proteins also promote or restrict access to DNA. Nuclear DNA is tightly packaged around protein histone complexes that form nucleosomes, the basis of chromatin. Organisation of DNA into chromatin prevents access to basal transcription machinery which generates an additional level of regulation (Cairns, 2009). Chromatin modification is achieved through covalent addition of chemical moieties to histone proteins or by nucleosome remodelling complexes (Kornberg and Lorch, 1999). These modifications result in the DNA becoming less compact (euchromatin) to permit access by transcription machinery (Sexton *et al.*, 2007). GR interacts with two types of chromatin remodeler; those that remodel nucleosomes in an ATP dependant manner and those that modify histones through addition or removal of acetyl groups. There are three families of ATP-dependent nucleosome remodelers, which comprise a large multiprotein complex with an ATPase catalytic core. The mechanism they utilise involves movement of the nucleosomes along the DNA. The SWI/SNF complex for example associates with GR through its AF1 domain to increase transactivation (Owen-Hughes *et al.*, 1996). Acetylation of

histones is increased in areas of active gene transcription. The large family of proteins that catalyse the addition/removal of acetyl groups to, or from, histones are histone acetyl transferases (HATs) and histone deacetylases (HDACs) respectively. Fundamentally, HATs are coactivators as their activity increases transcriptional machinery access to the DNA, whereas HDACs are corepressors that compact DNA repressing gene expression. Both the SRC family of coactivators and CBP possess some HAT activity. For example, GR associates with nuclear receptor corepressor (NCoR1) and silencing mediator for retinoic acid and thyroid hormone receptor (SMRT/NCoR2) that act as HDACs. Gc treatment also induces the expression of HDAC2 which represses NFκB driven gene expression (Jones and Shi, 2003;Ito *et al.*, 2006).

1.5 Regulation of GR by post translational modification

The interaction of GR with other proteins is regulated by post-translational modifications (PTM). The type of modification alters protein stability, localisation or conformation in the presence and absence of ligand. Understanding how PTMs regulate GR function is of great importance and could provide a potential therapeutic target.

1.5.1 Phosphorylation

The GR NTD contains several serine residues that are major targets for ligand-induced phosphorylation. Phosphorylation is reversible, covalent addition of organic phosphate (PO_4) to the hydroxyl side group of a serine, threonine or tyrosine residue. Addition of phosphate groups is catalysed by protein kinases, and their removal catalysed by phosphatases. Like other nuclear receptors, GR undergoes hyper-phosphorylation upon ligand binding. This, in turn induces a conformation change which directly alters protein stability, localisation, transactivation and transrepression activity. Interaction of GR with kinases and phosphatases is a critical regulatory step in the Gc response.

Early characterisation of GR phosphorylation examined rat and mouse GR. These protein orthologues are similar to human GR with equivalent phosphorylation sites, there are however some differences (Ismaili *et al.*, 2004). Human GR is phosphorylated on five serine residues (S113, S141, S203, S211 and S226) located in the AF1 domain within the NTD. Additionally Galliher-Beckley *et al* have described

S404 phosphorylation (Galliher-Beckley *et al.*, 2008b). Analysis of phosphorylation events in mitotic cells has also revealed that residues T8, S45, S134, S203, S211, S234 and S267A are phosphorylated during cell division (Daub *et al.*, 2008;Dephoure *et al.*, 2008). Antibodies raised against phosphorylated residues S203, S211 and S226 have enabled study into the function of these different modifications on the activity of the GR (Wang *et al.*, 2002;Ismaili *et al.*, 2004).

When compared to antagonists, GR agonists significantly increase phosphorylation at S211 and S226 (Wang *et al.*, 2002;Chen *et al.*, 2008). Phosphorylation at S203 is present when GR is unliganded and increases upon ligand binding (Ismaili *et al.*, 2004;Wang *et al.*, 2007). These phosphorylation events are interdependent since phosphorylation at one residue favours/inhibits modification of another (Wang *et al.*, 2007). Phosphorylation on S203 for example inhibits phosphorylation of S226 (Ismaili *et al.*, 2004;Wang *et al.*, 2007). In this way it is thought that modification of these residues represents the output/activity of various cellular kinases/phosphatases and therefore reflects an integrated response. These phosphorylation events induce conformational change in the GR producing novel interaction surfaces for cofactors. Phosphorylated GR sub-populations have preferential gene targets, supporting the role of phosphorylation in differential cofactor recruitment (Figure1.13) (Blind and Garabedian, 2008).

1.5.2 Regulators of phosphorylation

Protein kinases recognise a consensus amino acid sequence adjacent to the site of phosphorylation that further determines specificity. Phosphorylation sites within the GR have a common proline residue located after each serine, a consensus required for both cyclin-dependant kinase (CDK) and mitogen-activated kinase (MAPK) binding. Both families add phosphate groups to serine or threonine residues where the CDK consensus sequence is S/T-P-X-R/K and the MAPK consensus is ε-X-S/T-P (where ε = nonpolar aa). Residues S203 and S211 are modified by the CDK family whereas S226 is preferentially modified by the MAPK proteins (Ismaili *et al.*, 2004). CDK2/cyclin A kinase complex is able to phosphorylate both S203 and S211 whereas the CDK2/cyclin E kinase complex only modifies S203, but both lead to increased GR mediated transactivation (Krstic *et al.*, 1997). JNK, a MAPK enzyme,

phosphorylates S226 and negatively regulates GR mediated transactivation through driving its nuclear export (Itoh *et al.*, 2002).

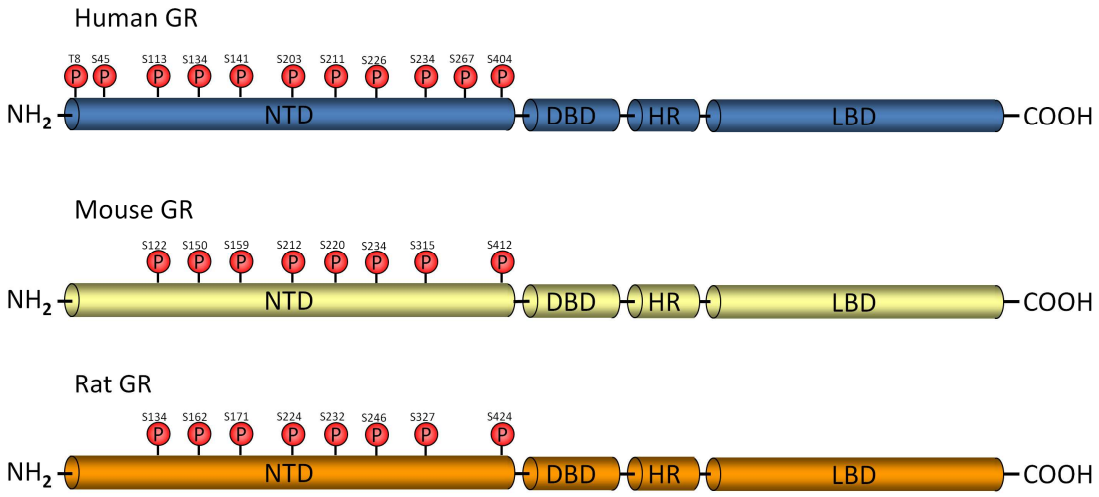

Figure 1.13: Phosphorylation of GR. The NTD of the GR is the site of phosphorylation events. The sites of phosphorylation differ between the human, mouse and rat GR orthologues. S: Serine; T: Threonine; P: Phosphorylation.

1.5.3 Functional consequences of phosphorylation

Ligand-induced phosphorylation events influence the subcellular location of the GR. Following addition of Gc, phosphorylation at S211 is associated with nuclear localisation of GR, whereas S203 is largely cytoplasmic (Wang *et al.*, 2002;Blind *et al.*, 2008). When in the nucleus, phosphorylation of GR on residue S404 by GSK3β triggers nuclear export, thereby prohibiting Gc-induced gene regulation (Galliher-Beckley *et al.*, 2008b). Work in our lab has recently shown that ligand-independent phosphorylation of GR in mitosis is linked to altered subcellular trafficking and that modification of GR on residues S203 and S211 facilitates distinct subcellular localisation. In fact, fluorescence microscopy has shown that phosphorylation at S203 targets GR to the centromere whereas phosphorylation at S211 is associated with the kinetochore during mitosis (Matthews *et al*, unpublished).

The phosphorylation status of GR also has strong links with GR stability. Phosphorylation on S203 for example promotes more rapid turnover in GR protein than phosphorylation on S211. Site directed mutagenesis of either of these sites confers greater protein stability, and site directed mutagenesis of S404 to an alanine

residue results in complete loss of degradation following treatment with Gc (Webster *et al.*, 1997;Wang *et al.*, 2002;Galliher-Beckley *et al.*, 2008b).

Additional regulation of GR activity is imposed by phosphatase enzymes that catalyse the removal of organic phosphate groups. Protein phosphatase 5 (PP5) associates with Hsp90 as part of the chaperone complex via its TPR domain and is linked with GR dephosphorylation (Silverstein *et al.*, 1997). Knockdown of PP5 drives increased phosphorylation at all three of the major sites in the AF1 domain, but most noticeably at S226 (Wang *et al.*, 2007). It is suggested that PP5 forms a bridge between GR and the transport protein dynein. This in turn serves to recycle GR exiting the nucleus, and returning it to conformation in the cytoplasm where it is free to rapidly bind hormone (Hinds, Jr. *et al.*, 2008). Treatment of cells with okadaic acid, an inhibitor of phosphatases, prevents GR from re-entering the nucleus following Gc treatment (DeFranco *et al.*, 1991). Reducing expression of PP5 through treatment with ISIS 15534 leads to accumulation of GR in the nucleus in the absence of ligand (Dean *et al.*, 2001). It has therefore been proposed that PP5 is a negative regulator of GR activity as its inhibition results in increased GR DNA binding, increased transcriptional activity of unliganded GR and augmented the response of ligand bound GR up to ten times of that found in the presence of PP5 (Zuo *et al.*, 1999).

1.5.4 Ubiquitination

Cellular proteins are targeted for degradation by the proteasome through the poly covalent addition of the 76 aa protein ubiquitin. This is a tightly regulated process, well conserved throughout eukaryotes consisting of three steps: activation of ubiquitin, transfer and ligation, each catalysed by E1, E2 and E3 enzymes respectively. GR is subject to ubiquitination and degradation by the proteasome following binding to Gc (Wallace and Cidlowski, 2001). Ubiquitination is targeted to lysine residues located within the PEST motif (Proline [P], Glutamine [E], Serine [S] and Threonine [T]), lysine 419 in GR (Rogers *et al.*, 1986). Proteasomal mediated degradation of GR was confirmed through *in vitro* application of proteasome selective inhibitor MG-132 which blocked downregulation of GR following Gc treatment, with a concomitant increase in transactivation. Substitution of lysine 419 to alanine abrogates degradation, supporting a role for ubiquitination in GR regulation (Wallace *et al.*, 2001). Interestingly S404 phosphorylation, which is mediated by GSK3β, is

located within the PEST degradation motif (Galliher-Beckley *et al.*, 2008b). The hyperphosphorylation that follows binding ligand could therefore regulate ubiquitination of the GR, since GR with all phosphorylation sites mutated to alanine does not undergo Gc mediated downregulation (Figure1.14) (Webster *et al.*, 1997).

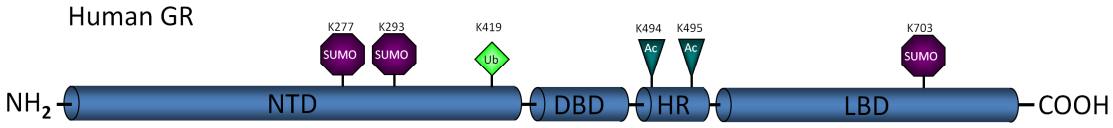

Figure 1.14: Post translational modifications of GR. The location of sites of sumoylation, ubiquitination, and acetylation with the GR are shown. K: Lysine; SUMO: Small ubiquitin-related modifier; Ub: Ubiquitination; Ac: Acetylation.

1.5.5 Sumoylation

Sumoylation is similar to ubiquitination but utilises a distinct pathway with a larger variety of functional outcomes. It comprises the reversible covalent addition of an 11 kDa protein called small ubiquitin-related modifier-1 (SUMO-1) to a lysine residue. This process is catalysed through the concerted effects of three enzymes, E1 activating enzyme, E2 conjugation enzyme (Ubc9) and an E3 ligase (Kaul *et al.*, 2002a). Within GR, the most well characterised addition of SUMO-1 occurs at a lysine residue within the ψ-K-X-E consensus sequence (where ψ = large hydrophobic aa) (Sternsdorf *et al.*, 1999;Johnson and Blobel, 1999). To date, three sites for sumoylation have been identified within the GR sequence; K277 and K293 within the NTD and K703 in the LBD. Addition of SUMO-1 to the NTD results in reduced transactivation from promoter regions suggesting that expression is regulated in a promoter dependent fashion (Tian *et al.*, 2002). In SUMO-1 over expression studies, Le Drean *et al* have reported that in the presence of Gc addition of SUMO-1 is capable of enhancing GR transactivation by up to 8-fold on GREs with multiple GR binding sites (Le Drean *et al.*, 2002). Over expression of SUMO-1 can also lead to GR protein instability, indicative of a role for sumoylation in GR turnover (Le Drean *et al.*, 2002). JNK mediated phosphorylation of S226, associated with transrepression, increases addition of SUMO-2 to the lysine residues in the NTD (Davies *et al.*, 2008). This suggests that the phosphorylation status of the GR could be key for influencing further post-translational modifications (Figure1.14).

1.5.6 Acetylation

Acetylation is the addition of an acetyl group to the amino group in the side chain of a lysine residue by a protein with HAT activity. This process has been well characterised as a regulatory mechanism imposed in gene expression through modification of histones, but is recognised to be present on other cellular proteins (Sadoul *et al.*, 2008). Acetylation following ligand binding has been observed on other steroid receptors such as the estrogen receptor and the androgen receptor and is required for effective transactivation of gene targets genes (Fu *et al.*, 2000). Much of the literature on acetylation relating to GR biology describes the indirect acetylation of cofactors that bind to GR such as Hsp90 (Murphy *et al.*, 2005;Aoyagi and Archer, 2005;Kovacs *et al.*, 2005). However, more recently it has been reported that the GR itself can be modified by acetylation causing alteration to its function. A putative acetylation motif was identified as K-X-K-K/R-X-K-K at aa 492-495 within the hinge region of the GR (Figure 1.14) (Wang *et al.*, 2004;Ito *et al.*, 2006). GR is acetylated after binding to Dex and mutation of either K494 or K495 results in loss of acetylation. Deacetylation of the GR by HDAC2 is required for GR interaction with NFκB (Ito *et al.*, 2006). The acetylation machinery has been found in association with enzymes of the ubiquitination pathway thereby providing a link between acetylation status and protein turnover (Fu *et al.*, 2004;Sadoul *et al.*, 2008). It was found by Nader *et al* that the transcription factor CLOCK and its partner BMAL-1, key constituents of the circadian pathway, repress GR transactivation (Nader *et al.*, 2009). These transcription factors form a negative feedback loop and are able to repress their own expression and therefore induce a oscillatory pattern of expression (Cermakian and Sassone-Corsi, 2000;Schibler and Sassone-Corsi, 2002). CLOCK/BMAL-1, which possesses HAT activity, physically interacts with the GR and acetylates the lysine residues in its hinge region, consequently reducing transactivation from GREs, and enhancing repression of NFκB induced gene expression (Nader *et al.*, 2009). These findings conflict the earlier results from Ito *et al* and together suggest that GR acetylation could have different outcomes dependent upon cell type or target gene. Clearly the effects of acetylation are complex and research into this is compounded by the difficulty of distinguishing acetylation of GR from its cofactors.

1.5.*7 Nitrosylation*

Nitric oxide (NO) is produced in large amounts during sepsis due to upregulation of nitric oxide synthase (NOS) enzyme. Gc administration can repress expression of NOS and thereby helps to prevent the onset of septic shock syndrome (Rees *et al.*, 1990). However Gc treatment is ineffective for treating septic shock and is thought to be due to NO reacting with cysteine side chains within the LBD of the GR, altering the affinity for Gc and binding to CBG (Huang *et al.*, 1987;Pugeat *et al.*, 1989;Simons, Jr. and Pratt, 1995). There are three cysteine residues located in close proximity to the steroid binding pocket of the GR (Stancato *et al.*, 1996). Galigniana *et al* showed that NO reduces binding of Gc to GR through S-nitrosylation of cysteine groups (Galigniana *et al.*, 1999). In this way this type of PTM is unlike the previously described as it is not catalysed by an enzyme and in this case only occurs in a disease state.

1.6 Glucocorticoid sensitivity.

Primary generalised glucocorticoid hypersensitivity (PGGH) and resistance (PGGR) are rare conditions linked to mutations in the GR. Due to the pleiotropic nature of GR and modular organisation, mutations within the receptor result in a wide range of phenotypic alterations. Most documented mutations render cells resistant to Gc action with varying degrees of severity, however some induce Gc hypersensitivity (Table 1.1).

Patients with PGGR lack sensitivity to Gc, resulting in overcompensation in the HPA axis and excess ACTH and cortisol production (Chrousos *et al.*, 1982;Chrousos *et al.*, 1993;Charmandari *et al.*, 2008b). The increased synthesis of ACTH and cortisol causes adrenocortical hyperplasia and elevated levels of other adrenal steroids. Overproduction of cortisol overrides the protective HSD2 mechanism in the kidneys and colon causing chronic activation of the MR which results in hypertension and hypokalemia (Charmandari, 2011). Increased production of cortisol also causes excessive production of androgens as they utilise the same precursor molecules. Hyperandrogenism can have various phenotypic results including hirsutism, male pattern hair loss, menstrual irregularities, female pseudohermaphroditism, precocious puberty and acne (Chrousos *et al.*, 1982;Chrousos *et al.*, 1993;Charmandari *et al.*, 2008b). The severity of the symptoms varies widely between cases with some

incidents of patients that are virtually asymptomatic (Bouligand *et al.*, 2010). The disparity of symptoms in patients with PGGR is likely due to the functional impact of the GR mutation and any compensation through differential expression of enzymes that control signalling pathways, such as HSD2 (Krozowski, 1999).

Table 1.1: Mutations conferring Gc resistance/hypersensitivity

Mutation	Outcome	Reference
GRβ 3' UTR	Gc resistance; rheumatoid arthritis	(Derijk *et al.*, 2001)
D401H	Gc hypersensitivity; hypertension; type 2 diabetes; visceral obesity; increased transactivation	(Charmandari *et al.*, 2008a)
C421Y	Gc resistance; resistance in leukaemia cell line	(Powers *et al.*, 1993)
4-bp deletion at exon/intron 6 splice site	Gc resistance; hypercortisolism; hirsutism; male pattern hair loss; menstrual irregularities	(Karl *et al.*, 1993)
R469STOP	Gc resistance; sub clinical hypercortisolism, bilateral adrenal hyperplasia	(Bouligand *et al.*, 2010)
R477H	Gc resistance; hirsutism; decreased transactivation	(Ruiz *et al.*, 2001)
I559N	Gc resistance; hypertension; decreased transactivation, Hyper androgenism, hypercortisolism, infertility, dominant negative; prevents GR nuclear import	(Karl *et al.*, 1996;Kino *et al.*, 2001)
V571A	Gc resistance; hypertension; hypokalemia; female pseudohermaphroditism; decreased ligand affinity; decreased transactivation	(Mendonca *et al.*, 2002)
D641V	Gc resistance; homozygous mutation; hypertension; decreased transactivation	(Chrousos *et al.*, 1982;Hurley *et al.*, 1991)
G679S	Gc resistance; hirsutism; decreased transactivation	(Ruiz *et al.*, 2001)
R714Q	Gc resistance; hypokalemia; hypertension; hypoglycemia; advanced bone age; decreased transactivation; dominant negative	(Nader *et al.*, 2010)
V729I	Gc resistance; homozygous mutation ; precocious puberty; hyperandrogenism; decreased affinity for ligand	(Malchoff *et al.*, 1993)
F737L	Gc resistance; hypertension; hypokalemia; decreased transactivation; dominant negative	(Charmandari *et al.*, 2007)
I747M	Gc resistance; hirsutism; cystic acne; oligo amenorrhea; dominant negative; prevents GR nuclear import	(Vottero *et al.*, 2002)
L773P	Gc resistance; hirsutism; acne; hypertension; anxiety; dominant negative; decreased transactivation	(Charmandari *et al.*, 2005)
2-bp deletion at nt 2318-9 773	Gc resistance; hypoglycemia; hypertension; decreased transactivation; no ligand binding	(McMahon *et al.*, 2010)

PGGH causes a hypersensitivity of tissues to Gc and compensation by the HPA axis. This heightened sensitivity to Gc results in visceral obesity, elevated circulating cholesterol and triglycerides, type 2 diabetes and hypertension (Charmandari *et al.*, 2008a). Mutations that cause PGGH are very rare, however the N363S polymorphism is linked to increased *in vivo* Gc sensitivity and correlates with increased BMI and male obesity (Huizenga *et al.*, 1998a;Lin *et al.*, 1999;Dobson *et al.*, 2001).

1.6.1 GR polymorphisms

In addition to the mutations within the GR other common changes in GR sequence known as polymorphisms have been identified. It is established that the sensitivity to Gc varies widely within the general population, as seen by responses to the 0.25mg dex suppression test (Huizenga *et al.*, 1998b). A number of studies have been carried out in order to determine whether or not GR polymorphisms are linked to Gc sensitivity (Derijk and de Kloet, 2008). Polymorphisms are common variations in genomic DNA sequence that occur in a population with a frequency greater than 1%. Many of the polymorphisms described for GR are found in the N terminal domain, probably due to the importance of maintaining the function of the C terminal ligand binding domain. Higher frequency polymorphisms can be expressed together, giving rise to haplotypes, which could potentially have different action when combined.

In exon 2 codons 22 and 23 have linked polymorphism where a glutamic acid and arginine (GAGAGG - ER) are switched for glutamic acid and lysine (GAAAAG - EK), known as the ER22/23EK polymorphism (Koper *et al.*, 1997). Studies further demonstrated that expression of ER22/23EK GR decreases Gc sensitivity. This resulted in improved physique at a young age, reduces excessive weight gain during pregnancy, lowers insulin and cholesterol levels, decreased risk of cardiovascular disease, decreased risk of dementia and increased survival rate in the elderly (van Rossum *et al.*, 2002;van Rossum *et al.*, 2004a;van Rossum *et al.*, 2004b;Russcher *et al.*, 2005a;Bertalan *et al.*, 2009). An alteration in the ratio of GR isoforms has been proposed as the mechanism for the observed decrease in Gc sensitivity with the ER22/23EK GR polymorphism. There was found to be a 15% increase in the expression of the less transcriptionally active GR-A isoform with no change in overall GR levels, most likely due to a stabilisation of GR-A mRNA (Yudt *et al.*, 2001;Russcher *et al.*, 2005b).

In a similar manner to ER22/23EK the A3669G polymorphism in the 3'UTR of exon 9β, discovered by Derijk and co-workers, associated with decreased sensitivity to Gc (Derijk *et al.*, 2001). ATTTA motifs act to destabilise mRNA and mutation of these motifs in c-fos and IL3 transcripts lead to an increased mRNA half life (Shyu *et al.*, 1991;Stoecklin *et al.*, 1994). The A3669G polymorphism removes a ATTTA motif in GRβ mRNA resulting in stabilisation of this transcript. Much like the isoform GR-A, GRβ is thought to have a dominant negative effect on GRα, therefore decreasing sensitivity to Gc (Oakley *et al.*, 1999). A study by van den Akker and co-workers suggests that the 9β A3669G specifically decreases transrepression with no effect on transactivation (van den Akker *et al.*, 2006b). Male carriers of this polymorphism were shown to have improved lipid profile whilst female carriers showed reduced central obesity (Syed *et al.*, 2006). In support of its role in decreasing sensitivity to Gc, carriers of 9β A3669G displayed a 68 % reduced risk of persistent nasel carriage of S.aureus (van den Akker *et al.*, 2006a). This polymorphism was first described in association with rheumatoid arthritis (RA) and was suggested to predispose individuals toward RA in the haplotype of ER22/23EK and 9β A3669G (van Oosten *et al.*, 2010). However further study has shown no association of GR polymorphisms with development of RA (Donn *et al.*, 2007).

As well as polymorphisms that confer reduced sensitivity to Gc there are examples that have been suggested to increase sensitivity. The substitution polymorphism of AAT for AGT at codon 363 (Asparagine to serine, N363S) in GR exon 2 was first identified in a Dutch family with hypercortisolism, but its function was not determined until sometime later (Karl *et al.*, 1993). The mutation leads to the creation of a new phosphorylation site (Huizenga *et al.*, 1998a). Carriers of N363S are thought to have greater sensitivity to Gc and studies have associated this polymorphism with increased BMI in normal and type II diabetes patients, cardiovascular disease and more recently in uncontrolled bronchial asthma (Lin *et al.*, 1999;Di Blasio *et al.*, 2003;Lin *et al.*, 2003;Roussel *et al.*, 2003;Panek *et al.*, 2012). However other studies did not support a role for N363S in disease (Rosmond *et al.*, 2001;Echwald *et al.*, 2001;Marti *et al.*, 2006). Further work in cell lines and PBMCs from carriers of N363S demonstrated a small increase in GR transactivation activity but no effect on

transrepression however microarray data showed significant regulation of genes by the N363S GR variant (Russcher *et al.*, 2005a;Jewell and Cidlowski, 2007).

The *Bcl*1 substitution polymorphism is a TGATGA to TCATGA in intron B, 647 basepairs downstream of GR exon 2. This polymorphism was discovered when enzyme digestion of the DNA region that encodes GR gave a 2.3kb fragment and a longer 4.5kb fragment from carriers, due to the loss of a *Bcl*1 cut site (Fleury *et al.*, 2003). There have been several reports that have suggested this polymorphism increases sensitivity to Gc and contributes to increased insulin production, increased abdominal fat, increased BMI and increased leptin (Weaver *et al.*, 1992;Buemann *et al.*, 1997;Rosmond *et al.*, 2000;Ukkola *et al.*, 2001;van Rossum *et al.*, 2003). It is not understood how the *Bcl*1 polymorphism alters sensitivity to Gc.

Identification, and characterisation of GR mutations and polymorphisms have highlighted the complexity of Gc biology. Mutations within the GR that lead to a clinical phenotype are very rare, but several cases of GR haploinsufficiency have been reported that are asymptomatic. This suggests that this type of mutation could be more common within the population and could in part explain the variation in Gc sensitivity. The frequency of GR polymorphisms varies considerably and much like GR mutations the genetic background upon which these are expressed undoubtedly alters an individuals' response. Understanding the reasons for development of symptoms in patient cohorts is obviously very important, but *in vitro* study of GR mutations is also a valuable tool to dissect GR function, and inform rational drug design.

1.7 Modulating GR function by ligand structure

Design of synthetic GR ligands with increased potency did not lead to a diminished side effect profile. Therefore ligands with the ability to separate the beneficial GR actions from the unwanted off target effects, termed selective glucocorticoid receptor agonists (SEGRAs) or dissociated steroids, have been sought. Originally it was assumed that the anti-inflammatory actions of Gc were due to transrepression of pro-inflammatory genes via interaction of monomeric GR with other transcription factors such as NFκB or AP1. Off target actions of Gc are attributed to the transactivation activity of dimeric GR binding to target DNA. Indeed mice with a specific mutation

that prevents GR from dimerising ($GR^{dim/dim}$) lose the ability to transactivate metabolic genes that contain a GRE, yet the anti-inflammatory action of Gc treatment is retained (Reichardt *et al.*, 1998;Frijters *et al.*, 2010). Comparison of gene expression in the livers of wild type and $GR^{dim/dim}$ mice treated with Gc revealed the importance of dimerisation for target gene induction. This observation prompted the search for ligands that would favour the transrepressive action of GR. This has yielded lots of potential candidate therapeutics, examples of which are detailed in table 1.2 below.

Table 1.2: Dissociative GR ligands

Δ-9,11 analog	↓ TNFα potentiated NFκB signalling. No transactivation of GRE reporters. Muscular dystrophy mouse model ↓ inflammation. Reduced side effects.	(Baudy *et al.*, 2012)
Ginsenoside (Rg1)	Stabilisation of IκB. ↓ MAPK activation in response to LPS. ↓ Inflammation in mouse models of acute and chronic inflammation with no hyperglycaemia or osteoporosis.	(Lee *et al.*, 1997;Chung *et al.*, 1998;Leung *et al.*, 2006;Du *et al.*, 2011)
Compound A (phenyl aziridine precursor)	Non steroidal. ↓ GR protein down regulation. Favours GR monomer formation. ↓ IL6, ↓ E-selectin, IL1β and inhibits NFκB. ↓ TNFα driven nuclear translocation and DNA binding of p65 and ↓ MAPK activation. No transactivation of GRE reporters. No hyperglycaemia or hyperinsulinemia Adverse side effects due to decomposition in vivo	(De *et al.*, 2005;Dewint *et al.*, 2008;Gossye *et al.*, 2009;Wust *et al.*, 2009;van *et al.*, 2010;Robertson *et al.*, 2010;Reber *et al.*, 2012)
AL-438	Non steroidal. ↓ IL6 and ↓ E-selectin. ↓ hyperglycaemia ↓ osteoporosis Differential cofactor recruitment ↓ peroxisomal proliferator-activated receptor gamma coactivator-1	(Coghlan *et al.*, 2003;Owen *et al.*, 2007)
LGD5552	Non steroidal ↓ IL6, ↓ E-selectin, ↓ Monocyte chemoattractant protein-1, ↑ IL10 ↓ Inflammation in mouse models of arthritis No fat disposition, no osteoporosis and no loss of adrenal weight	(Miner *et al.*, 2007;Lopez *et al.*, 2008)

Although some promising dissociative GR ligands have been discovered, recent research has highlighted that not all the anti-inflammatory actions of GR are due to transrepression. Contrary to earlier understanding, it is becoming clear that the anti-inflammatory actions of GR rely on both its transactivation and transrepression activity. The direct binding of GR to DNA through interaction with nGREs that suppress pro-inflammatory genes and GREs upstream of anti-inflammatory proteins is vital for effective resolution of inflammation. Therefore the design of SEGRA's/dissociative steroids that solely target GR transrepression will only mediate part of its anti-inflammatory action.

1.8 Summary

Glucocorticoids are the most potent anti-inflammatory agent known, yet there is still much to understand about how they work. Gc actions are mediated via the ubiquitously expressed GR which is a ligand activated transcription factor. GR mediates temporally and spatially distinct effects within the cell activating signalling cascades in the cytoplasm within minutes and translocating to the nucleus to regulate gene expression over hours. GR has a dynamic structure and adopts a different conformation depending upon which ligand is bound, generating ligand specific protein interaction surfaces. GR is also modified on multiple residues by phosphorylation, acetylation, sumoylation and ubiquitination in response to ligand binding. The magnitude and combination of these events dictates GR stability, compartmentalisation and activity. The gene targets for ligand activated GR are diverse, due to its role in glucose metabolism and the immune response, meaning the side effect profile of Gc therapy is large and diverse. Sensitivity to Gc therapy also varies between individuals with the resultant outcome of treatment being unpredictable. Ultimately understanding how GR mediates its diverse effects, will allow screening for sensitivity and development of specific treatment regimens.

1.9 Hypothesis and aims of PhD

This work aims to address two major issues that limit the use of Gc. These are the genetic factors underlying Gc resistance, and the quest for selective drugs with limited side effects.

i) Genetic factors that underlie Gc resistance

The genetic factors that contribute to Gc resistance are poorly understood. Familial Gc resistance is rare and several cases have been attributed to mutations in the GR. A case of familial Gc resistance has been identified. I plan to obtain DNA samples from these patients and sequence GR using tiled primers. Any mutations identified will be generated for *in-vitro* studies. The effects of the mutation on GR activation, trafficking and function will be determined using combinations of immunoblotting, immunofluorescence, synthetic reporters and qPCR assays. An immortalised cell line will also be generated which will enable further study into Gc resistance in these individuals.

ii) The selectivity of Gc actions

The discovery of potent non-steroidal Gc (NSG) for the GR has provided a completely new line of research for drug design. GlaxoSmithKline have designed a panel of NSGs that bind to GR with high affinity. In preliminary studies it was noted that two ligands failed to induce transcription of Per1 which is rapidly induced following Dex treatment. It is possible therefore that these NSGs may be selective Gc ligands. This project will determine whether these NSGs differ mechanistically from standard synthetic Gc. Given the GR has distinct roles in the nucleus and cytoplasm the compartment GR resides in is an important determinant of the cells response to Gc. As it is unknown whether these NSGs alter the trafficking of the GR upon binding, this will be investigated using real time using fluorescent microscopy. The ability of GR to activate kinases within the cytoplasm and regulate target genes within the nucleus in response to these ligands will be measured by specific phospho-immunoblot and qPCR respectively. Screening the novel NSGs in this way will enable identification of drugs with restricted selectivity of action. In turn their structure could then be used as a basis for development of a new generation of synthetic Gc, which offer equal potency with a diminished side effect profile.

Chapter 2: Familial Glucocorticoid Resistance

Familial glucocorticoid resistance caused by a novel frameshift glucocorticoid receptor mutation

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2.1 Abstract

Context: Familial glucocorticoid resistance is a rare condition with a typical presentation of women with hirsutism and hypertension, with or without hypokalemia.

Objective: The aim was to determine the cause of apparent glucocorticoid resistance in a young woman.

Patients and Methods: We studied a family with a novel glucocorticoid receptor (GR) mutation and a surprisingly mild phenotype. Their discovery resulted from serendipitous measurement of serum cortisol with little biochemical or clinical evidence for either hyperandrogenism or mineralocorticoid excess.

Results: The causative mutation was identified as a frameshift mutation in exon 6. Transformed peripheral blood lymphocytes were generated to analyze GR expression *in vitro*. Carriers of the mutation had less full-length GR, but the predicted mutant GR protein was not detected. However, this does not exclude expression *in vivo*, and so the mutant GR (Δ612GR) was expressed *in vitro*. Simple reporter gene assays suggested that Δ612GR has dominant negative activity. Δ612GR was not subject to ligand-dependent Ser211 phosphorylation or to ligand-dependent degradation. A fluorophore-tagged construct showed that Δ612GR did not translocate to the nucleus in response to ligand and retarded translocation of the wild-type GR. These data suggest that Δ612GR is not capable of binding ligand and exerts dominant negative activity through heterodimerization with wild-type GR.

Conclusion: Therefore, we describe a novel, naturally occurring GR mutation that results in familial glucocorticoid resistance. The mutant GR protein, if expressed *in vivo*, is predicted to exert dominant negative activity by impairing wild-type GR nuclear translocation.

2.2 Introduction

Familial glucocorticoid resistance is a rare endocrine syndrome characterized by reduced cortisol activity (van Rossum and Lamberts, 2006). This results in a compensatory increase in the tone of the hypothalamic-pituitary-adrenal axis, increased concentrations of ACTH, and hypertrophic adrenal glands. Adrenal stimulation by ACTH, in a manner analogous to that of congenital adrenal hyperplasia, results in elevated circulating concentrations of mineralocorticoids (including precursors such as deoxycorticosterone and corticosterone) and adrenal androgens (van Rossum et al., 2006). Due to such hormone excess, patients present with hypertension and/or hypokalemic alkalosis and, in females, hirsutism, male pattern balding, and menstrual irregularities. Although in healthy individuals, the type II 11-βhydroxysteroid dehydrogenase enzyme protects the renal mineralocorticoid receptor from binding by cortisol, in the syndrome of familial glucocorticoid resistance, the much higher circulating cortisol concentrations apparently overwhelm the enzyme's barrier function and so illicitly activate the mineralocorticoid receptor (van Rossum et al., 2006).

The syndrome of familial glucocorticoid resistance is rare and has been ascribed to mutations in the glucocorticoid receptor (GR) gene. Inactivating mutations in the ligand binding domain, DNA binding domain, and a splice site mutation have been described (Hurley et al., 1991;Karl et al., 1993;Malchoff et al., 1993;Karl et al., 1996;Kino et al., 2001;Ruiz et al., 2001;Mendonca et al., 2002;Vottero et al., 2002;Charmandari et al., 2005;Charmandari et al., 2006;Charmandari et al., 2007;Charmandari et al., 2008a;Charmandari et al., 2008b;McMahon et al., 2010). Familial glucocorticoid resistance may be inherited as an autosomal dominant or recessive trait. Dominant inheritance may be due to dominant negative activity of the expressed mutant GR or to haploinsufficiency (Karl et al., 1993;Kino et al., 2001;Vottero et al., 2002). However, the heterozygous parents of a child presenting with complete generalized glucocorticoid resistance due to homozygous carriage of a null GR mutation have no endocrine abnormality, suggesting phenotype heterogeneity (McMahon et al., 2010).

We describe a family with glucocorticoid resistance due to a frameshift mutation in the GR gene, which results in expression of a truncated GR protein. The three affected women have a mild phenotype, because indeed the family was only discovered as a result of a serendipitous serum cortisol assay. The mutation introduces a premature stop codon after insertion of 15 novel amino acids. *In vitro* studies demonstrated that the truncated receptor was unresponsive to ligand, but exerted modest ligand-independent anti-nuclear factor κB (NFkB) activity and template specific dominant negative action on transactivation by the wild-type GR. The truncated receptor remained cytoplasmic before and after ligand addition and delayed nuclear translocation of the wild-type receptor, suggesting heterodimerization.

2.3 Subjects and methods

2.3.1 Clinical diagnosis of familial glucocorticoid resistance

A 20 year old female presented to her primary care physician complaining of fatigue. A 0900 hours serum cortisol concentration of 1636 nmol/l was obtained, raising the possibility of Cushing's syndrome, hence she was referred for an endocrine opinion (Table 2.1). On examination she had no clinical features of Cushing's syndrome. Treatment with a combined oral contraceptive pill in the form of Cilest (ethinylestradiol 35 mcg/norgestimate 250 mcg) was thought to be responsible but despite stopping for 6 weeks a repeat 0900 hours cortisol remain elevated at 1003 nmol/l. After obtaining informed consent the patient (index) was investigated, as was her 18 year old sister and parents (Table 2.1). The index's sister and mother had both received laser treatment for facial hirsutism. The only possible relevant family history was that of facial hirsutism in the deceased maternal grandmother. The results of a dexamethasone suppression test revealed failure of cortisol suppression in all three women (Table 2.1), so fulfilling the criteria for diagnosis of familial Gc resistance. Investigations on the father were normal. The only apparent phenotypical abnormality was mild facial hirsutism in the index's sister, as evidenced by a minimally raised Ferriman and Gallwey score. The mother reported previous hirsutism, but did not have a raised Ferriman and Gallwey score, possibly as a result of the combined oral contraceptive pill which she had taken for over 10 years only stopping 6 weeks prior to investigation. All three women had raised androstenedione concentrations, and minimally elevated calculated free testosterone (Table 2.1). One sister, the proband, had suppressed renin concentration, suggesting apparent mineralocorticoid excess, likely due to her raised cortisol, but all three were normotensive and normokalaemic, and none were taking any medication (Table 2.1).

Table 2.1: Biochemical investigations.

Investigations	**Mother**	**Sister 1 (index)**	**Sister 2**
Weight (Kg)	58.8	55.2	64.2
BMI (Kg/m^2)	24	23.5	24.5
Hirsutism score (Ferriman-Gallwey)	3 (<8)	5 (<8)	**11** (<8)
0900h ACTH (ng/l)	**91.2** (7-63)	**89.1** (7-63)	48 (7-63)
0900h cortisol (nmol/l)	**1126** (176-536)	**915** (176-536)	**780** (176-536)
ACTH/cortisol profile 0900h 1100h 1400h 1600h	33.2/853 37.9/672 28.6/520 22/350	43.1/811 21.8/412 31.0/436 19.0/393	24.2/1165 7.2/594 17.0/708 22.2/711
Testosterone (nmol/l)	1.5 (<2.9)	2.4 (<2.9)	2.7 (<2.9)
Androstenedione (nmol/l)	**14.1** (4.0-10.2)	**16.3** (4.0-10.2)	**22.5** (4.0-10.2)
DHEAS (µmol/l)	6.0 (0.96-6.95)	5.5 (4.02-11)	9.5 (1.77-9.99)
17-hydroxyprogesterone (nmol/l)	3.4 (<20nmol/l)	2.6 (<20nmol/l)	6.3 (<20nmol/l)
Urinary free cortisol (nmol/24hr)	212 (<290)	207 (<290)	**460** (<290)
Plasma renin activity (nmol/l/h)	2.2 (1.5-3.5)	**0.8** (1.5-3.5)	1.8 (1.5-3.5)
Aldosterone (pmol/l)	285 (100-450)	320 (100-450)	490 (100-450)
SHBG (nmol/l)	42 (26.1-110)	83.6 (26.1-110)	33.6 (26.1-110)
FAI	3.57 (<7.5)	2.87 (<7.5)	**8.04** (<7.5)
Free Testosterone (pmol/l)	**23.1** (2.82-21.86)	**22.7** (2.82-21.86)	**47.8** (2.82-21.86)
Bio available testosterone (nmol/l)	0.506	0.545	1.17
1mg dexamethasone test 0900h cortisol(nmol/l)	**129** (<50nmol/l)	**272** (<50nmol/L)	**321** (<50nmol/L)

The characteristics and relevant biochemical investigations for the index family are shown. Abnormal results are highlighted in bold, normal range shown in brackets. DHEAS, dehydroepiandrosterone sulfate; SHBG, sex hormone binding globulin; FAI, free androgen index.

2.3.2 Research design

Anti-GR (clone 41) was obtained from BD Biosciences (Oxford, UK); anti-phospho-(Ser211)-GR, from Cell Signaling Technology (Danvers, MA); horseradish peroxidase conjugated antimouse and antirabbit, from GE Healthcare (Buckinghamshire, UK); dexamethasone, from Sigma (Dorset, UK); and mifepristone (RU486), from Sigma. AH3-Luciferase and NRE-luciferase have been previously described (Matthews *et al.*, 2008;Matthews *et al.*, 2009).

2.3.3 Amplification of genomic DNA by PCR

PCR of patient DNA was performed in a final volume of 25µl containing 500 ng genomic DNA, 50 pmol of each forward and reverse oligonucleotide primer, 0.25 µl of each 25 µM deoxynucleotide triphosphate (Bioline, London, UK), 2.4 µl x10 NH4 buffer (Bioline), 0.75 µl of 50 mM $MgCl_2$ (Bioline), and 1.25 U Taq polymerase (Bioline). The PCR consisted of 30 cycles, with each cycle made up of a denaturation step of 1 min at 94 ^{o}C, an annealing step of 1-min gradient between 54 or 60 ^{o}C, and a primer extension step of 1 min at 72 ^{o}C. Primer sequences are shown in table 2.2. Before the initial cycle, the temperature was increased to 95 ^{o}C for 4 min; after the final cycle, an extension step of 8 min was added. Each PCR was placed in the gradient at a temperature that corresponded to the annealing temperature of the primers (exon 4, 55 ^{o}C; exon 7, 55 ^{o}C; exon 3, 56 ^{o}C; exon 6, 57 ^{o}C; exon 9, 57 ^{o}C; exon 8, 58 ^{o}C; and exon 5, 59 ^{o}C). Single products were confirmed by agarose gel electrophoresis.

Table 2.2: Primers to amplify GR from genomic DNA.

	Forward primer	**Reverse primer**
Exon 3	tgctagcacttgaagccaga	ttagcctttcatgggctttg
Exon 4	accggaaacaaagacagagg	tcccatttttattgggcagt
Exon 5	cgcagaccttcccattacag	ttcacctgactctccccttc
Exon 6	ttggcaattcccacagagat	gccccaagcactcataactc
Exon 7	cagccaagatgcaggaagtt	ggccttcatatttcatgcttt
Exon 8	tcaagtgcagaatggcagac	caccaacatccacaaactgg
Exon 9	tgagatgttcccactgacca	caactgcttctgttgccaag

2.3.4 Sequencing and cloning

A QIAquick Spin PCR Purification Kit (QIAGEN, Crawley, UK) was used to purify PCR product according to the manufacturer's instructions. Sequencing was carried out at the University of Manchester core facility using the corresponding forward or reverse primer with BIG Dye Terminator cycle sequencing chemistry (Applied Biosystems, Foster City, CA) and an Applied Biosystems 3730 DNA Analyzer. The obtained sequencing data were analyzed using Chromas Lite 2.01 (www.technelysium.com.ac/chromas_lite.html). The amplified PCR product of exon 6 was cloned into a pGEM-T–Easy vector (Promega, Madison, WI) as described by the manufacturer. Purified plasmid was subsequently sequenced using T7 forward or SP6 reverse sequencing primers.

2.3.5 Site-directed mutagenesis

pcDNA3 Δ612GR was created by a single base deletion (C1835) from the pcDNA3 GR plasmid using a Quick Change Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA) according to the manufacturer's instructions (primers: forward, ttgctctggggtggagatatatagacaatcaagtgc; reverse, gcacttgattgtctatatatctccaccccagagcaa). A fragment spanning the region containing the deletion (C1835) Δ612GR was excised from pcDNA3 Δ 612GR using EcoRI (Roche Diagnostics, Burgess Hill, UK) and subcloned into pcDNA3-green fluorescent protein (GFP) using a rapid ligation kit (Roche Diagnostics). Correct orientation was confirmed by DNA sequencing using tiled primers to cover the entire 2.5-kb sequence (primer sequences shown in table 2.3).

2.3.6 Cell culture and maintenance

Human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Teddington, UK) were cultured in low glucose (1 g/liter) DMEM from PAA Laboratories (Yeovil, UK) with stable glutamine (2 mM; PAA) supplemented with 10% heat inactivated fetal bovine serum (Invitrogen, Paisley, UK) or 10% charcoal dextran stripped fetal calf serum (sFCS; Invitrogen) in a humidified atmosphere of 5% carbon dioxide at 37 ^{o}C. The human osteosarcoma cell line (U20S; American Type Culture Collection) was cultured in DMEM with stable glutamine 2 mM supplemented with 10% fetal bovine serum or 10% sFCS in a humidified atmosphere

of 5% carbon dioxide at 37 ^{o}C (Matthews *et al.*, 2008;Matthews *et al.*, 2009). Lymphocyte cultures were immortalized using Epstein-Barr virus infection in the regional clinical genetics laboratory after full patient consent.

Table 2.3: Primers to verify construction of Δ612GR-GFP expression vector

	Forward primer	**Reverse primer**
Δ612GR	caccaaaatcaacgggactt	cacactgctggggttttctt
Δ612GR GFP	cgacaaccactacctgagca	tccatcacatctcccctctc
Tiling primer set 1	tgaaggtttctgcgtcttca	ttgcttactgagccttttgga
Tiling primer set 2	catccactgctgtgtctgct	gggacccagaagaaaactcc
Tiling primer set 3	gggtccccaggtaaagagac	gttttcacttggggcagtgt
Tiling primer set 4	cccctggggtaattaagca	gcttctgatcctgctgttga
Tiling primer set 5	aggaccacctcccaaactct	tgtttttcgagcttccaggt
Tiling primer set 6	ccctaccctggtgtcactgt	agggtcatttggtcatccag
Tiling primer set 7	ggcaataccaggtttcagga	tggtcgtacatgcagggtag
Tiling primer set 8	acggtctgaagagccaagag	ccacttcatgcatagaatccaa
Tiling primer set 9	attccccgagatgttagctg	gatggctggcaactagaagg

2.3.7 Immunoblot analysis

Cell lysates (20 µg protein) in RIPA buffer [50mM Tris Cl (pH 7.4), 1% Nonidet P-40 (Igepal), 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA] containing protease and phosphatase inhibitors were electrophoresed on Tris/Glycine 4–12% gels (Invitrogen) and transferred to 0.2 µm nitrocellulose membranes (Bio-Rad Laboratories, Hertfordshire, UK) overnight at 4 ^{o}C. Membranes were blocked for 1 h (0.15 M NaCl, 2% dried milk, 0.1% Tween 20) and incubated with primary antibodies (diluted in blocking buffer) overnight at 4 ^{o}C. After three 10-min washes [88 mM Tris (pH 7.8), 0.25% dried milk, 0.1% Tween 20], membranes were incubated with a species-specific horseradish peroxidase-conjugated secondary antibody (diluted in wash buffer) for 1h at room temperature, and washed three more times for 10 min each. Immunoreactive proteins were visualized using enhanced chemiluminescence (ECL Advance; GE Healthcare) (Matthews *et al.*, 2008;Matthews *et al.*, 2009).

2.3.8 Reporter gene assay

Cells were cotransfected with 3 µg AH3-luciferase or NFkBluciferase reporter gene construct together with 0.1µg cytomegalovirus- Renilla luciferase (to correct for transfection efficiency) and either 3 µg full length GR or 3 µg Δ612GR using Fugene 6 (Roche Diagnostics), as described before (Matthews *et al.*, 2009). Twenty-four hours after transfection, cells in DMEM containing sFCS were treated as specified before lysis, then assayed for luciferase activity using a dual-luciferase reporter assay system, following the manufacturer's instructions (Promega, Southampton, UK) as we have previously described (Matthews *et al.*, 2008;Kayahara *et al.*, 2008;Matthews *et al.*, 2009).

2.3.9 Immunofluorescence

After 24 h in DMEM containing sFCS, U20S cells transfected (Fugene 6) with GR-GFP, GR-ASRed, or Δ612GR-GFP were treated as specified. Cells were fixed with 4 % paraformaldehyde for 30 min at 4 ^{o}C, and subsequently the cells were stained with Hoeschst (Sigma) in PBS (2 µg/ml) for 20 min at 4 ^{o}C. After three washes in PBS, coverslips were mounted using Vectamount AQ (Vector Laboratories, Peterborough, UK). Images were acquired on a Delta Vision RT (Applied Precision Inc., Issaquah, WA) restoration microscope using a 40X/0.85 Uplan Apo objective and the Sedat Quad filter set (Chroma 86000v2). The images were collected using a Coolsnap HQ (PhotoMetrics Inc., Huntington Beach, CA) camera with a Z optical spacing of 0.5 µm. Raw images were then deconvolved using the Softworx software, and average intensity projections of these deconvolved images were processed using Image J. Images for Hoeschst and GFP were excited with the 405 and 488 nm laser lines, respectively, as previously described (Matthews *et al.*, 2009).

2.4 Results

2.4.1 Identification of a GR truncation mutant, Δ612GR

Blood samples were taken from the index, her sister, and both parents and the genomic DNA was extracted. Exons 2 through to 9 of the GR gene were amplified, and sequenced. Analysis revealed that exon 6 did not correlate with the wild type sequence (Figure 2.1 A), which was confounded due to heterozygous peaks within the sequence trace indicative of a heterozygous genotype (Figure 2.1 B). Subcloning of the PCR products into a pGEM-T – Easy Vector followed by a second round of sequencing revealed deletion of a single base (C 1835), causing a frameshift at amino acid 612 and introducing a stop codon at position 627 (Figure 2.1 C, D). The predicted GR truncation lacks a significant portion of the ligand binding domain, with an additional, novel 15 amino acid sequence (Figure 2.1 D). Site directed mutagenesis removed C1835 from pcDNA3 hGR forming pcDNA3 GRΔ612. U2OS cells, which are deficient in endogenous GR expression, were transfected with Δ612GR to determine if the mutant GR is produced. In U20S cells transfected with GR, an intense band with the same migration as the endogenous GR was seen, and those cells transfected with Δ612GR had a new protein species, with lower apparent molecular mass (Figure 2.1 E). After 1 hour treatment with 100 nM Dexamethasone, there was a significant reduction in steady state protein expression, an expected consequence of ligand-dependant protein degradation. There was no ligand-dependent reduction in Δ612GR expression (Figure 2.1 E). The wild-type GR undergoes rapid ligand-dependent phosphorylation on multiple residues, but the best defined is serine 211. Following Dexamethasone treatment, untransfected U2OS or those expressing GR showed enhanced phosphorylation of the GR at serine 211. In contrast Δ612GR showed no change (Figure 2.1 E). This suggests that Δ612GR is not activated by ligand.

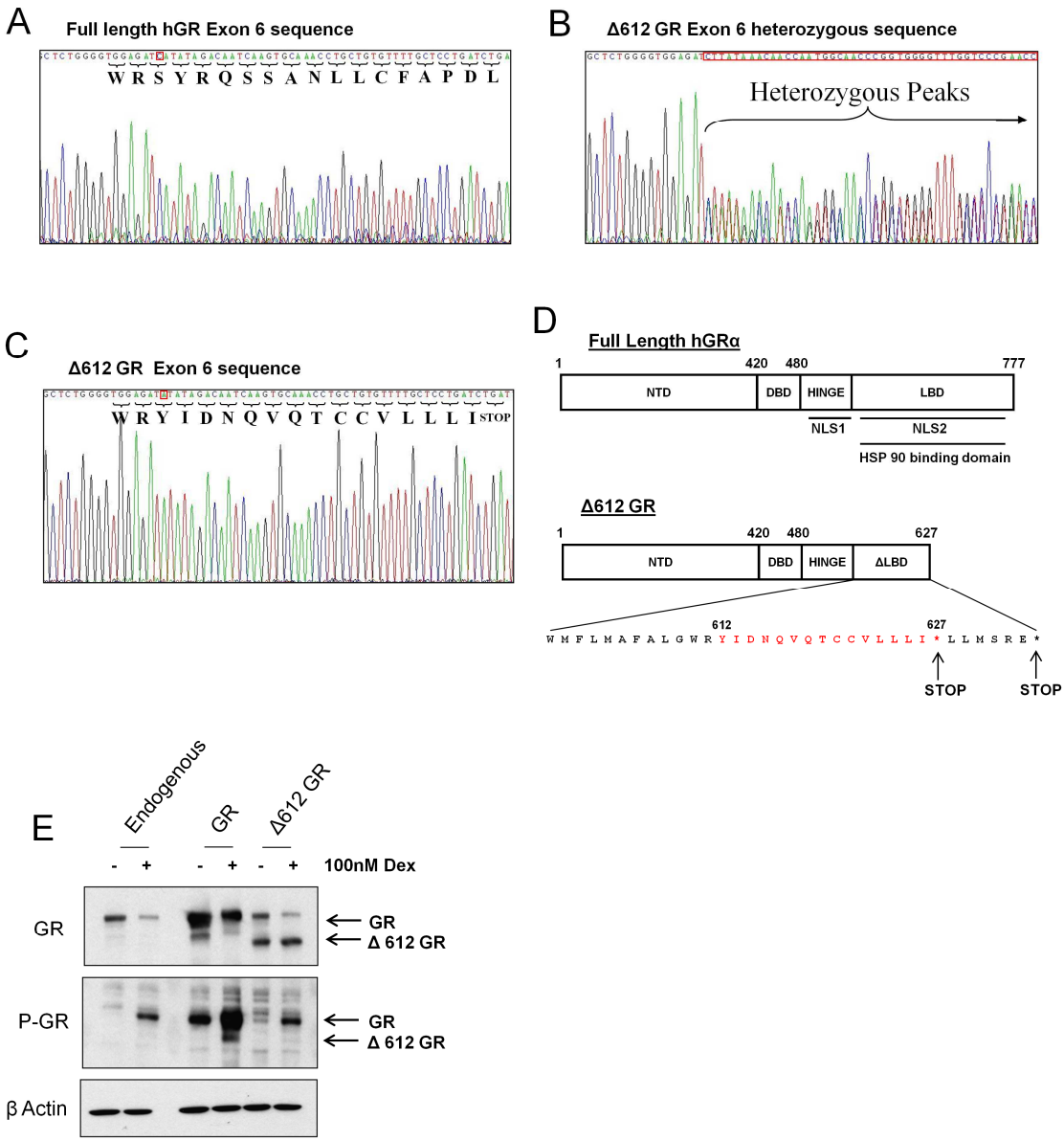

Figure 2.1: Sequencing of GR Exon 6 with subsequent identification of deletion mutation; Δ612GR. (A-C) GR exon 6 was amplified from DNA isolated from patient blood samples using PCR. The PCR product was subsequently sequenced. The sequence trace is shown for GR (A) and the initial heterozygous Δ612GR exon 6 (B). Following cloning into the pGEM-T – Easy vector, the sequence trace of the Δ612GR is shown (C) with the deletion mutation highlighted in red. (D) Schematic demonstrating that a receptor truncation is caused by introduction of a stop codon at residue 627. (E) U2OS cells were transfected with GR or Δ612GR. After treatment with 100 nM Dexamethasone for 1 hour, cells were lysed in RIPA buffer containing phosphatase and protease inhibitors and analysed by immunoblotting for GR abundance and phosphorylation on Ser211 (as indicated). β-actin was used as a loading control. Wildtype and truncated GR is indicated with arrows. Representative images are shown.

2.4.2 Screening for the Δ612GR mutation

Screening of other family members including the index's sister, mother, father, maternal uncle and aunt identified the same mutation in two other subjects, the index's sister and mother (Figure 2.2 A). Consistent with the clinical data outlined in Table 2.1, the father was not affected. Blood samples were subsequently taken from the index case, her sister, and both parents, and immortalised lymphocyte cell lines were generated. Cells were lysed and immunoblotted for GR (Figure 2.2 B). There was no detectable expression of Δ612GR in any of the three affected individuals. Quantification of GR expression did reveal that the index, sister and mother expressed only approximately half the amount of GR compared to the father (Figure 2.2 C).

2.4.3 Steady state expression of GR and ligand activation

Expression of GR was down-regulated by ligand treatment in all family members tested (Figure 2.3 A), and GR was serine 211 phosphorylated by ligand (Figure 2.3 B). Basal phosphorylation was higher in the unaffected father, and the lower molecular weight bands seen are alternatively translated isoforms.

2.4.4 Glucocorticoid-induced gene regulation by Δ612GR

Although we were unable to detect expression of the truncated GR protein isoform in transformed lymphocytes, the protein may be expressed below the limit of detection, or in other tissues, and so its function was determined. In GR-deficient HEK293 cells co-transfected with GR and the glucocorticoid activated mouse mammary tumor virus reporter plasmid (AH3-luc), treatment with dexamethasone induced a significant and robust response, whereas Δ612GR transfected cells were unresponsive (Figure 2.4 A). In cells co-transfected with GR and Δ612GR, there was significant inhibition of GR transactivation (Figure 2.4 B). Similar effects were observed using the partial GR agonist RU486 (Figure 2.4 C and D).

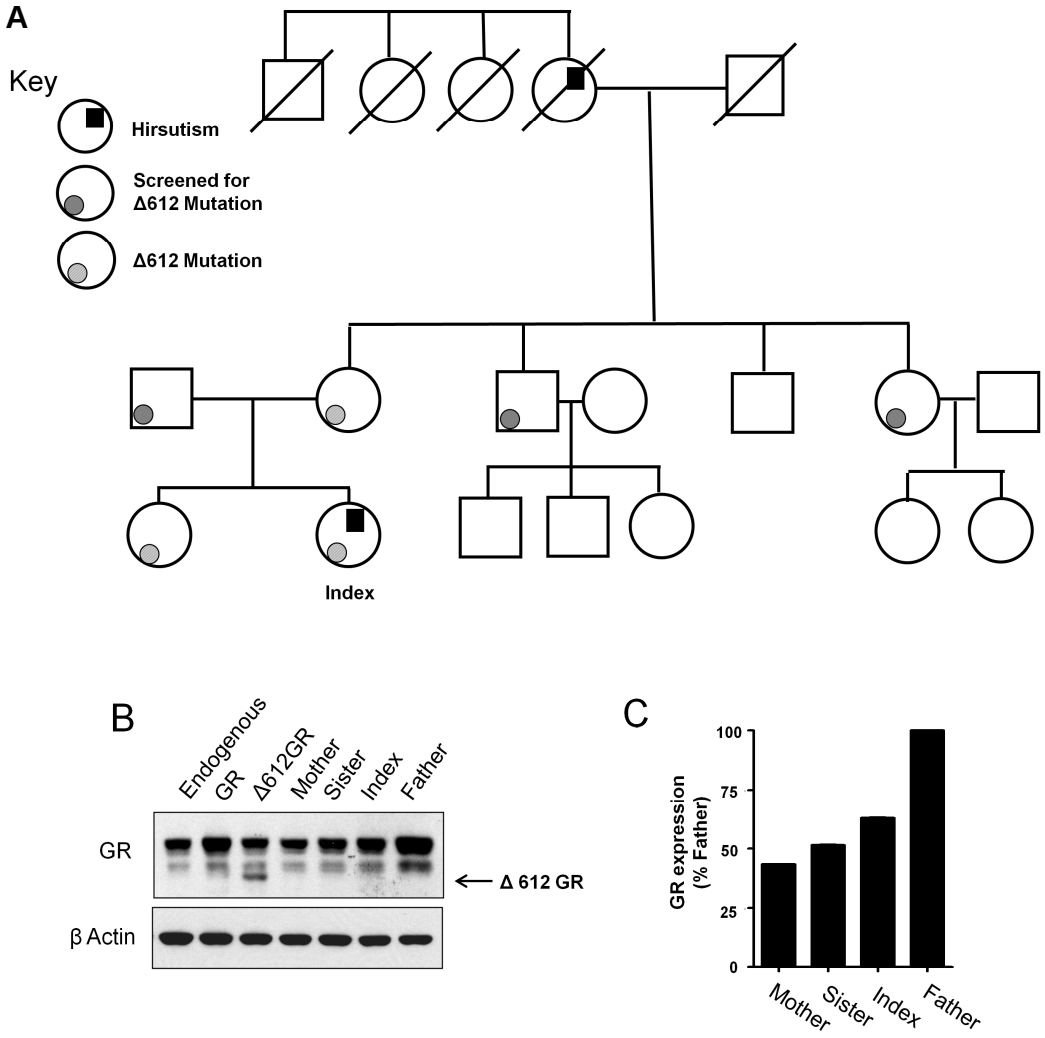

Figure 2.2: Family Tree. (A) A family tree was constructed. The mother and sister of the index case carried the Δ612GR mutation, but not her father. Two of her mother's three siblings agreed to be screened; however they did not have the mutation. (B) Immortalised cell lines were generated from blood taken from the index, her sister, mother and father. Cells were lysed and immunoblotted for GR and actin, alongside lysates shown in figure 2.1E. Representative immunoblots shown. (C) Immunoreactive bands were quantified using ImageJ. Graph depicts GR/actin ratio and is expressed as a percentage of the father's GR expression.

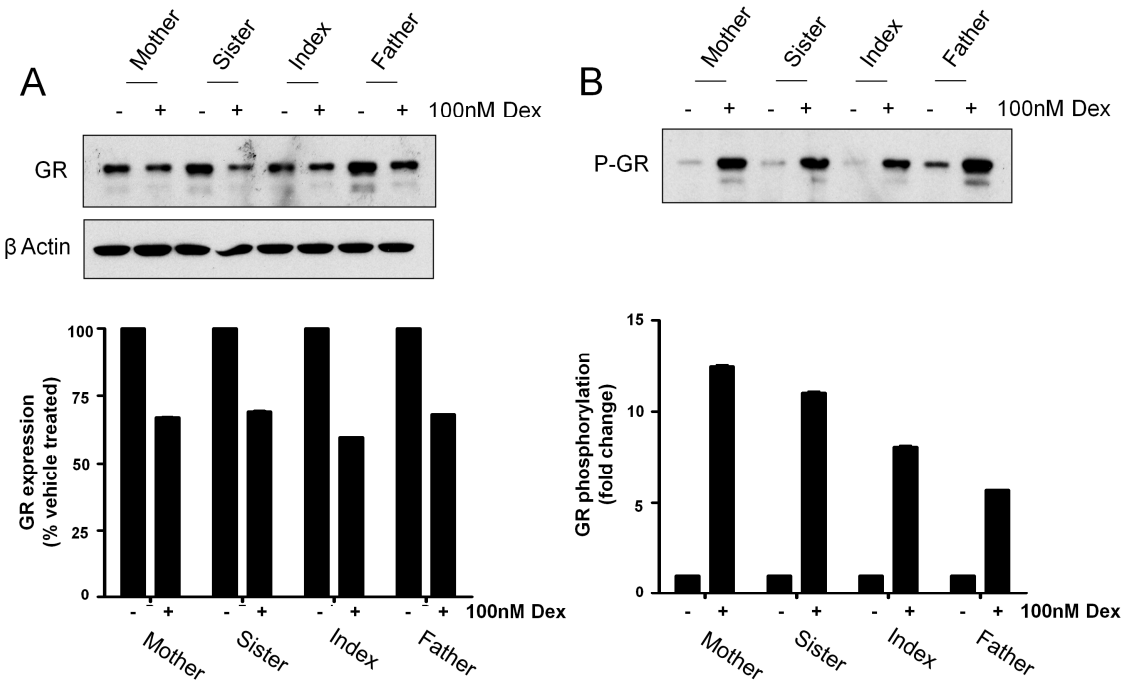

Figure 2.3: Glucocorticoid sensitivity is template specific. (A, B) Immortalised lymphocytes were treated with 100 nM Dexamethasone for 1 hour then lysed in RIPA buffer containing phosphatase and protease inhibitors and analysed by immunoblotting for GR abundance (A) and phosphorylation on Ser211 (B). β-actin was used as a loading control. Immunoreactive bands were quantified using ImageJ. GR/actin and PGR/GR ratios were calculated and shown as percentage vehicle treated and fold increase over vehicle treated respectively.

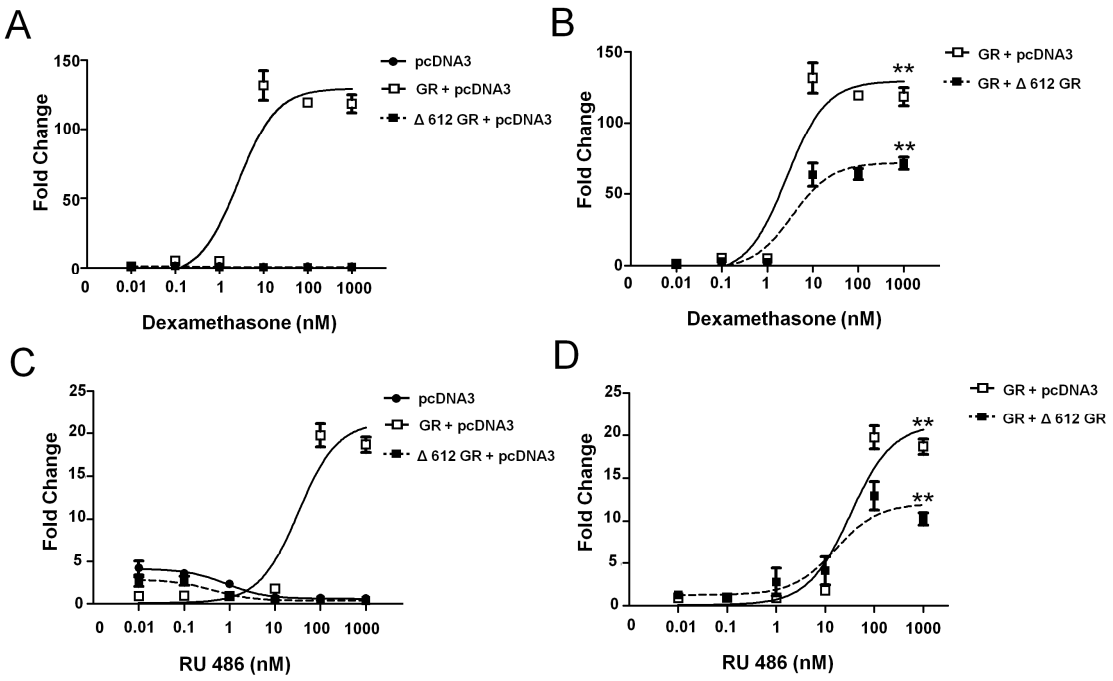

Figure 2.4: Δ612GR does not mediate transactivation. (A-D) HEK cells were cotransfected with a positive GR reporter gene (pAH3-luc) and either empty, GR or Δ612GR expression vectors alone or in combination. Twenty-four hours after transfection, cells were treated with 0.01-100 nM Dexamethasone (A and B) or 0.01-100 nM RU486 (C, D) for 18 hours. Cells were then lysed and subjected to analysis by luciferase assay. Graphs (mean ± SEM) show the fold change in luciferase readings compared to baseline from one of three representative experiments performed in triplicate. ** Indicates $P < 0.01$

Modulation of target gene expression by non-DNA binding or tethering mechanisms is important in physiology. Therefore, Δ612GR effects on NFkB driven gene expression were measured. In cells expressing GR and NRE-luc, treatment with dexamethasone significantly inhibited TNF induction of the NFkB reporter (Figure 2.5 A). In comparison to GR, Δ612GR had no effect (Figure 2.5 A). Co-transfection of Δ612GR with GR had no significant effect when compared to GR only transfected cells (Figure 2.5 B). Treatment of GR expressing cells with RU486 induced significant repression of NFkB (Figure 2.5 C), whereas RU486 was without effect in Δ612GR expressing cells (Figure 2.5 D). Interestingly, expression of Δ612GR consistently potentiated the TNFα transactivation of an NFkB reporter gene (Figure 2.5 E).

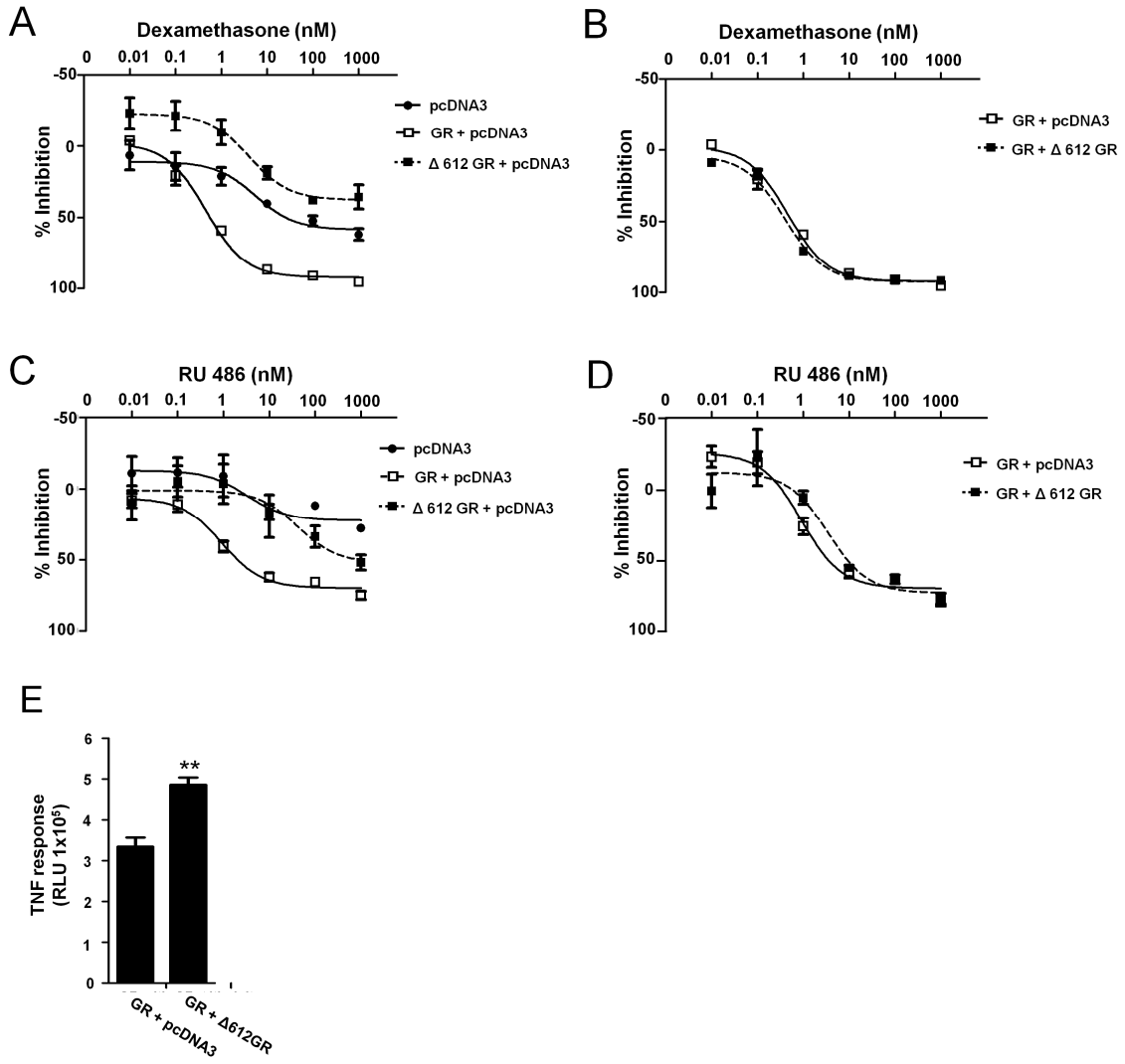

Figure 2.5: Δ612GR does not mediate transrepression. (A-E) HEK cells were cotransfected with a glucocorticoid repressed NFkB reporter gene (NRE-luc) and either empty, GR or Δ612GR expression vectors alone or in combination. Twenty-four hours after transfection, cells were pretreated with TNF α (0.5ng/ml) for 30 minutes then treated with 0.01-100 nM Dexamethasone (A and B) or 0.01-100 nM RU486 (C and D) for 18 hours. Cells were then lysed and subjected to analysis by luciferase assay. Graphs (mean ± SEM) show % inhibition. (E) TNF induction in the absence of Gc is also shown. One of three representative experiments performed in triplicate are shown. ** Indicates $P < 0.01$.

2.4.5 Subcellular localisation of Δ612GR

GFP-tagged GR and Δ612GR were also expressed in U2OS cells and migrated with molecular weights of 30 kDa higher than their untagged counterparts. To determine whether Δ612GR was constitutively cytoplasmic, U20S cells were transfected with Δ612GR-GFP. In untreated cells, the GR-GFP localises predominantly to the cytoplasm (Figure 2.6 A). Dexamethasone induced near complete nuclear translocation of GR-GFP by 30 min, which was sustained over the 120 min assay period (Figure 2.6 A). In contrast, transfected Δ612GR-GFP was only ever observed in the cytoplasm of transfected cells (Figure 2.6 B).

To determine whether Δ612GR influenced wild-type GR trafficking when co-expressed, U20S cells were cotransfected with GR-Asred together with either GR-GFP or Δ612GR-GFP. When coexpressed with GR-GFP, GR-Asred was nuclear in 50% of the cells after 10 minutes treatment with 100 nM dexamethasone (Figure 2.6 C). Near complete nuclear translocation of GR-Asred was evident by 20 minutes (Figure 2.6 C). In contrast, in cells co-expressing Δ612-GFP, dexamethasone induced nuclear translocation of GR-Asred in 50% of the cells after 30 minutes, where near complete nuclear translocation was not evident until 60 minutes (Figure 2.6 D).

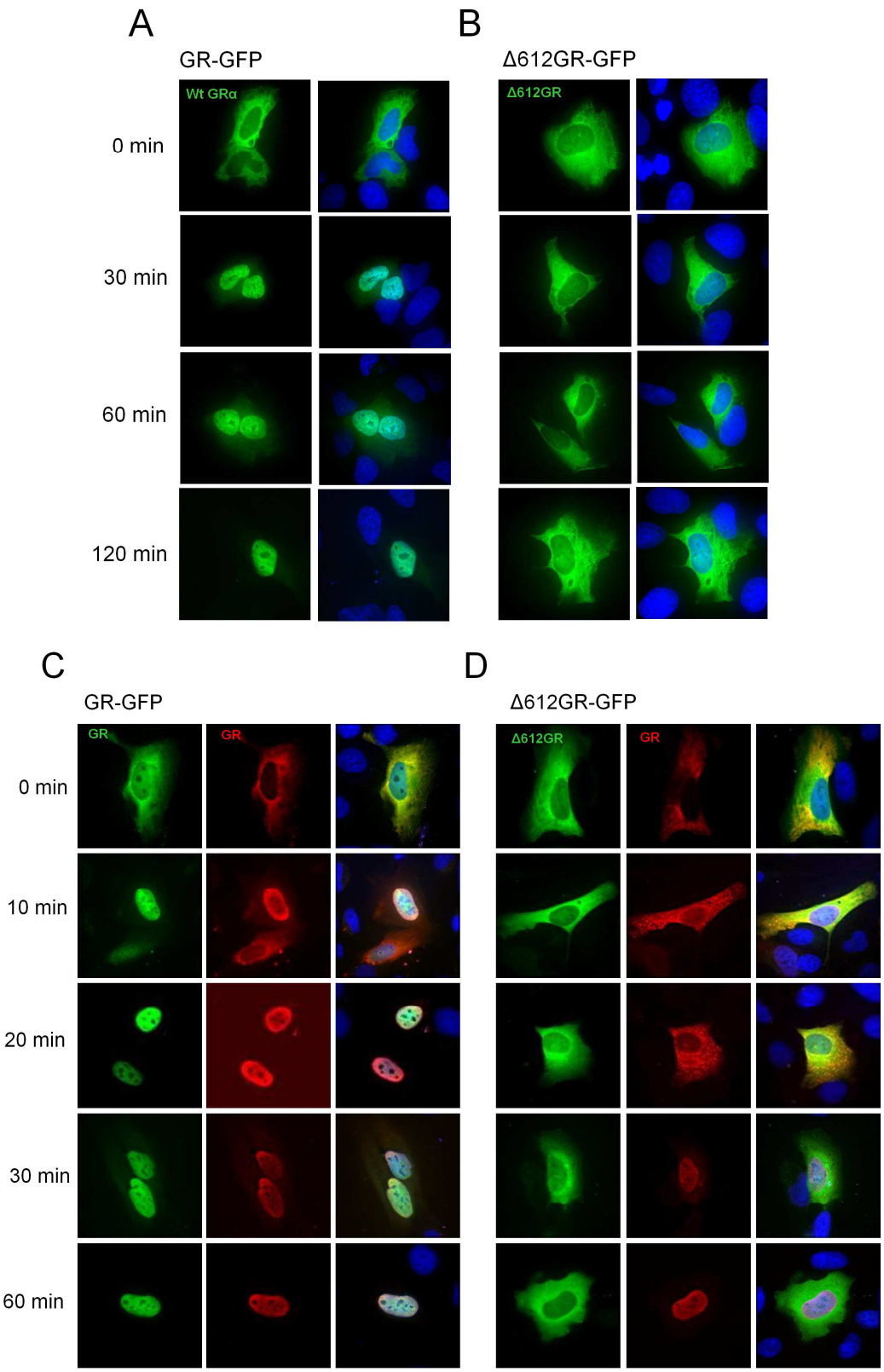

Figure 2.6: Δ612GR-GFP does not translocate to the nucleus in response to ligand. After transfection with GR-GFP (A), Δ612GR-GFP (B), GR-Asred with GR-GFP (C) or GR-Asred with Δ612GR-GFP (D), serum starved U20S cells were incubated with 100 nM Dexamethasone (10, 20, 30, 60 or 120 minutes, as indicated), fixed with paraformaldehyde and analysed for subcellular localisation of the ASRed (red) or GFP tag (green). Nuclei were counterstained using Hoescht (blue). Images are representative of four independent experiments.

2.5 Discussion

Familial Gc resistance due to mutations in the GR gene is thought to be rare, and to present with a distinct clinical phenotype (Charmandari *et al.*, 2004;van Rossum *et al.*, 2006). Therefore it is important that the range of clinical manifestation, as in the kindred reported here, is broadened to include apparently normal women with normal reproductive potential. In the kindred described here, we were unable to document the presence of the mutation in the grandparents of the index because they were deceased, and her surviving mothers siblings were mutation negative. However, there was no menstrual irregularity, nor were problems with conception reported by the mother of the index case. There was clear biochemical evidence of Gc resistance, with raised serum cortisol concentrations following dexamethasone suppression. In addition, androstenedione and testosterone levels were high normal or high. However, these biochemical changes were not accompanied by significant hirsutism, as measured by Ferriman-Gallwey score, supporting the importance of other genetic background effects in determining the phenotype of mild androgen excess in women, which is a frequent clinical observation in women with polycystic ovarian syndrome.

The clinical diagnosis prompted sequencing of the GR gene, which identified a novel mutation in exon 6. This led to a frame shift mutation. This would be predicted to induce nonsense mediated RNA decay, and so result in GR haploinsufficiency. However, haploinsufficiency has previously been reported to be without endocrine phenotype in the parents of the first reported complete Gc resistance patient (McMahon *et al.*, 2010), or with hirsutism and hypertension in an affected young woman (Karl *et al.*, 1993), indicating phenotypic heterogeneity. In addition, alternative splicing in the GR 3' region has previously been reported to result in expression of truncated GR proteins capable of affecting Gc sensitivity (GR-P, or GRδ) (De Lange *et al.*, 2001), and so further analysis was undertaken. Epstein-Barr transformed B lymphoblasts were established from the family members to allow measurement of GR protein expression. These studies showed reduced GR protein in the carriers of the mutation, with impaired basal serine 211 phosphorylation. However, in these studies no truncated GR protein was observed. The process of nonsense mediated RNA decay is complex, and relies on cellular expression of key proteins, which may vary between cell types; reviewed in (Silva and Romao, 2009).

Indeed, previous studies have shown significant expression of proteins even in the presence of premature stop codons (Dash *et al.*, 2009). Therefore, the predicted protein product of the mutant allele was expressed, and its function analysed.

The predicted GR has 15 novel amino acids and a premature stop codon, giving rise to a 75kD protein, that did not undergo ligand-dependent phosphorylation on serine 211, nuclear translocation, or acute ligand-dependent protein degradation, in contrast to wild-type GR (Sommer *et al.*, 2007;Kayahara *et al.*, 2008;Matthews *et al.*, 2009). However, the lack of evidence of a ligand-dependent action does not exclude biologically relevant activity. Therefore reporter gene studies were undertaken initially using a transactivation reporter (Matthews *et al.*, 2008;Matthews *et al.*, 2009). The Δ612GR did not show any ligand-dependent transactivation of AH3-luc, as would be predicted based on the disrupted ligand binding domain, and lack of evidence of ligand activation of GR. However, Δ612GR consistently inhibited the transactivation seen with wild-type GR. This was an unexpected finding, possibly reflecting a cytoplasmic effect on wild-type GR as Δ612GR was constitutively cytoplasmic. Previous reports suggest that cytoplasmic GR can exert an anti-NFκB effect, possibly through protein kinase A (Bledsoe *et al.*, 2004;Grad *et al.*, 2006). Therefore, the effect of Δ612GR on TNFα driven NFκB transactivation was measured. There was no ligand- independent inhibition of NFκB activity, and no evidence of ligand-dependent repression. The small inhibition seen at higher ligand concentrations was similar to that seen in vector transfected cells, and is attributable to the low level endogenous expression of GR in these cells.

In contrast to the dominant negative action on wild-type GR seen with the transactivation assay there was no impact of Δ612GR expression on repression of NFκB by the wild-type GR. Therefore, the effects of Δ612GR are mechanism specific. Δ612GR expression resulted in consistent, significant potentiation of TNFα transactivation of an NFκB reporter gene, suggesting opposition to endogenous limiting factors, but the mechanism and implications remain uncertain. The dominant negative effects of Δ612GR on wild-type GR transactivation were interesting because previous reports have suggested that the GRβ splice isoform of GR, similarly incapable of binding glucocorticoid agonists, can exert dominant negative effects on

GR transactivation (Bamberger *et al.*, 1995;Hamid *et al.*, 1999;Hauk *et al.*, 2002;Yudt *et al.*, 2003;Pujols *et al.*, 2004;Lu and Cidlowski, 2004;Lewis-Tuffin *et al.*, 2007), although this is controversial (Otto *et al.*, 1997;Brogan *et al.*, 1999). A further GR splice variant, GR-P, which is similar to Δ612GR, although including exon 7 in addition to exons 2-6, has been analysed and found to exert a wild-type GR potentiating effect, again in the absence of ligand binding (De Lange *et al.*, 2001). This suggests the presence of modulating activity in exons 5 and 6, possibly by competing with wild-type GR for binding to heat shock protein complexes in the cytoplasm, required for ligand binding competency (Pratt and Toft, 1997;Pratt, 1998;Pratt *et al.*, 1999;Galigniana *et al.*, 2001;Kayahara *et al.*, 2008). A further point mutation in the GR C terminal has been reported to cause dominant negative activity by interference with co-activator recruitment in the nucleus, not a feasible mechanism for the Δ612GR as it is retained in the cytoplasm (Vottero *et al.*, 2002), and another potential mechanism is regulation of wild-type GR nuclear translocation (Charmandari *et al.*, 2004). Indeed, co-transfection studies showed that the truncated GR significantly slows the rate of nuclear accumulation in response to ligand, potentially a mechanism for dominant negative action.

In conclusion, we report a family with Gc resistance due to a novel mutation in exon 6. The phenotype is mild and is intermediate between carriers of ligand binding domain missense mutations, who present with features of androgen excess, and hypertension, and carriers of some null, or hypofunctioning mutations who are apparently without any endocrine phenotype (Mendonca *et al.*, 2002;Charmandari *et al.*, 2008b;McMahon *et al.*, 2010). The expressed mutant GR exhibited dominant negative activity on wild-type GR, which may explain the difference between the family reported here, and that recently described from Australia (McMahon *et al.*, 2010). It is clear that the spectrum of clinical manifestation in heterozygous carriers of deleterious mutations in the GR gene is broad, but it is possible that dominant negative activity of the mutant GR plays a role in clinical manifestation.

2.6 Acknowledgments

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Chapter 3: Novel Non-steroidal Glucocorticoids

A ligand-specific kinetic switch regulates glucocorticoid receptor trafficking and function

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3.1 Abstract

The ubiquitously expressed glucocorticoid receptor (GR) is a major drug target for inflammatory disease, but issues of specificity and target tissue sensitivity remain. We now identify high potency, non-steroidal GR ligands, GSK47867A and GSK47869A, which induce a novel conformation of the GR ligand-binding domain (LBD) and augment the efficacy of cellular action. Despite their high potency, GSK47867A and GSK47869A both induce surprisingly slow GR nuclear translocation, followed by prolonged nuclear GR retention, and transcriptional activity following washout. We reveal that GSK47867A and GSK47869A specifically alter the GR LBD structure at the Hsp90-binding site. The alteration in the Hsp90-binding site was accompanied by resistance to Hsp90 antagonism, with persisting transactivation seen after geldanamycin treatment. Taken together, our studies reveal a new mechanism governing GR intracellular trafficking regulated by ligand binding that relies on a specific surface charge patch within the LBD. This conformational change permits extended GR action, probably because of altered GR–Hsp90 interaction. This chemical series may offer anti-inflammatory drugs with prolonged duration of action due to altered pharmacodynamics rather than altered pharmacokinetics.

3.2 Introduction

Synthetic glucocorticoids are potent anti-inflammatory drugs used to treat multiple conditions including asthma and rheumatoid arthritis (Schett *et al.*, 2008;Krishnan *et al.*, 2009). Unfortunately glucocorticoid treatment also carries a wide range of serious side effects including hyperglycaemia and osteoporosis (Canalis *et al.*, 2002). In recent years a significant effort has been made to design dissociative ligands with the anti-inflammatory potency of conventional glucocorticoid, but with a reduced spectrum of side-effects (Lin *et al.*, 2002;Bledsoe *et al.*, 2004;Cerasoli, Jr., 2006;Wang *et al.*, 2006;McMaster *et al.*, 2007;McMaster *et al.*, 2008b;van Lierop *et al.*, 2012).

Glucocorticoid actions are mediated by the ubiquitously expressed glucocorticoid receptor (GR; NR3C1) a member of the nuclear hormone receptor superfamily with a conserved modular structure consisting of an N-terminal regulatory domain, a DNA binding domain (DBD) and a C-terminal ligand binding domain (LBD) (Hollenberg *et al.*, 1985;Encio *et al.*, 1991). The unliganded GR resides in the cytoplasm in a complex with heat-shock proteins and immunophilins (Grad *et al.*, 2007). Ligand binding triggers rapid activation of cytosolic kinase signalling cascades and concomitantly results in exposure of two nuclear localisation signals (NLS1, and NLS2) enabling nuclear import (Picard and Yamamoto, 1987). This is accompanied by replacement of the immunophilin FKBP51 with FKBP52 (Davies *et al.*, 2002) which associates with dynein to drive GR along microtubules (Czar *et al.*, 1994;Harrell *et al.*, 2004). The process of translocation to the nucleus post ligand binding occurs rapidly, with the majority of cellular GR being nuclear 30 minutes after treatment with 100nM Dex (Nishi *et al.*, 1999). In addition cell cycle phase is able to regulate the subcellular localisation of unliganded GR, but with far slower kinetics of nuclear accumulation (Matthews *et al.*, 2011). In the nucleus GR binds to cis-elements to activate or repress target gene expression, recruiting co-modulator proteins from distinct classes to effect chromatin remodelling, and recruitment of the basal transcriptional machinery (Ford *et al.*, 1997;Jones *et al.*, 2003;Ito *et al.*, 2006;Johnson *et al.*, 2008).

GR recruits co-modulator proteins via its transcriptional activation function domains (AF1, and AF2) (Warnmark *et al.*, 2000;Kumar *et al.*, 2001;Bledsoe *et al.*, 2002). The GR AF1 is the site of various post translational modifications including phosphorylation, both in the presence and absence of ligand. (Wang *et al.*, 2002;Ismaili *et al.*, 2004;Galliher-Beckley *et al.*, 2008a). Phosphorylation directs GR function by impacting protein stability and recruitment of specific co-modulator proteins such as MED14 (Chen *et al.*, 2006;Chen *et al.*, 2008). In addition, co-modulators bind to the GR AF2 domain, within the LBD (Heery *et al.*, 1997). Structural information about bound ligand is transmitted through differential folding of the LBD, which directs GR function by offering differentially attractive signals for co-modulator recruitment. Both GR agonists and antagonists provoke similar rapid kinetics of nuclear translocation, but differ in the profile of co-modulator proteins recruitment, providing a mechanism for their different modes of action (Bledsoe *et al.*, 2002;Kauppi *et al.*, 2003;Stevens *et al.*, 2003).

Here we identify a novel switch mechanism that regulates GR trafficking in response to ligand binding, distinct from an effect attributable to ligand potency. We identify two novel, non-steroidal GR ligands that regulate the GR surface to greatly reduce rates of nuclear translocation and reduce reliance on heat-shock protein for continuing activity. The difference in GR conformation induced by the novel GR ligands reveals a patch of positive charge on the surface of the LBD. We propose that this prevents efficient engagement with the active nuclear translocation mechanism, subsequent export, and protein degradation mechanisms for the GR. The result is generation of ligands with greatly prolonged duration of action as a consequence of altered pharmacodynamics rather than pharmacokinetics.

3.3 Materials and methods

Anti-hGR (clone 41, BD Biosciences, Oxford, UK); Anti-phospho-(Ser211)-GR, anti αTubulin (Cell Signalling Technology, MA, USA); Horseradish peroxidase conjugated anti-mouse and anti-rabbit (GE Healthcare, Buckinghamshire, UK); Dexamethasone, Hydrocortisone and Fluticasone Propionate (Sigma, Dorset, UK). TAT3-Luciferase, and NRE-luciferase have been previously described (Matthews *et al.*, 2008;Matthews *et al.*, 2009).

3.3.1 Cell culture and maintenance

HeLa cells and A549 cells (ATCC, Teddington, UK) were cultured in low glucose (1 g/l) Dulbecco's modified Eagle's medium (DMEM; PAA, Yeovil, UK) supplemented with stable 2 mM glutamine (PAA) and 10 % heat inactivated fetal bovine serum (FBS; Invitrogen, Paisley, UK) or 10 % charcoal dextran stripped fetal calf serum (sFCS; Invitrogen). A549's stably transfected with GRE-Luc and NRE-Luc were also supplemented with 1% Non essential amino acid (NEAA; Invitrogen) and 1% Geneticin (Invitrogen). All cells were grown in a humidified atmosphere of 5 % carbon dioxide at 37 ^{o}C.

3.3.2 Immunoblot analysis

Following treatment cells were lysed in RIPA buffer (50 mM TrisCl pH7.4, 1 % NP40 (Igepal), 0.25 % Na-deoxycholate 150 mM NaCl, 1 mM EDTA) containing protease and phosphatase inhibitors (Sigma), and 10 μg protein was electrophoresed on Tris/Glycine 4-12 % gels (Invitrogen) and transferred to 0.2 micron nitrocellulose membranes (BioRad Laboratories, Hertfordshire, UK) overnight at 4 °C. Membranes were blocked for 2 hours (NaCl 0.15 M, 2 % dried milk, 0.1 % Tween 20) and incubated with primary antibodies (diluted in blocking buffer) overnight at 4 ˚C. After three 10 minute washes (88 mM Tris pH 7.8, 0.25 % dried milk, 0.1 % Tween 20), membranes were incubated with a species-specific horseradish peroxidase-conjugated secondary antibody (diluted in wash buffer) for 1 hour at room temperature, and washed a further three times, each for 10 minutes. Immunoreactive proteins were visualised using enhanced chemiluminescence (ECL Advance, GE Healthcare).

3.3.3 Reporter gene assays

HeLa cells seeded in DMEM containing sFCS were co-transfected with 2 μg reporter gene and 0.5 μg CMV-renilla luciferase (to correct for transfection efficiency) using Fugene 6 (Roche Diagnostics, West Sussex, UK) at a ratio of 3:1 (v/w). 24 hours post transfection, cells were treated as specified in results prior to lysis, then assayed for luciferase activity using a dual-luciferase reporter assay system following the manufacturer's instructions (Promega, Southampton, UK).

Stable A549 GRE-Luc or NRE-Luc cells were seeded in DMEM containing sFCS into 96 well plates and incubated overnight. Cells were treated as specified in results and 18 hours later each well washed twice with PBS (first without Mg^{2+}, Ca^{2+}, then with Mg^{2+}, Ca^{2+}). Renilla Glo (Promega, E2720) or Bright Glo (Promega E2620) lysis buffer was added the GRE cells or the NRE cells respectively according to the manufacturer's instructions. Cell lysates were read using a luminometer (Wallac 1450 MicroBeta Trilux Liquid Scintillation counter and luminometer). Ten one second reads were taken per well and the average RLU determined.

3.3.4 Immunofluorescence

3.3.4.1 Fixed cells

Following 24 hours in DMEM containing sFCS, HeLa cells were transfected (Fugene 6) with hGR-GFP and treated as specified in results. Cells were fixed with 4 % paraformaldehyde for 30 minutes at 4 ˚C, and subsequently stained with Hoeschst (Sigma) in PBS (2 μg/ml) for 20 minutes at 4 ˚C. Following three 5 minute washes in PBS, coverslips were mounted using Vectamount AQ (Vector Laboratories, Peterborough, UK). Images were acquired on a Delta Vision RT (Applied Precision, GE Healthcare) restoration microscope using a 40X/0.85 Uplan Apo objective and the Sedat Quad filter set (Chroma 86000v2, VT, USA). The images were collected using a Coolsnap HQ (Photometrics, AZ, USA) camera with a Z optical spacing of 0.5μm. Raw images were then deconvolved using the Softworx software (GE Healthcare) and average intensity projections of these deconvolved images processed using Image J (Rasband, 1997).

3.3.4.2 Live cells

Following 24 hours in DMEM containing sFCS, HeLa cells were transfected (Fugene 6) with 5µg GR-GFP and transferred to a glass bottomed 24 well plates. Alternatively HeLa cells were plated into a glass bottomed 24 well plate in DMEM containing sFCS. Each well was transfected (Fugene 6) with 0.5µg HaloTag-GR (Catalog number FHC10483, Promega) and incubated for 16 hours with 0.25µl Halo ligand (HaloTag TMRDirect, Catalog number G2991, Promega) to enable visualisation. Subcellular GR trafficking was tracked in real time at 37^{o}C with 5% CO_2. Images were acquired on a Nikon TE2000 PFS microscope using a 60x/ 1.40 Plan Apo or 40x/1.25 Plan Apl objective and the Sedat filter set (Chroma 89000). The images were collected using a Cascade II EMCCD camera (Photometrics). Raw images were then processed using Image J.

3.3.5 Fluorescent recovery after photobleaching (FRAP)

HeLa cells were transfected (Fugene 6) with 5 µg hGR-GFP then seeded into a glass bottomed 24 well plate. Cells were maintained at 37^{o}C and 5% CO_2 and images collected on a Leica TCS SP5 AOBS inverted confocal (Leica, Milton Keynes, UK) using a 63x/ 0.50 Plan Fluotar objective and 7x confocal zoom. The confocal settings were as follows, pinhole 1 airy unit, scan speed 1000Hz unidirectional, format 1024 x 1024. Images were collected using the following detection mirror settings; FITC 494-530nm using the 488nm (13%).

3.3.6 MTS Assay

Cells were seeded into a 96 well plate were treated as described in the results. Upon completion of the treatment 10 µl of MTS reagent (Promega) was added to each well. Cells were incubated for 4 hours, reading at 490nm every hour.

3.3.7 Q-RTPCR

Cells were treated as required, then lysed and RNA extracted using an RNeasy kit (Qiagen). 10 ng RNA was reverse transcribed, and subjected to qPCR using Sybr Green detection in an ABI q-PCR machine (Applied biosystems, CA, USA) and data analysed by δδCT method (Livak and Schmittgen, 2001).

3.3.8 Bioluminescence real-time recording

HeLa cells transfected (Fugene 6) with 2µg TAT3-luc plasmid were grown to 80% confluency in 35-mm tissue culture dishes in phenol red free DMEM with 10% FCS and 1% glutamine. Prior to the experiment, cells were supplemented with 0.1 mM Luciferin substrate (Izumo *et al.*, 2003;Yamazaki and Takahashi, 2005). Each dish lid was replaced with a glass cover then sealed with vacuum grease before being placed in a light-tight and temperature-controlled (37°C) environment. Light emission (bioluminescence) was measured continuously using a Photomultiplier tube (PMT, H6240 MOD1, Hamamatsu Photonics, Hertfordshire, UK). Baseline measurements (photon counts per minute) were taken for each PMT prior to treatment and then deducted from the experimental values attained.

3.3.9 Measurement of ligand uptake using mass spectroscopy

A549 cells were grown to 90% confluency in 6 well plates. Following treatment the media was removed from the cells and retained for analysis. The cells were washed three times with PBS and lysed in 300µl of M-Per mammalian protein extraction reagent (#78503, Thermoscientific, Essex, UK) on the shaker at 750rpm at room temperature for 5 minutes. The whole cell lysate was collected, then centrifuged at 10000rpm for 10 minutes, then the supernatant collected and analysed by mass spectrometry.

3.3.10 Measurement of cytokine production

A549 cells were seeded into a 96 well plate into DMEM with 10% FCS and incubated overnight. In order to slow cell proliferation and prevent any interference from steroid present in FCS the media was changed to DMEM with 1% sFCS prior to ligand treatment. Following treatment supernatents were collected and assayed for IL6 and IL8 concentration using a Luminex 100 (Merck Millipore, MA, USA) with StarStation software according to the manufacturer's instructions.

3.3.11 Computational modelling of GR crystal structure

Crystal structures of GR bound to Dex (1M2Z) and GSK47866A (3E7C) (Madauss *et al.*, 2008) were downloaded from the RCSB Protein Data Bank (PDB) (Berman *et al.*, 2007). The structures were imported into Maestro (Schrodinger, 2012) and prepared using the Protein Preparation module. Each Ligand was extracted and scrambled conformationally before docking back into the native active site models to verify that the docking program (GLIDE) (Schrodinger, 2009) was competent at reproducing the x-ray pose for each complex.

Models of compounds GSK47866A, GSK47867A and GSK47869A (S-isomers) were prepared using the Ligprep module and a set of 272 conformers generated using the confgen module of Maestro. This set of conformers was docked in the 3E7C active site model yielding 62 successful poses. Again, as found in the bootstrapping exercise, GSK47866A best scoring pose was extremely close in conformation and position within the active site pocket (RMSD ~0.2), indicative of a robust model. Crystal structures 1M2Z and 3E7C were superposed and conformations of residues within 6 Angstrom of the Dex ligand in 1M2Z were compared visually. Any differing substantially were coloured differently (Figure 3.4 A, B), and these atom colours projected onto a molecular surface to reveal regions of the protein surface impacted by the residue movements induced by binding of GSK47866A (Figure 3.7 A,C). The regions of surface modification thus highlighted guided where to look for differences in electrostatic potential, projected onto the same molecular surface (Figure 3.7 B,D)

3.3.12 Modelling of GR mutant with impaired Hsp90 interaction

The original 1M2Z x-ray coordinates, already optimised for use with the OPLS forcefield in Maestro, were used to mutate M604 to Threonine. The built-in residue mutation building tool was employed for this. The mutated structure was optimised using the Protein Preparation Wizard option to perform a restrained, all-atom minimisation. Surface and electrostatic potential colouring was calculated as for all other examples, ensuring a consistent range of electrostatic potential values of -0.2 to 0.2 for the blue-white-red colour ramp.

3.4 Results

3.4.1 GSK47867A and GSK47869A, are highly potent GR agonists

There is wide interest in understanding how variation in ligand structure (Figure 3.1 A) affects the function of GR. Here, we use novel, non-steroidal glucocorticoid receptor ligands (NSG) with very high potency, and specificity for GR to determine how ligand structure impacts receptor function (Figure 3.1 B-C, Figure 3.2). Transient GR transactivation and transrepression models in HeLa cells were used initially to compare the NSGs to conventional synthetic glucocorticoid ligands. We find that both GSK47867A and GSK47869A were approximately 30 times more potent that Dexamethasone (Dex, Figure 3.1 B-C, Table 3.1). Similar results were also obtained using A549 cells with stably integrated GRE-Luc or NFκB-Luc templates (Figure 3.3 A-B). The steroidal glucocorticoid Fluticasone Propionate (FP) had similar potency to GSK47867A and GSK47869A. Hydrocortisone was significantly less potent than all the synthetic ligands tested (Figure 3.1).

To rationalise subsequent matched analyses, saturating concentrations of the ligands were selected, calculated as 10 times the measured EC50 for transactivation (Table 3.1). At these concentrations all ligands showed similar repression of IL6 and IL8 secretion (Figure 3.3 C-D), and inhibition of cell proliferation (Figure 3.3 E-F).

Table 3.1: Saturating concentration of ligands calculated from EC_{50}

	Dex	67A	69A
Average EC_{50}	6.26	0.29	0.28
StDev	± 3.8	± 0.13	± 0.06
10X (EC_{50}+StDev)	100 nM	3 nM	3 nM

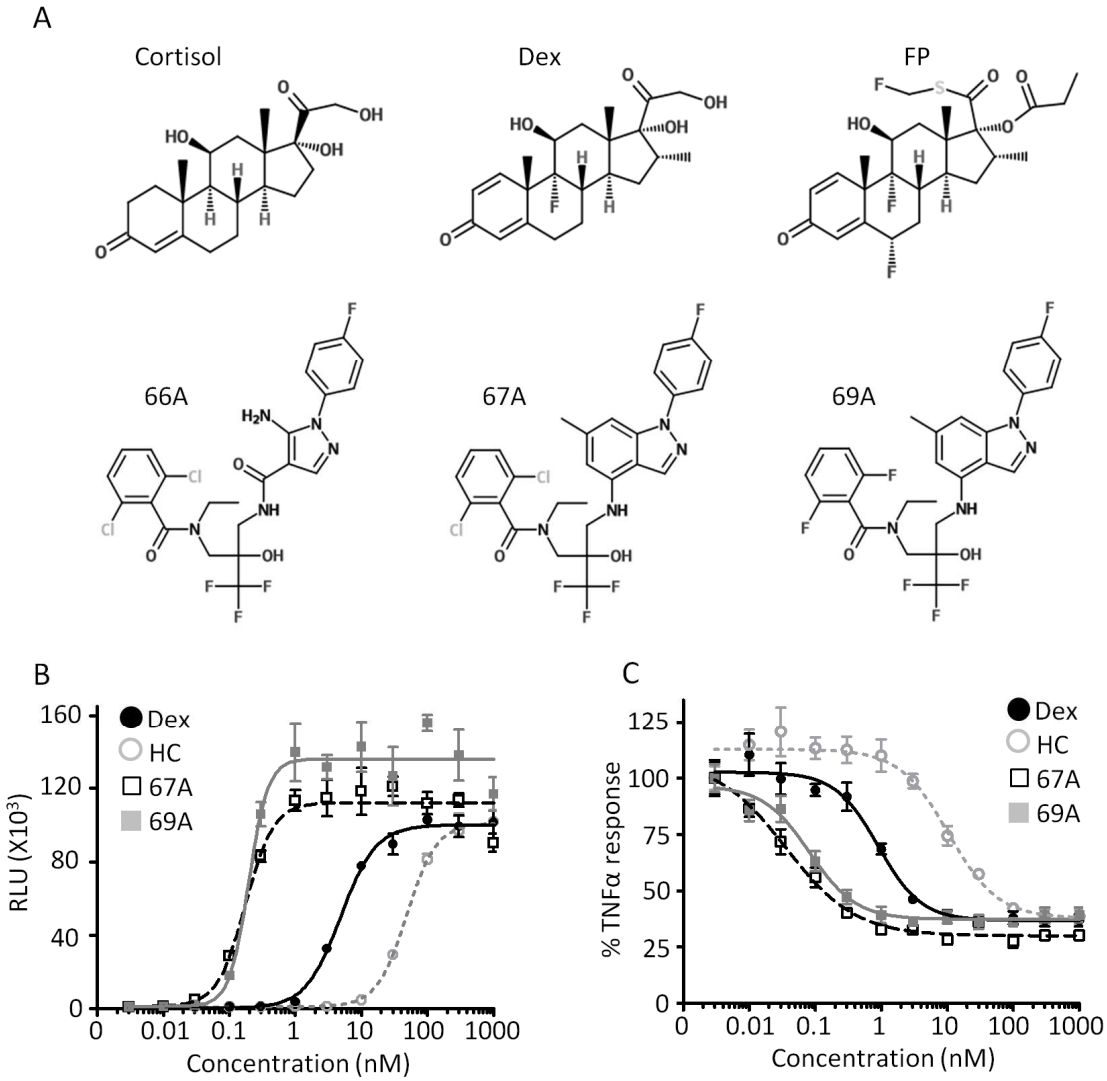

Figure 3.1: GSK47867A and GSK47869A are highly potent GR agonists. Structure of steroidal and non steroidal glucocorticoids (A). HeLa cells were transfected with a positive GR reporter gene (TAT3-luc) (B) or with a glucocorticoid repressed NFκB reporter gene (NRE-luc) (C). At 24 hours post-transfection, NRE-Luc transfected cells were pre-treated with TNF α (0.5 ng/ml) for 30 minutes. Subsequently all transfected cells were treated with 0.01-1000 nM Dex, hydrocortisone (HC), GSK47867A (67A) or GSK47869A (69A) for 18 hours, and were then lysed and subjected to analysis by luciferase assay. The graphs (mean ± SD) show the relative light units (RLU) (B) or percentage inhibition (C) from one of three representative experiments performed in triplicate.

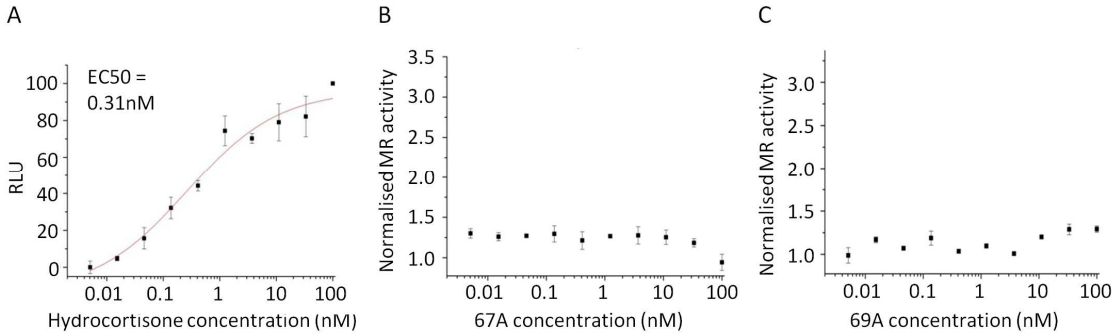

Figure 3.2: NSGs are highly GR specific. MR transactivation assays for GSK47867A (67A) and GSK47869A (69A) (B and C) relative to a hydrocortisone control (A). The EC50 value for hydrocortisone is given in the upper left. RLU are normalised between 0 (no activity) and 100 (full activity) and the EC50 values were determined by GraphPad Prism software, n=3.

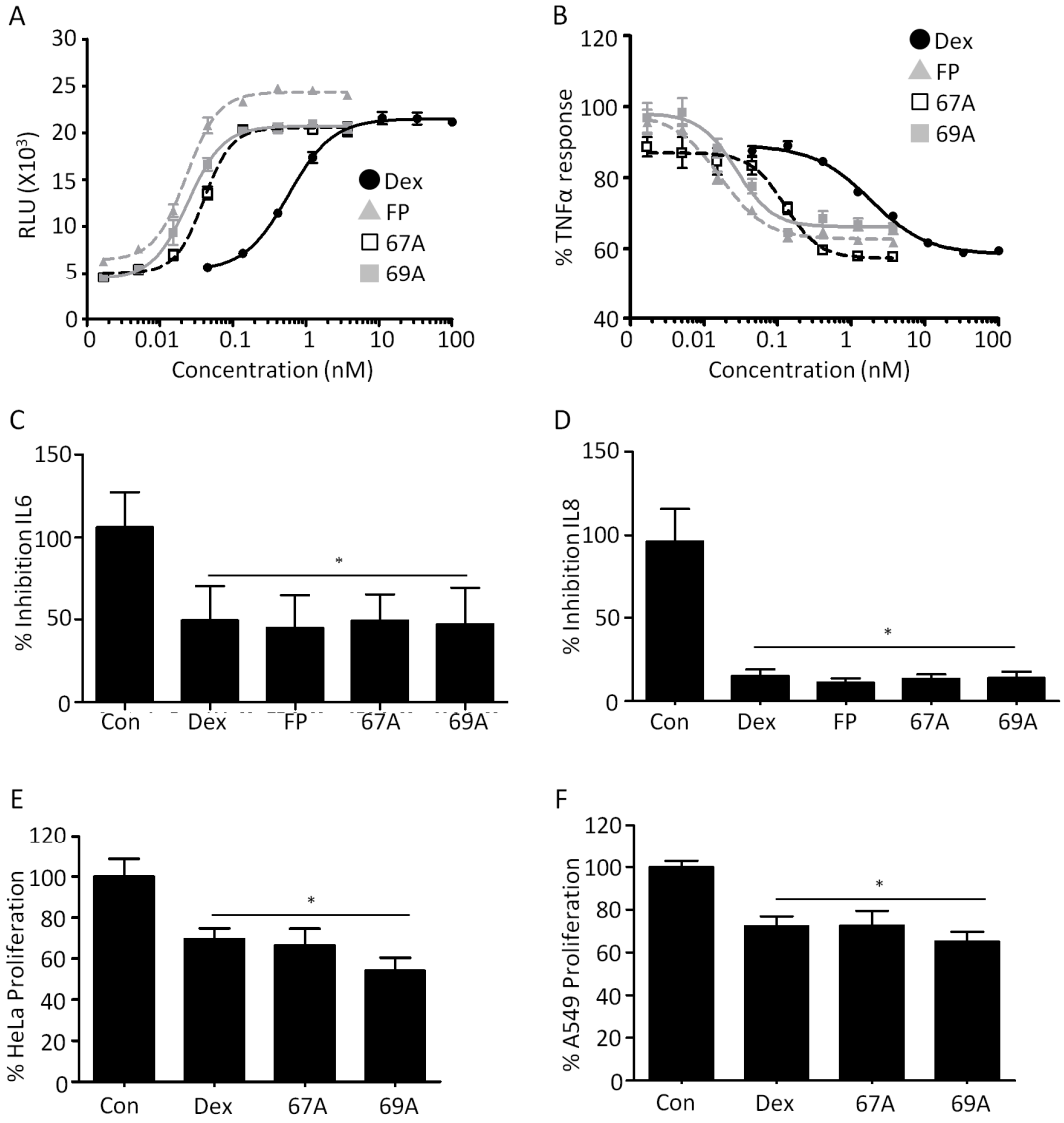

Figure 3.3: The NSGs are highly potent. A549 cells stably expressing a GRE-Luc (A) or NFκB-Luc (B) were used to confirm the results found in HeLa (Figure 1 B,C). A549 cells were treated with 0.01-1000 nM Dex, FP, GSK47867A (67A) or GSK47869A (69A) for 18 hours then lysed and subjected to analysis by luciferase assay. Graph (mean ± SD) shows the relative light units (RLU) (A) or percentage TNFα response (B) from one of three representative experiments performed in triplicate. A549 cells were incubated with DMSO, 100 nM Dex, 3 nM FP, 3 nM 67A or 3 nM 69A for 18 hours. Following treatment supernatants were collected and assayed for IL6 (C) and IL8 (D) concentration using a Luminex 100 with StarStation software. Graphs (mean ± SEM) show percentage inhibition of IL6 and IL8 production. HeLa and A549 cells were treated with DMSO vehicle, 100 nM Dex, 3 nM 67A or 3 nM 69A for 72 hours. Subsequently MTS reagent was added to the treated cells and the formazan production was measured after 2 hours at a wavelength of 490 nm. Graphs show formazan production for each treatment which directly correlates to the number of viable cells (E,F). Statistical significance was evaluated by one way ANOVA followed by Tukey post-test. Asterisks indicate: $*p < 0.001$ significantly different from vehicle treated control.

3.4.2 GR crystal structure reveals ligand-specific altered surface charge

To identify conformational differences in the GR ligand binding domain (LBD), we first compared the structures of GR-Dex (1M2Z) and GR-GSK47866A (3E7C) a non-steroidal GR ligand similar in structure to GSK47867A (Figure 3.1 A and Figure 3.4). An active site model derived from the coordinates of deposited structure 3E7C was used to dock GSK47867A and GSK47869A. Both GSK47867A and GSK47869A are similar to GSK47866A and gave very high scoring fits in the binding pocket formed by GSK47866A bound to the GR LBD (Figure 3.5). Inspection of the poses showed sensible, well fitting conformers, indicating that structure 3E7C was a suitable surrogate to compare with 1M2Z.

Observation of the ligand binding pocket in each crystal structure revealed that amino acids in closest proximity to each ligand demonstrated significant movement compared to Dex at the head (A ring, Figure 3.4 C-D) and tail (D ring, Figure 3.6 C-D). The greatest displacement was seen in amino acids Gln570 and Arg611 (Figure 3.4 C-D). Less displacement was seen at the opposite end of the ligand; most noticeable here was the movement of Gln642 (Figure 3.6 C-D).

The effect of residue movements in the GR LBD upon binding of GSK47866A was examined by visualisation of the molecular surface (Figure 3.7, 3.8 and 3.9). This revealed a distinct surface electrostatic potential difference, highlighting a patch of positive charge in the GR-GSK47866A structure resulting from displacement of Arg611 (Figure 3.7 B,D). This demonstrates that the structural difference between Dex and the NSGs results in a different GR surface charge upon binding, with potential for altered for protein-protein interactions.

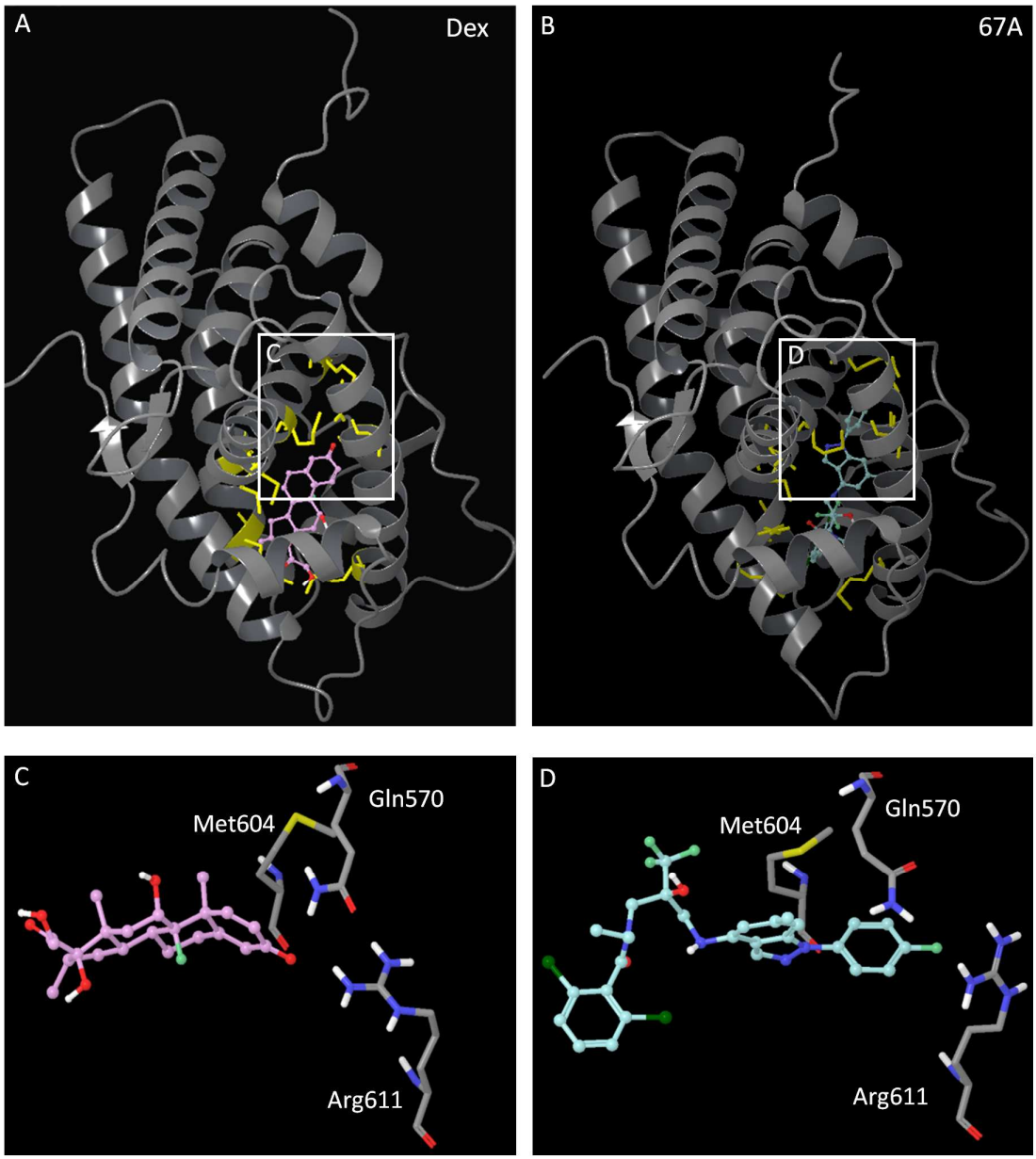

Figure 3.4: Dex and GSK47867A binding induces different GR LBD structures. Comparison of the crystal structures of the GR LBD bound to Dex (A, purple) and GSK47867A (67A) (B, blue). The residues in the binding pocket that show significant movement upon 67A binding are highlighted in yellow. When 67A binds to the GR LBD the head region causes movement of residues Gln570, Met604 and Arg611 (D) when compared with Dex binding (C).

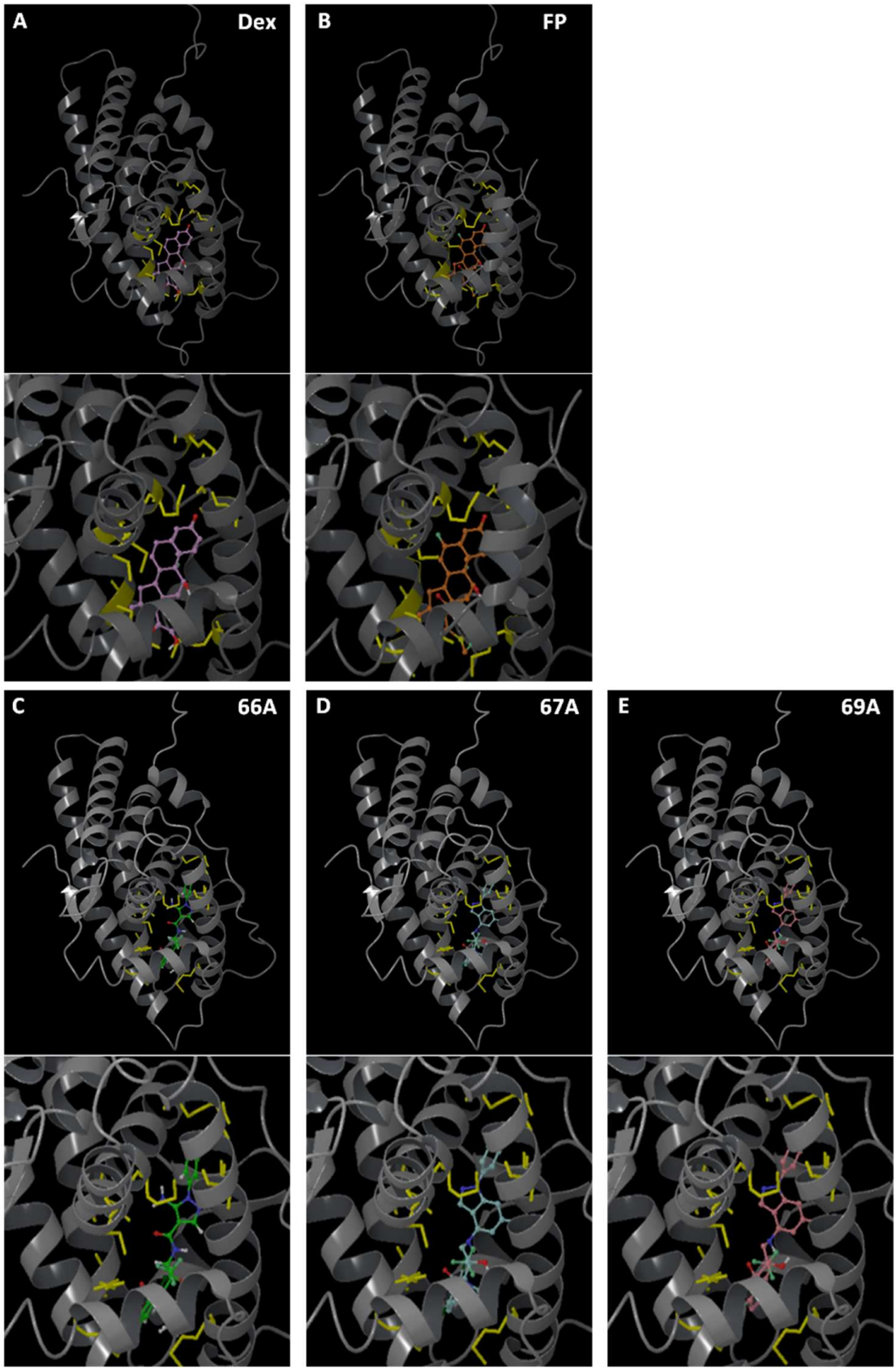

Figure 3.5: Docking NSG into GR LBD crystal structure. Comparison of the crystal structures of the GR LBD bound to Dex (A, purple), FP (B, orange) and GSK47866A (66A) (C, green). GSK47867A (67A) (D, blue) and GSK47869A (69A) (E, Pink) were docked into the binding pocket of 66A and gave a very high scoring fit. The residues in the binding pocket that show significant movement with NSG binding are highlighted in yellow.

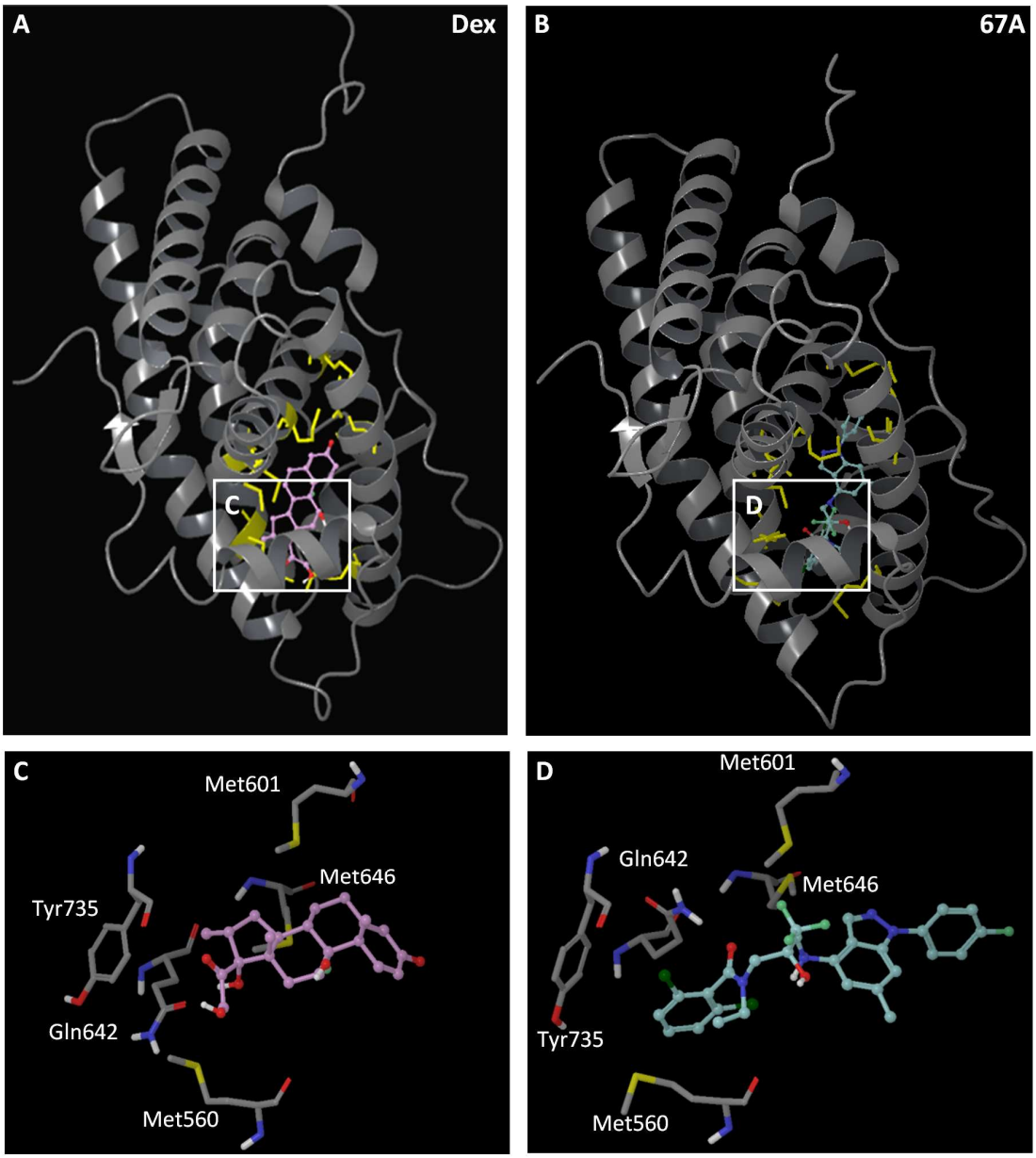

Figure 3.6: Movement of residues at tail of ligand (D ring). Comparison of the crystal structures of the GR LBD bound to Dex (A, purple) and GSK47867A (67A) (B, Blue). The residues in the binding pocket that show significant movement upon 67A binding are highlighted in yellow. When 67A binds to the GR LBD the tail region of the ligand causes movement of residues Met560, Met601, Gln642, Met 646 and Tyr 735 (D) when compared with Dex (C).

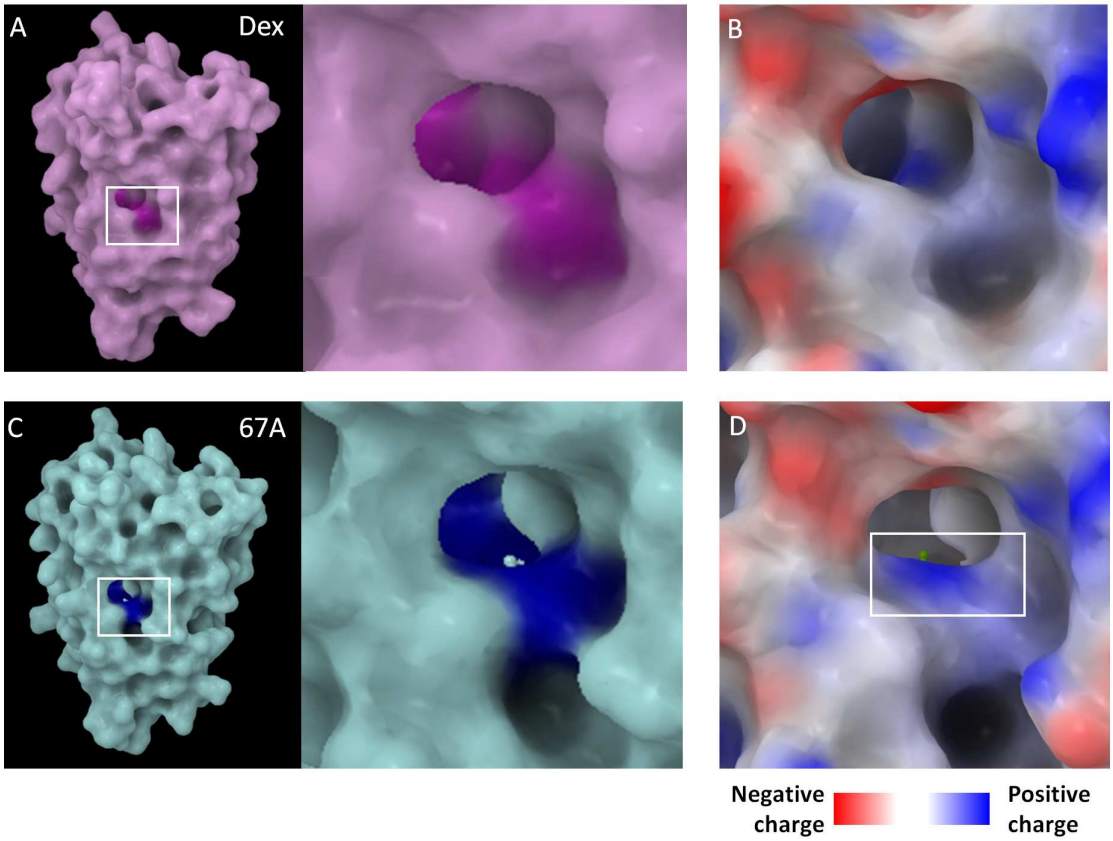

Figure 3.7: GR LBD surface charge is altered by GSK47867A binding. (A, C) The region of the GR LBD surface where residues Gln570, Met604 and Arg611 are exposed is highlighted [A, with Dex in purple and C, GSK47867A (67A) in blue]. (B, D) A close up of this region is shown with an electrostatic charge map that reveals the creation of a patch of positive surface charge due to the movement of Arg611 upon 67A binding.

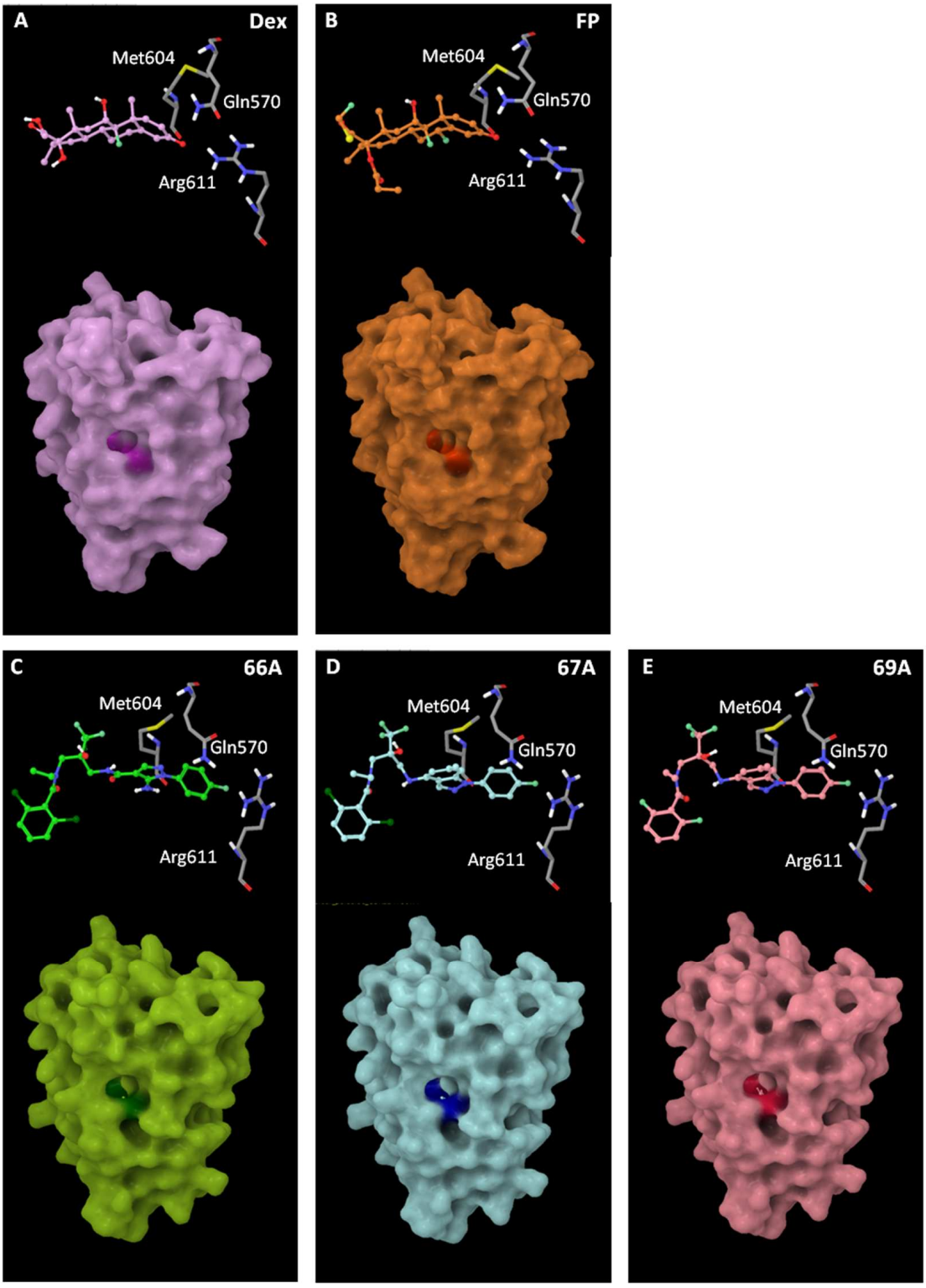

Figure 3.8: Comparison of GR LBD surface with steroidal and non-steroidal ligands at the head end of the ligand (A ring). Comparison of the crystal structures of the GR LBD bound to Dex (A, purple), FP (B, orange), GSK47866A (66A) (C, green), GSK47867A (67A) (D, blue) and GSK47869A (69A) (E, pink). The region of the GR LBD surface where residues Gln570, Met604 and Arg611 are exposed is highlighted.

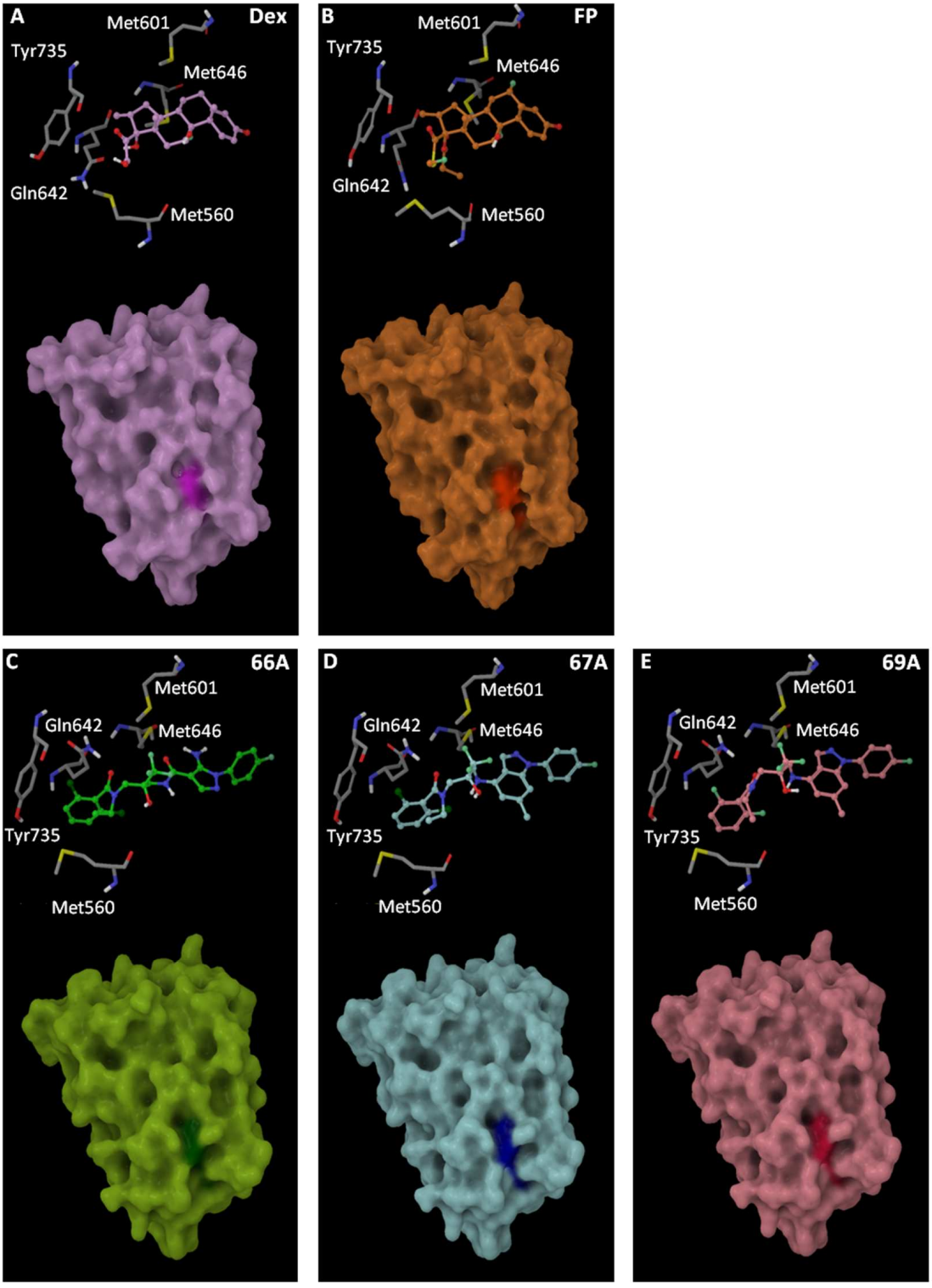

Figure 3.9: Comparison of GR LBD surface with steroidal and non-steroidal ligands at the tail end of the ligand (D ring). Comparison of the crystal structures of the GR LBD bound to Dex (A, purple), FP (B, orange), GSK47866A (66A) (C, green), GSK47867A (67A) (D, blue) and GSK47869A (69A) (E, pink). When the NSGs bind to the GR LBD the tail region of the ligands cause movement of residues Met560, Met601, Gln642, Met 646 and Tyr 735. The region of the GR LBD surface where residues Met560, Gln642 and Tyr735 and are exposed is highlighted.

3.4.3 NSG induce different kinetics of endogenous Gc target gene regulation

To determine whether the alteration in GR surface charge upon binding NSG had any functional consequence, transcript levels of endogenous glucocorticoid induced (GILZ and FKBP5) and glucocorticoid repressed (IL6 and IL8) target genes were quantified at multiple time points (Figure 3.10 A-B, Figure 3.11 A-B). Both the steroidal and NSG ligands displayed equivalent kinetics of FKBP5 induction (Figure 3.10 A). Although NSG treatment did not induce GILZ transcript at 1 hour, similar induction was observed at later time points (Figure 3.10 B). Similarly NSG treatment did not repress IL6 or IL8 transcripts at 1 hour but comparable repression was observed at later time points (Figure 3.11 A-B).

3.4.4 NSG treatment results in delayed kinetics of GR 211 phosphorylation

Transactivation of IGFBP1 is reliant on Ser211 phosphorylation of the GR, a signal to recruit the co-activator protein MED14. Dex treatment resulted in significant induction of IGFBP1 transcript by 1 hour (Figure 3.12 A), but the NSG ligands failed to induce transcript at this early time point. This lack of transcript regulation at an early time point was similarly seen with GILZ, IL6 and IL8. Ligand induction of GR Ser211 phosphorylation was compared. Treatment with Dex resulted in rapid phosphorylation of GR at both serine residues 203 and 211 (Figure 3.12 B). The NSG ligands induced slower onset of phosphorylation of both serine residues 203 and 211 (Figure 3.12 B).

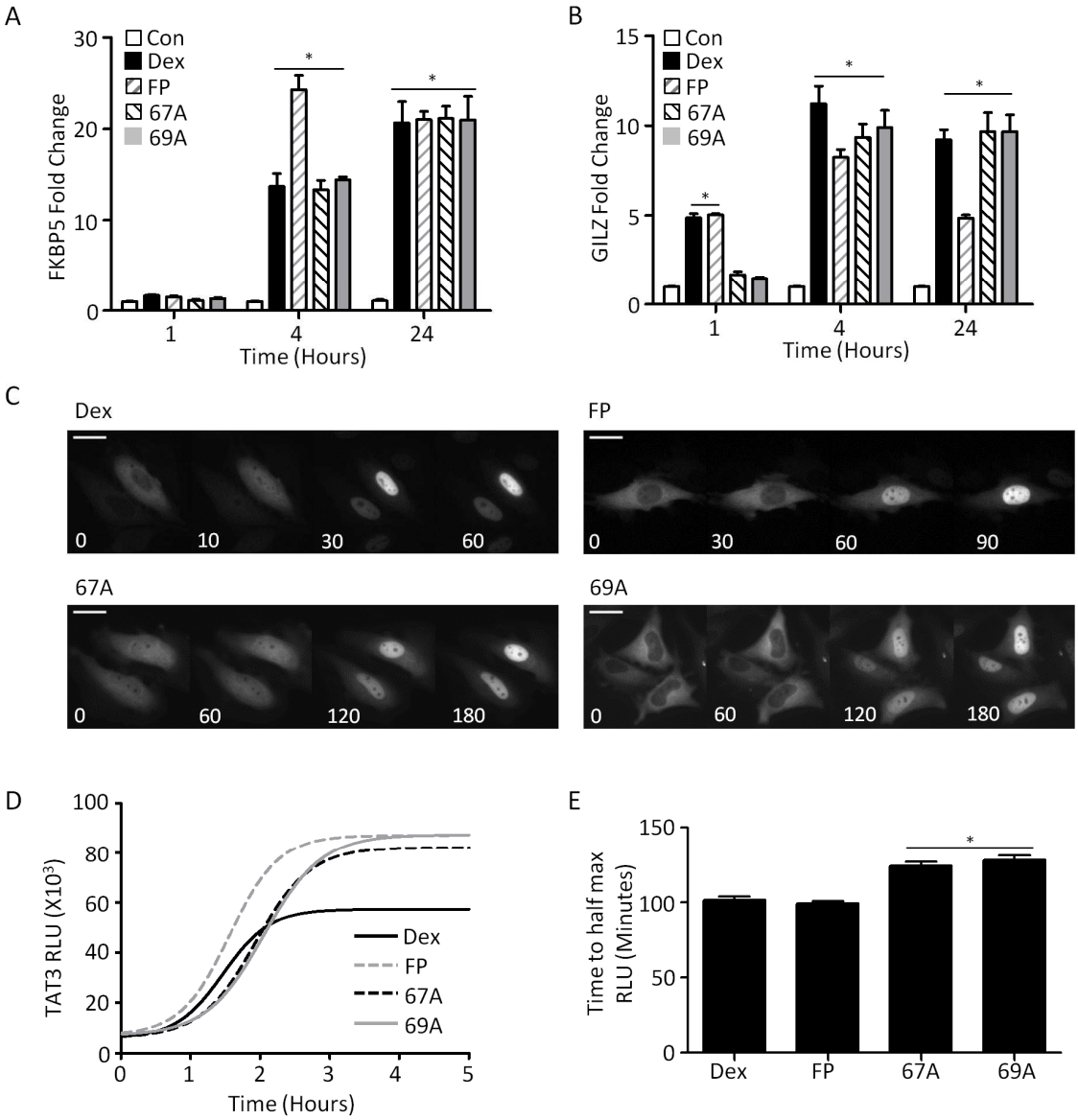

Figure 3.10: GSK47867A and GSK47869A induce slow kinetics of GR activation. HeLa cells were treated with DMSO vehicle, 100 nM Dex, 3 nM GSK47867A (67A) or 3 nM GSK47869A (69A) for 1, 4 or 24 hours. Cells were then lysed and RNA was extracted using an RNeasy kit. RNA was reverse transcribed and subjected to qPCR for FKBP5 (A) and GILZ (B) using Sybr Green detection in an ABI q-PCR machine with data analysed by the δδ CT method. Graphs (mean ± SEM) combine data from three separate experiments and display fold change over that in vehicle-treated control. (C) Following transfection with HaloTag-GR, HeLa cells were incubated with 100 nM Dex, 3 nM FP, 3 nM 67A or 69A. Cells were imaged in real time at 37°C to determine the subcellular localisation of the GR (white) at the times indicated. Scale bar, 25μm. Images are representative of three independent experiments. (D) HeLa cells transfected with a TAT3-Luc reporter plasmid were treated with 100 nM Dex, 3 nM FP, 3 nM 67A or 69A for up to 24 hours. The production of luciferase was tracked by measuring the relative light units (RLU) emitted from each sample, graph D tracks RLU production over the first 5 hours following addition of treatment and is representative of three separate experiments. The time taken to reach half the maximal light output was measured for all treatments (E). Statistical significance was evaluated by one way ANOVA followed by Tukey post-test. Asterisks indicate: *$p <$ 0.005 compared with control, **$p <$ 0.001 compared with Dex.

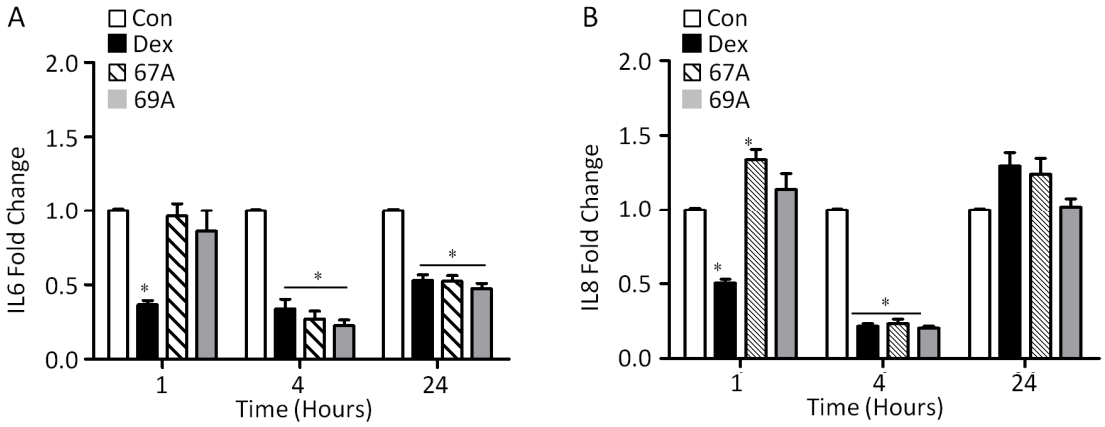

Figure 3.11: Regulation of endogenous genes IL6 and IL8. HeLa cells were treated with TNF α 5 ng/ml, DMSO vehicle, 100 nM Dex, 3 nM GSK47867A (67A) or 3 nM GSK47869A (69A) for 1, 4 or 24 hours then lysed and RNA extracted using an RNeasy kit. RNA was reverse transcribed and subjected to qPCR for IL6 (A) or IL8 (B) using Sybr Green detection in an ABI q-PCR machine and data analysed by the δδ CT method. Graphs (mean ± SEM) combine data from three separate experiments and display fold change over vehicle treated control. Statistical significance was evaluated by one way ANOVA followed by Tukey post-test. Asterisks indicate: *p < 0.005 significantly different from control.

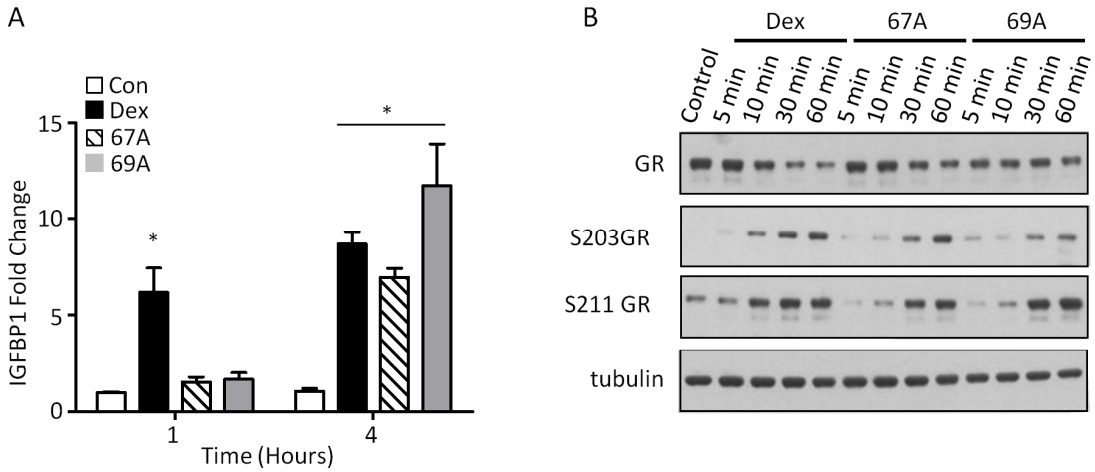

Figure 3.12: Kinetics of GR phosphorylation. HeLa cells were treated with DMSO vehicle, 100 nM Dex, 3 nM GSK47867A (67A) or 3 nM GSK47869A (69A) for 1 or 4 hours then lysed and RNA extracted using an RNeasy kit. RNA was reverse transcribed and subjected to qPCR for IGFBP1 (A) using Sybr Green detection in an ABI q-PCR machine and data analysed by the δδ CT method. Graph (mean ± SEM) combines data from two separate experiments and displays fold change over vehicle treated control. HeLa cells were treated either with 100 nM Dex or 3 nM 67A or 69A for up to 60 minutes then lysed in RIPA buffer containing phosphatase and protease inhibitors and analysed by immunoblotting for GR abundance, GR ser 203 and GR ser 211 phosphorylation (B). α-Tubulin was used as a loading control. Statistical significance was evaluated by one way ANOVA followed by Tukey post-test. Asterisks indicate: *p < 0.005 significantly different from control.

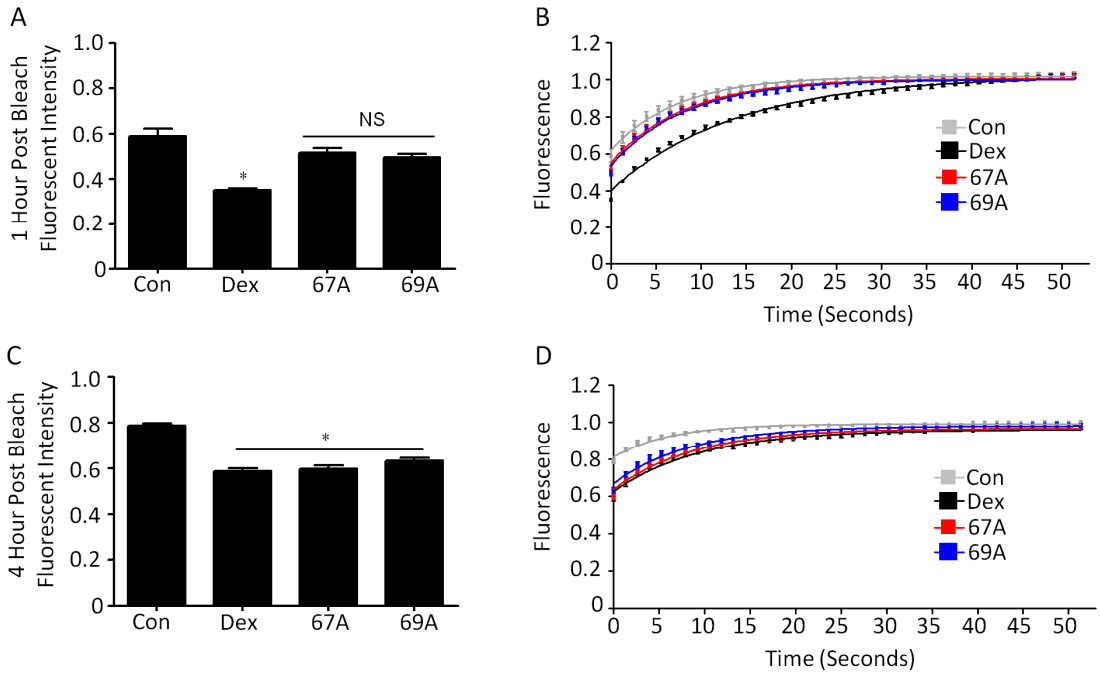

Figure 3.13. GR nuclear mobility. HeLa cells were transfected with GR-GFP and incubated with 100 nM Dex, 3 nM GSK47867A (67A) or 3 nM GSK47869A (69A) for 1 (A,B) or 4 hours (C,D). The nuclei of the treated cells were subjected to photobleaching and the level of bleach (A, C) and time to recovery (B, D) was measured for each treatment. Statistical significance was evaluated by one way ANOVA followed by Tukey post-test. Asterisks indicate: $*p < 0.001$ significantly different from control.

3.4.5 NSG treatment results in slow rate of GR nuclear translocation

The delay in endogenous gene transactivation and receptor phosphorylation seen with NSG treatment suggested that nuclear translocation may also be delayed. Use of a halo tagged GR clearly demonstrated a slower rate of nuclear translocation with both GSK47867A and GSK47869A (Figure 3.10 C). Ligand-bound nuclear GR has a signature FRAP signal, with reduced intranuclear mobility resulting in delayed recovery from photobleaching. FRAP studies revealed that at 1 hour following NSG treatment nuclear GR displayed characteristics of an unliganded receptor (Figure 3.13 A-B). However with 4 hour NSG treatment nuclear GR displayed the typical signature of liganded receptor, indicative of a delay in adoption of the activated GR conformation (Figure 3.13 C-D).

Altered kinetics of GR phosphorylation may explain the observed differences in nuclear translocation rate and transactivation of endogenous glucocorticoid target genes. Therefore, we made GR mutants Ser211Ala (phosphodeficient) and Ser211Asp (phosphomimetic) to assess the importance of this phosphorylation site (Figure 3.14 A). However, the phosphomimetic GR did not significantly increase the rate of GR translocation with either GSK47867A or GSK47869A treatment (Figure 3.14 C-D). Likewise the phosphodeficient GR had no significant impact on the rate of translocation seen with Dex treatment (Figure 3.14 B, D).

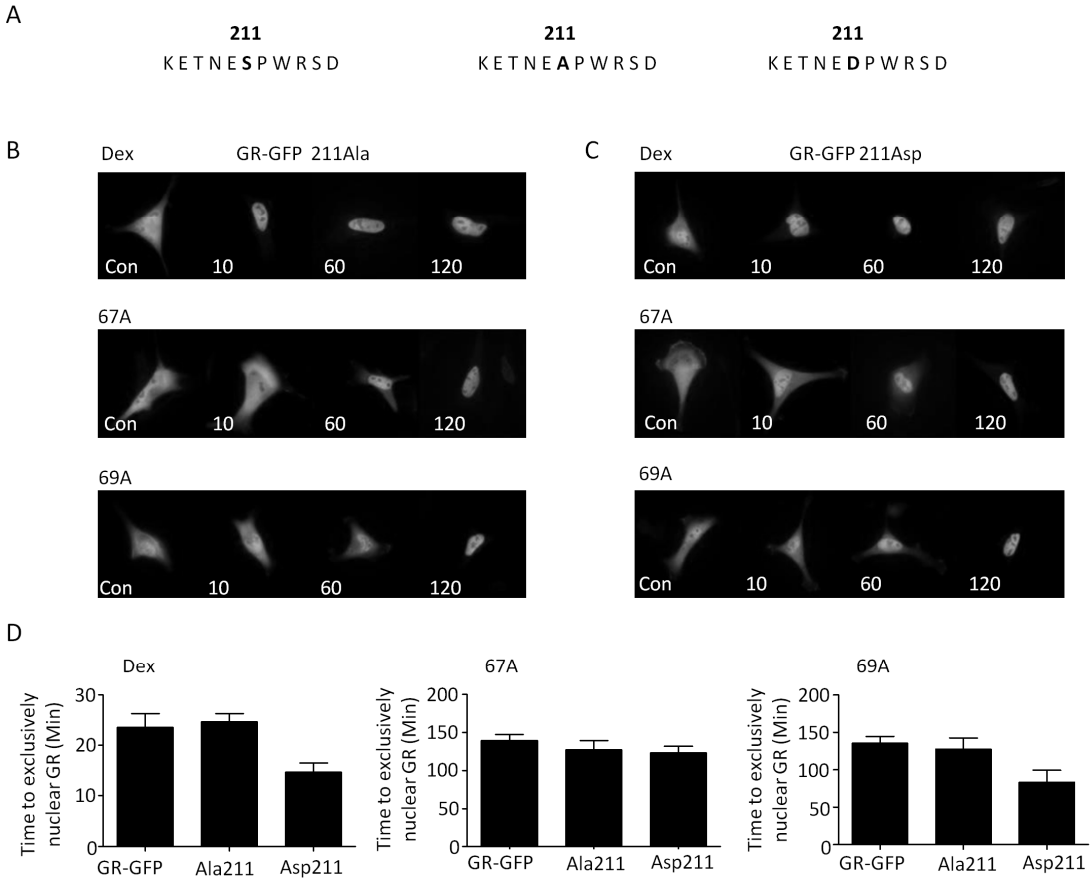

Figure 3.14: GR translocation with 211 phospho mutants. Schematic of plasmids for GR-GFP, GR-GFP 211Ala and GR-GFP 211Asp (A). HeLa cells were transfected with plasmids containing either GR-GFP, GR-GFP 211Ala (B) or GR-GFP 211Asp. (C) Transfected cells were incubated with 100 nM Dex, 3 nM GSK47867A (67A) or 3 nM GSK47869A (69A). Cells were fixed with PFA and analysed for subcellular localisation of the GR-GFP (white) at the times indicated. Images are representative of three independent experiments. Time (mean +/- SEM) taken for the GR to become exclusively nuclear was measured across both the fixed and live cell imaging experiments (D). Statistical significance was evaluated by one way ANOVA followed by Tukey post-test. Asterisks indicate: *p < 0.001 significantly different from GR-GFP.

3.4.6 NSG treatment results in slower onset of GR transactivation

Treatment with NSG results in slowed GR nuclear translocation and delayed transactivation of endogenous glucocorticoid target genes. To measure the kinetics of GR transactivation more precisely, real-time luciferase analysis was used (Meng *et al.*, 2008;McMaster *et al.*, 2008a) (Figure 3.10 D). This revealed that the NSG ligands consistently took longer to reach half-maximal transactivation compared to either Dex, or the higher potency FP (Figure 3.10 E). Interestingly all three high potency ligands resulted in greater maximal transactivation (Figure 3.10 D).

3.4.7 Delayed action of NSG cannot be explained by impaired cellular uptake

One possible explanation for these observations is altered ligand access to the intracellular GR. Initially mass spectroscopy analysis of cell lysates was performed after 10 minutes ligand exposure (Figure 3.15 A). A 10μM concentration of each ligand was compared, to permit detection of the ligand by mass spectrometry in cell lysates. Strikingly, the NSG ligands showed greater than 10 fold increased concentrations within the cells compared to Dex, effectively ruling out delayed ligand penetration.

To further evaluate cell pharmacokinetics, cells were incubated with 100 nM Dex or 3 nM FP, GSK47867A or GSK47869A for 10 minutes, washed and then incubated in ligand-free medium for 4 hours. These samples were compared to cells treated with ligand continuously for 4 hours (Figure 3.15 B-D). Short exposure to both NSG ligands resulted in greater induction of GILZ and FKBP5 although not IGFBP1 compared to Dex, again demonstrating rapid cellular accumulation of ligand. Furthermore cells incubated with NSG on ice for 1 hour to permit ligand access in the absence of GR activation still showed delayed nuclear translocation (Figure 3.15 E-F), implicating a post receptor mechanism of action. The observed differences could not be attributed to Dex activation of mineralocorticoid receptor, as the mineralocorticoid receptor antagonist Spironolactone did not affect the Dex induction (Figure 3.16 A-B).

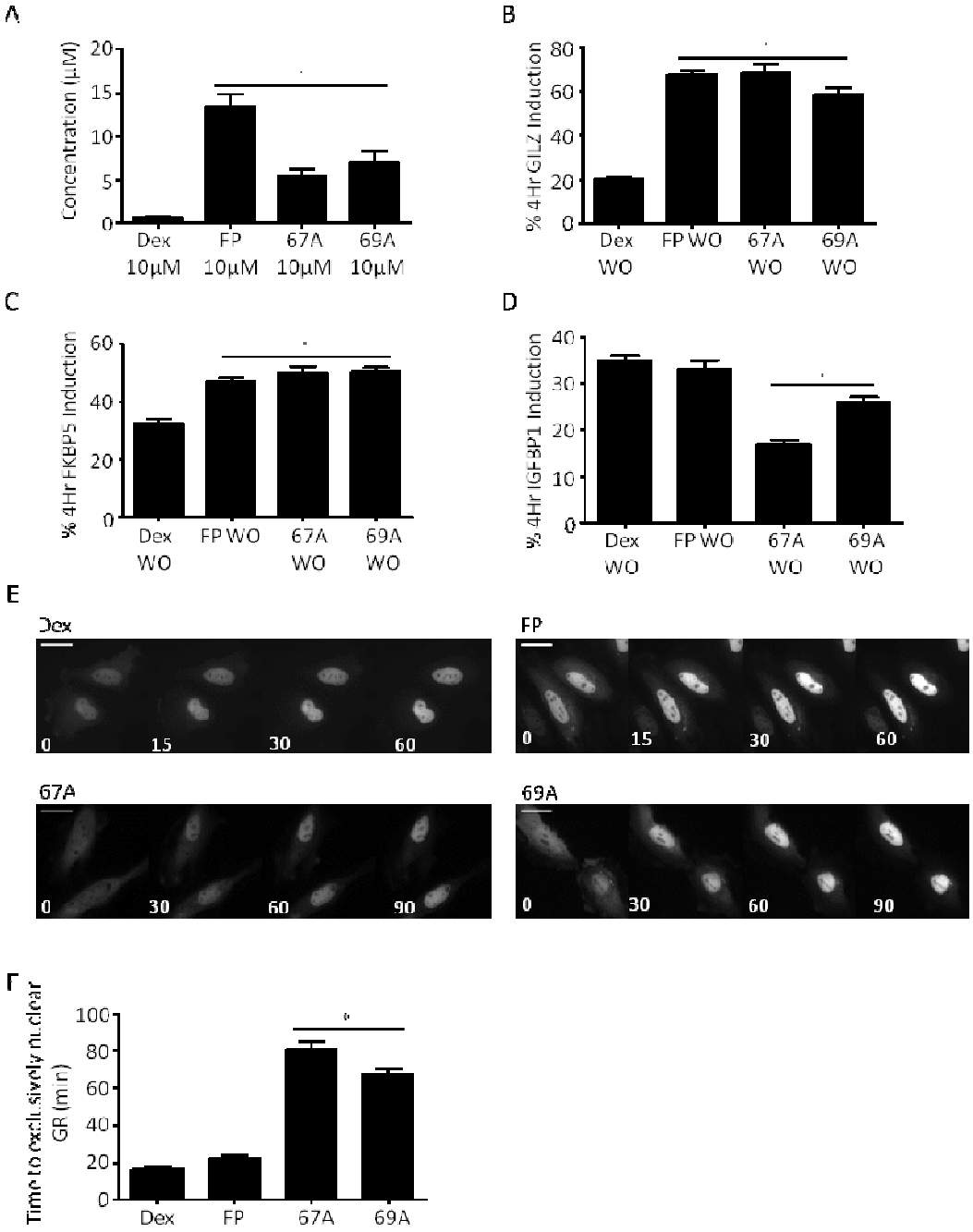

Figure 3.15: GSK47867A and GSK47869A rapidly accumulate in cells. A549 cells were treated with 10 µM Dex, FP, GSK47867A (67A) or GSK47869A (69A) for 10 minutes and subsequently washed and lysed. The cell samples were analysed for ligand uptake by mass spectrometry (A). HeLa cells were treated with DMSO vehicle (not shown), 100 nM Dex, 3 nM FP, 3 nM 67A or 3 nM 69A either for 4 hours or for 10 minutes followed by washout (WO) and cultured in ligand-free medium for 4 hours. Subsequently cells were lysed and RNA extracted using an RNeasy kit. RNA was reverse transcribed and subjected to qPCR for GILZ (B), FKBP5 (C) and IGFBP1 (D) using Sybr Green detection in an ABI q-PCR machine and data were analysed by the δδ CT method. Graphs (mean ± SEM) combine data from three separate experiments and display percentage induction compared with the equivalent 4 hours of constant treatment. Following transfection with HaloTag-GR HeLa cells were placed on ice for 10 minutes and subsequently incubated with 100 nM Dex, 3 nM FP, 3 nM 67A or 69A for 1 hour on ice. Following treatment, cells images were captured in real time at 37°C to determine the subcellular localisation of the GR (white, E). Scale bar, 25µm. Graph F displays average time to exclusively nuclear GR following 1 hour with ligand on ice, calculated from three separate experiments. Statistical significance was evaluated by one way ANOVA followed by Tukey post-test. Asterisks indicate: *$p < 0.001$ compared with Dex.

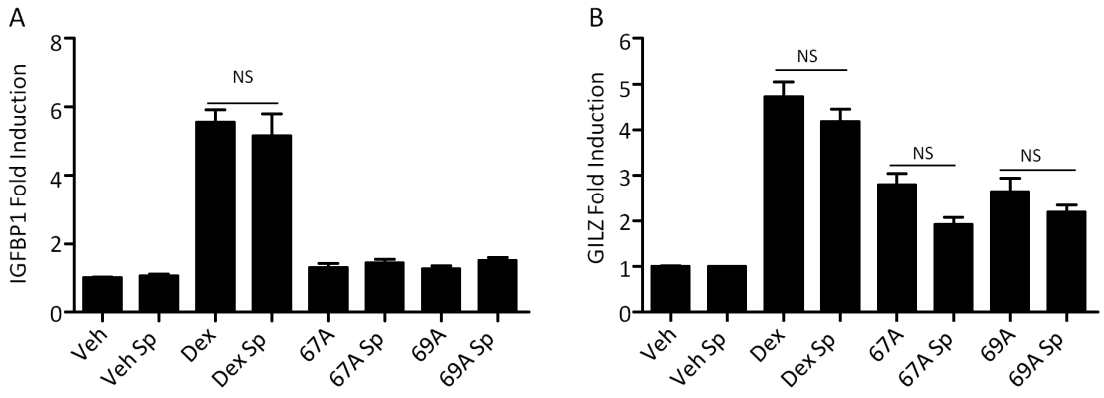

Figure 3.16: Endogenous gene regulation in the presence of an MR inhibitor. HeLa cells were pretreated for 30 minutes with 1 uM spironolactone and subsequently treated with DMSO vehicle, 100 nM Dex, 3 nM GSK47867A (67A) or 3 nM GSK47869A (69A) for 1 hour then lysed and RNA extracted using an RNeasy kit. RNA was reverse transcribed and subjected to qPCR for IGFBP1 (A) and GILZ (B) using Sybr Green detection in an ABI q-PCR machine and data analysed by the δδ CT method. Graphs (mean ± SEM) combine data from three separate experiments and display fold change over vehicle treated control. Statistical significance was evaluated by one way ANOVA followed by Tukey post-test. NS = not significantly different from treatment without spironolactone.

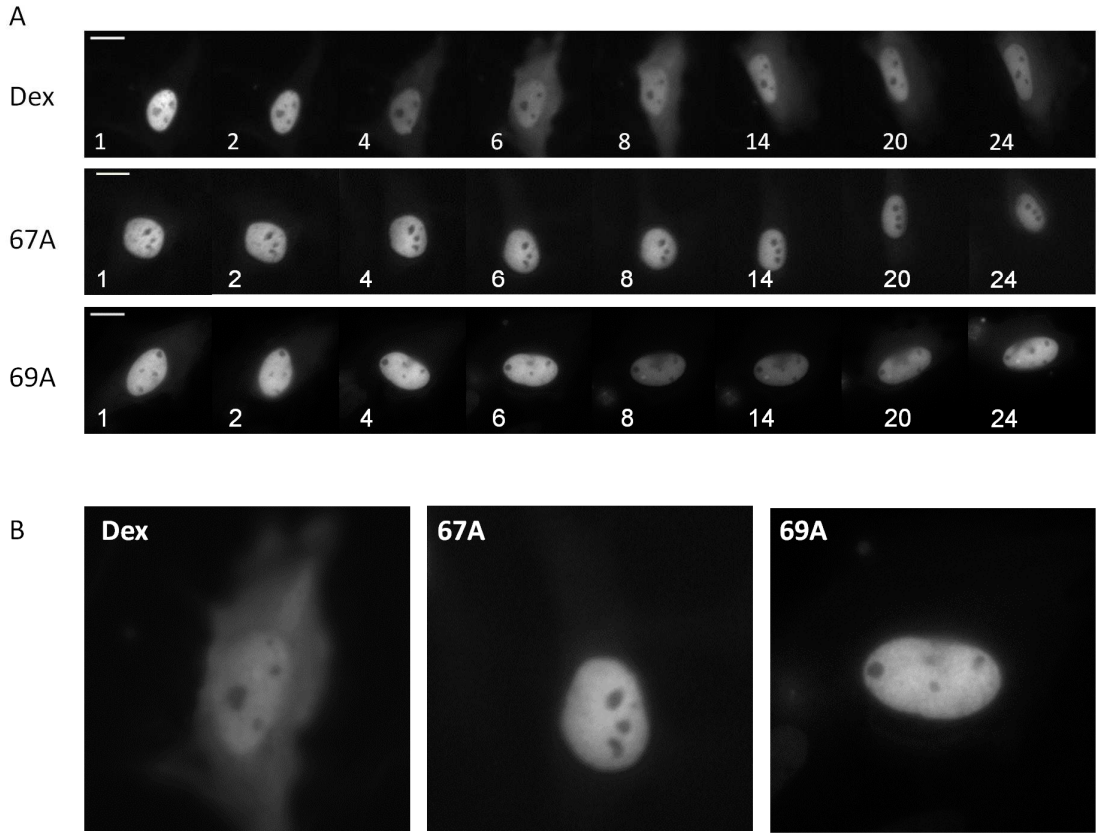

Figure 3.17: Nuclear export of the GR. HeLa cells were transfected with GR-GFP and incubated with 100 nM Dex, 3 nM GSK47867A (67A) or 3 nM GSK47869A (69A) for 2 hours then subsequently washed and imaged for the following 24 hours to assess subcellular GR localisation (white). Magnification of the 6 hour time point demonstrates the difference in subcellular GR localisation (B). Scale bar, 25µm. Images are representative of three independent experiments.

3.4.8 NSG bound GR shows prolonged nuclear retention

As treatment with both NSG ligands results in delayed nuclear translocation, we investigated whether nuclear export of GR may also be slower. To measure GR export HeLa cells were treated with 100 nM Dex or 3 nM NSGs for 1 hour then washed and placed in serum free media and imaged over 24 hours (Figure 3.17 A). In cells treated with NSG the GR-GFP was not exported from the nucleus during the 24 hour wash-out period, but Dex treated cells exported GR from the nucleus within 6 hours (Figure 3.17 B).

3.4.9 Structural modelling suggests that NSGs modify the Hsp90 interaction surface

Our data clearly demonstrates that when bound to NSG there is altered interaction of GR with the translocation machinery resulting in delayed nuclear import, delayed transcriptional activity and receptor export. The chaperone heat shock protein 90 (Hsp90) is known to play key roles in this aspect of GR biology, including maintaining GR structure, ligand binding activity, and trafficking of GR between nucleus and cytoplasm (Segnitz and Gehring, 1997;Tago *et al.*, 2004;Kakar *et al.*, 2006;Grad *et al.*, 2007;Echeverria *et al.*, 2009). GR residues identified by Ricketson and co-workers (Ricketson *et al.*, 2007) as important for Hsp90 interaction were mapped onto the crystal structure of GR bound to Dex (Figure 3.18 A). Surface map analysis of GR following replacement of Met604 with Thr604, which has been shown to inhibit Hsp90 recruitment, was in the same part of the GR structure that was differentially affected by NSG binding (Figure 3.18 B, C).

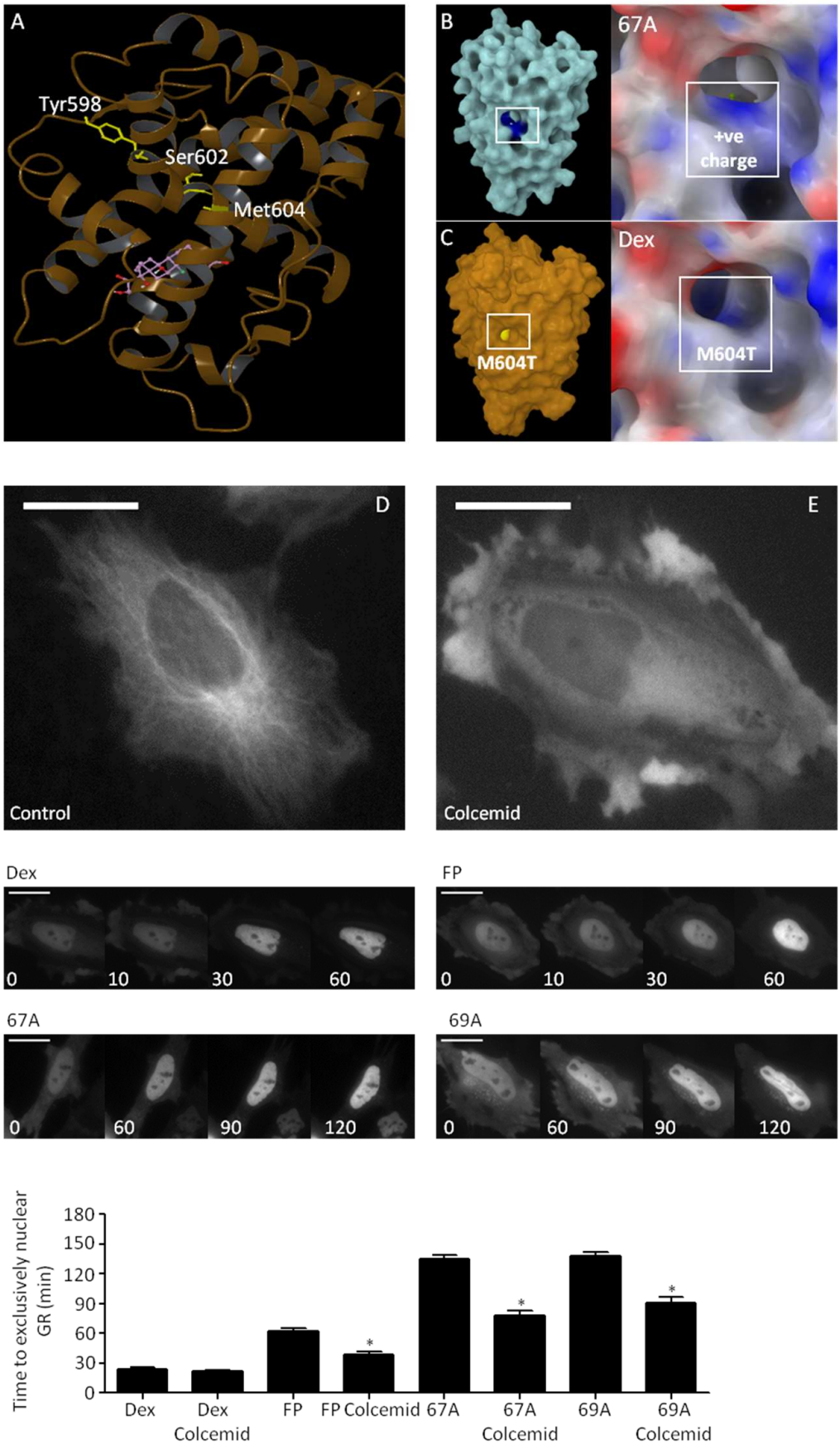

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Figure 3.18: Disruption of the microtubule network increases the rate of GR translocation in a ligand specific manner. (A) The ribbon structure of the GR LBD bound to Dex. The residues highlighted in yellow were identified by Ricketson *et al* as important for interaction between GR and Hsp90. The region of the GR LBD surface where the NSGs cause an alteration in surface charge is shown in panel B. The region of the GR LBD surface where Met604 is exposed is highlighted in panel C in yellow. This area overlaps the region identified as having altered surface charge upon binding NSG, supporting the lack of Hsp90 engagement with NSG treatment. (D) Untreated HeLa cells with GFP-labelled microtubules. Incubation for 1 hour with 2 μM colcemid disrupts the microtubule network (E). Following transfection with a halo tagged GR, HeLa cells were incubated with 2 μM Colcemid for 1 hour then subsequently co treated with 100 nM Dexamethasone, 3 nM FP, 3 nM GSK47867A (67A) or 3 nM GSK47869A (69A) (F). Cells were imaged in real time and analysed for subcellular localisation of the GR (white). Scale bar, 25μm. The average time taken (mean ± s.e.m) for the GR to be exclusively nuclear. Statistical significance was evaluated by one way ANOVA followed by Tukey post-test. Asterisks indicate: * p <0.005 compared with treatment without colcemid

3.4.10 Microtubule disruption improves nuclear translocation rate

Hsp90 anchors the GR to the microtubule network, so permitting rapid, energy-dependent nuclear translocation. Hsp90 antagonism slows the rate of nuclear translocation (Galigniana *et al.*, 1998). However, in addition, GR can translocate using a diffusion mechanism (Nishi *et al.*, 1999). Disruption of the microtubule network using colcemid restores rapid GR translocation even in the presence Hsp90 inhibitor geldanamycin (Segnitz *et al.*, 1997;Galigniana *et al.*, 1998). Therefore, we used colcemid to determine if the microtubule architecture was slowing NSG mediated nuclear translocation. Colcemid significantly increased the rate of NSG-driven nuclear translocation, but had no effect on that promoted by Dex (Figure 3.18 D-G), suggesting a diffusion mechanism for translocation

3.4.11 NSGs mediate prolonged duration of action

The duration of ligand-dependent activity depends on continuing presence of ligand, and maintaining GR in a ligand-binding compatible conformation. To investigate these phenomena we initially undertook washout studies, using real time reporter gene luciferase analysis. These revealed a striking prolongation of transactivation following NSG ligand withdrawal compared to either Dex or FP, which was not explained by increased ligand potency (Figure 3.19 A).

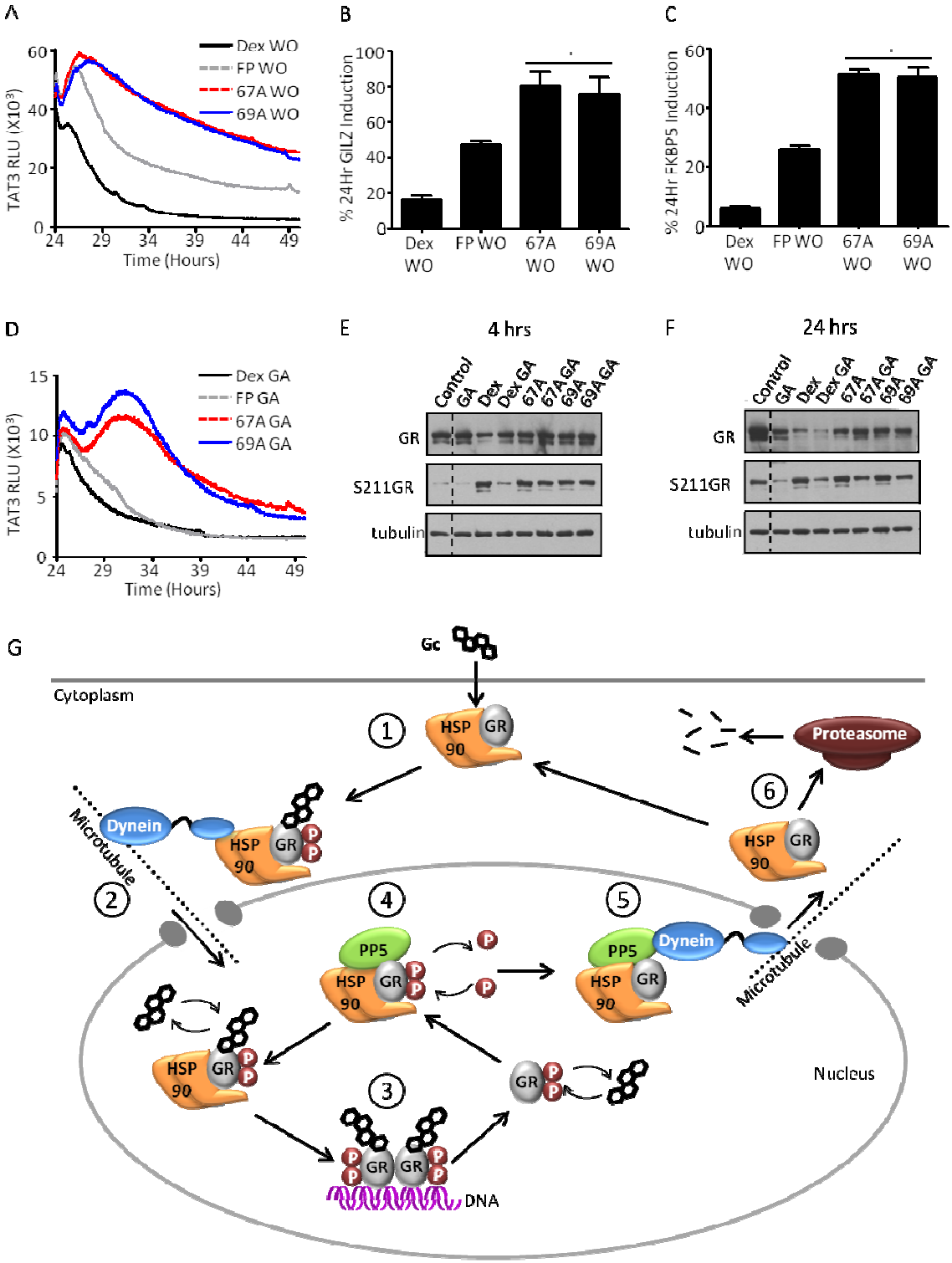

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Figure 3.19: Antagonism of Hsp90 has less impact on the activity of NSG ligands. HeLa cells transfected with a TAT3-Luc reporter plasmid were treated with 100 nM Dex, 3 nM GSK47867A (67A) or 3 nM GSK47869A (69A) for 24 hours. Subsequently cells were either co-treated with 10 mM geldanamycin (GA) (D) or washed (WO) and placed in serum-free recording medium (A) for a further 24 hours. The production of luciferase was tracked by measuring the relative light units (RLU) emitted from each sample. Graphs tracks RLU production for 24 hours following GA addition or ligand removal. Graphs are representative of three separate experiments. HeLa cells were treated with DMSO vehicle (not shown), 100 nM Dex, 3 nM FP, 3nM 67A or 3 nM 69A for 24 hours or 1 hour followed by washes (WO) and then cultured in ligand-free medium for 24 hours. Subsequently cells were lysed and RNA was extracted using an RNeasy kit. RNA was reverse transcribed and subjected to qPCR of GILZ (B) and FKBP5 (C) using Sybr Green detection in an ABI q-PCR machine and data analysed by the δδ CT method. Graphs (mean ± s.e.m) combine data from three separate experiments and display percentage induction compared to equivalent 24 hour constant treatment. HeLa cells were treated with 100 nM Dex, 3 nM 67A or 69A for 2 hours and then co treated with 10 mM GA for a further 2 hours (E) or 22 hours (F), and a constant 4 hour or 24 hour treatment was used as a comparison. Following treatment, cells were lysed in RIPA buffer containing phosphatase and protease inhibitors and analysed by immunoblotting for GR abundance and GR ser 211 phosphorylation. α-Tubulin was used as a loading control. Statistical significance was evaluated by one way ANOVA followed by Tukey post-test. $*p < 0.01$ compared with both Dex and FP. Mechanism of GR action (G). Upon binding glucocorticoid (Gc) (1) the GR interacts with the translocation machinery enabling nuclear import (2). In the nucleus GR binds to cis-elements to activate or repress target gene expression (3). The GR undergoes dynamic cycles of dissociation, and re-binding of ligand, which occurs in a Hsp90 dependent manner (4). Interaction with PP5 facilitates nuclear export of the GR (5) enabling it to be recycled or targeted for degradation by the proteasome (6).

To corroborate these observations with endogenous genes a two hour ligand exposure was chased with a 24 hour washout before measurement of GILZ and FKBP5 transcripts (Figure 3.19 B-C). There was significantly enhanced preservation of transactivation seen with both the NSGs compared to the potency matched control steroid FP.

To determine the role of Hsp90 in mediating prolonged GR transactivation, geldanamycin was used. As Hsp90 activity is required for initial GR ligand binding, these studies were performed sequentially, adding geldanamycin after ligand activation. The geldanamycin was added to cells at the time of maximal transactivation, in the presence of continuing ligand exposure (Figure 3.19 D). Both FP and Dex showed exponential decay of transactivation, as predicted. However, the NSG ligands showed a striking biphasic response, with initial potentiation, followed by decay (Figure 3.19 D).

As Hsp90 is also essential for maintaining GR protein stability investigation of receptor abundance and phosphorylation was undertaken. Inhibition of Hsp90 preserves GR protein levels following Dex treatment for 4 hours (Figure 3.19 E), but not at a later time point (Figure 3.19 F). Strikingly, the NSG ligands did not show such a ligand-dependent loss of GR protein (Figure 3.19 E,F), again identifying differences in Hsp90 interaction with the novel NSGs. Additionally treatment of cells with Dex in the presence of geldanamycin results marked dephosphorylation of GR at serine 211 (Figure 3.19 E). However treatment with the NSG was protective for serine 211 phosphorylation (Figure 3.19 E). Collectively, these studies suggest that GR-Hsp90 interactions can be modulated by ligand structure, to influence the properties of the glucocorticoid response.

3.5 Discussion

Understanding how the GR interprets its ligands to permit appropriate cellular responses is of vital interest in both physiology and pharmacology, as the GR remains an important drug target in inflammation and malignancy (Barnes, 2011;De *et al.*, 2011). The advent of drug design based on the crystal structure predicted pharmacophore has permitted new generations of ligands to be synthesised, including those studied here (Kauppi *et al.*, 2003;Bledsoe *et al.*, 2004). Our initial findings identified that although highly potent, the NSG ligands surprisingly result in slowed kinetics of GR phosphorylation, nuclear import and delayed onset of GR-dependent gene transactivation. Our data suggests that the NSG ligands fundamentally alter the mechanism of GR activation.

A possible explanation for the delayed kinetics of cellular response to GSK47867A and GSK47869A is reduced efficiency of cellular uptake of ligand. Although the NSGs retain the highly lipophilic characteristics of steroidal ligands, they may interact differentially with membrane components. However our mass spec studies in fact showed an accelerated ligand accumulation with the NSGs compared to Dex. We also undertook a functional assay, washing off ligand after a short incubation, and tracking response of glucocorticoid target genes. Again, the NSGs produced enhanced target gene transactivation compared with Dex, indicating rapid ligand accumulation. Furthermore treatment of cells with ligand for 1 hour on ice allowed for saturation of the receptor without translocation. When the cells were returned to 37^{o}C the GR rapidly translocated with both Dex and FP but translocation was slower for both the NSG ligands, supporting defective interaction with the nuclear translocation machinery post ligand binding.

To explain these observations we interrogated the crystal structure of GR LBD bound to GSK47867A and GSK47869A. This revealed a very similar conformation to that seen with Dex, but there was a single difference, namely the addition of a patch of positive charge on the external surface of the LBD. Ricketson and co-workers were able to demonstrate, through amino acid substitution, that this surface is required for Hsp90 interaction (Ricketson *et al.*, 2007). Hsp90 recognises the GR LBD through two, defined hydrophobic sites and binds to a solvent accessible major groove maintaining GR stability and permitting high-affinity ligand binding (Fang *et al.*,

2006), as depicted in Figure 3.19 G. Following ligand binding Hsp90, undergoes a conformation change to bind to the same region of the GR LBD, but with a different motif. This is required to couple the GR to the dynein active transport mechanism through the bridging effect of immunophilins (Harrell *et al.*, 2004)(Figure 3.19 G). Hsp90 remains associated with the GR in the nucleus, where binding to the major groove of the GR LBD competes with recruitment of co-activators (Caamano *et al.*, 1998;Kang *et al.*, 1999;Fang *et al.*, 2006), and also promotes nuclear retention (Tago *et al.*, 2004;Kakar *et al.*, 2006). Binding of NSGs to the GR LBD forces the movement of Arg611, leading to the creation of a novel interaction surface which could be the mechanism by which interaction with Hsp90 is altered. Therefore, we measured the impact of Hsp90 manipulation on GR function with both the steroidal ligands, and NSGs.

GR is anchored to the microtubule network through interaction with Hsp90 to facilitate nuclear translocation. Antagonism of Hsp90 therefore reduces the rate of GR nuclear translocation and can be overcome by disrupting the microtubule network (Galigniana *et al.*, 1998;Nishi *et al.*, 1999). Here we show that the absence of an intact microtubule network significantly increases the rate of GR translocation in response to the NSGs but not Dex, which suggests an impaired interaction of GR-NSG with Hsp90. Evidence has emerged that persisting glucocorticoid action requires cycles of dissociation, and re-binding of ligand to the GR, which occurs in a Hsp90 dependent manner (Stavreva *et al.*, 2004;Conway-Campbell *et al.*, 2011)(Figure 3.19 G). To test the role of Hsp90 we used the inhibitor geldanamycin (Segnitz *et al.*, 1997). As predicted, geldanamycin curtailed the glucocorticoid transcriptional response rapidly, irrespective of ligand potency, for the two steroid agonists. However, in keeping with the hypothesis that Hsp90 binding was disrupted by the final conformation adopted by the NSG bound GR there was greatly prolonged transactivation observed, with a gradual decay likely due to degradation of GR protein. It was, however, striking that the pattern of response for both NSGs included an initial augmentation of response, which is compatible with displacement of Hsp90 from the major groove, and subsequent promotion of co-activator recruitment. It is also possible that disruption of the Hsp90 interaction surface also affects interaction between GR, and co-modulator protein partners (Caamano *et al.*, 1998;Kang *et al.*, 1999;Fang *et al.*, 2006).

Altered NSG-driven nuclear translocation, and interaction with Hsp90 may also affect GR nuclear export, and the duration of cellular response. Indeed, our washout studies showed a dramatic difference between the steroidal and NSG ligands, with marked reduction in GR export rate and prolongation of action seen with the NSGs, observed with both transfected reporter genes, and endogenous gene transcripts. A similar prolongation of action was seen in cells treated with geldanamycin which may result from stabilised GR-ligand interaction, due to altered engagement with Hsp90, and its associated protein complex, including enzymes such as protein phosphatase 5 (PP5). PP5 is responsible for removing phosphate modification from GR Ser211, and promoting GR nuclear export (DeFranco *et al.*, 1991;Silverstein *et al.*, 1997;Galigniana *et al.*, 2002;Hinds, Jr. *et al.*, 2008)(Figure 3.19G).

Geldanamycin treatment resulted in loss of the Dex ligand-dependent GR Ser211 phosphorylation. However NSG-liganded GR was not dephosphorylated under the same conditions, implying altered recruitment of PP5. PP5 also associates with Hsp90 as part of the chaperone complex (Silverstein *et al.*, 1997;Hinds, Jr. *et al.*, 2008) (Figure 3.19 G), and contains a peptidylprolyl isomerase domain that is capable of dynein interaction and therefore forming a bridge between the GR and the nuclear export machinery (DeFranco *et al.*, 1991;Galigniana *et al.*, 2002)(Figure 3.19 G). Therefore, as PP5 has been implicated in the nuclear export of the GR, the lack of dephosphorylation seen with NSG treatment is compatible with a broader change in protein recruitment with the NSG ligands. Interestingly, it was also observed that NSG treatment preserved GR protein expression compared with Dex treatment. This would further suggest that the conformation adopted by GR following NSG binding decouples protein recruitment required for terminating the GR transcriptional signal (Nawaz and O'Malley, 2004)(Figure 3.19 G).

In conclusion we have identified two NSGs that bind to GR with high specificity but paradoxically result in profoundly slowed kinetics of cellular response. Analysis of the structural effects of these NSGs bound to GR suggests a change to the GR surface, through the movement of Arg611 in the ligand binding pocket of the GR, resulting in an alteration in the GR surface charge. The change in electrostatic charge is close to the known binding site for Hsp90, and co-modulator proteins. This alteration carries with it the consequence of delayed GR phosphorylation and nuclear translocation,

which in turn results in delayed early glucocorticoid target gene regulation. The ability to manipulate the kinetics of GR activation by designing novel NSGs has implications for therapy, by targeting cellular pharmacodynamics rather than organismal pharmacokinetics.

3.6 Acknowledgements

We thank Bill Leavens and Midori Kayahara for technical assistance. Special thanks to Peter March and Roger Meadows for their help with the microscopy. Peter Trebble is supported by a BBSRC CASE studentship. Laura Matthews is supported by a FMHS Stepping Stones Fellowship. The Bioimaging Facility microscopes used in this study were purchased with grants from BBSRC, Wellcome and the University of Manchester Strategic Fund.

Chapter 4: General discussion

4.1 Overview

Glucocorticoids (Gc) are potent anti-inflammatory agents, offering significant therapeutic benefit in the treatment of a range of inflammatory diseases. Although Gc are effective medicines in the majority of patients, their clinical efficacy is limited by variation in response and the development of serious side effects over time. The structure of the glucocorticoid receptor (GR) is dynamic, where the final receptor conformation is directed by the ligand to which it is bound. Insight into how ligand structure influences GR conformation can be informed using chemical biology approaches, through experimental mutation of the GR and also through the characterisation of naturally occurring GR mutations. Much like the changes coordinated by ligand structure, genetic mutations alter the receptor conformation to develop unique activity profiles. Understanding how receptor mutations and receptor-ligand interactions direct specific cellular effects is an essential step towards improving current therapies.

4.2 GR mutations and PGGR

Primary generalised glucocorticoid resistance (PGGR) is assumed to be rare, because of the crucial role GR signalling plays in development, metabolism and resolution of inflammation. The spectrum of PGGR symptoms is surprisingly broad with wide variation in severity, ranging between patients that are relatively asymptomatic to those that present with serious biochemical and phenotypic abnormalities. Biochemically, patients with PGGR present with increased plasma ACTH, raised serum cortisol and elevated urinary free cortisol without the characteristic symptoms of hypercortisolism. Although a normal circadian rhythm of cortisol release is maintained (albeit at higher levels), the HPA axis does not respond normally to a Dex suppression test (Chrousos *et al.*, 1993). The classic clinical phenotype of PGGR manifests as apparent mineralocorticoid excess; hypertension and hypokalemia. Androgen excess is also often reported, causing female pseudohermaphroditism, premature onset of puberty in children, acne, hirsutism, hypofertility, male pattern balding, menstrual irregularities, oligo-anovulation in women and oligospermia in men (Charmandari *et al.*, 2008b). Inhibition of gonadotropin release through excess production of androgens most likely accounts for the impaired fertility seen in patients. In some cases severe anxiety is also reported which is linked to the elevated production of ACTH (Charmandari *et al.*, 2005). Treatment of PGGR constitutes high

doses (1-3mg once daily) of synthetic Gc such as Dex (which has low mineralocorticoid activity), to suppress excess production of ACTH (Chrousos *et al.*, 1982). The reduction in ACTH in turn decreases adrenal production of steroids alleviating symptoms (Charmandari and Kino, 2010).

PGGR is caused by a mutation within the GR gene that impairs receptor function. Many GR mutations have been tested experimentally *in vitro* to define the impact on Gc binding affinity, GR trafficking and transcriptional activity. They are summarised in table 4.1 below.

Table 4.1: GR mutations.

Mutation	Gc Binding	Translocation	Transactivation	Severity	Index Gender	Genotype	Dominant negative
R469STOP	None	None	None	Mild	Male	Heterozygous	N.D.
R477H	Normal	20 min	Minimal	Moderate	Female	Heterozygous	Yes
I559N	None	180 min	Minimal	Severe	Male	Heterozygous	Yes
V571A	6 x less	25 min	10-50 x less	Very Severe	Female	Homozygous	No
4-bp deletion at exon 6 splice site	N/A	N/A	N/A	Severe	Female	Heterozygous	N.D.
S612Y	N.D.	None	None	Mild	Female	Heterozygous	Yes
D641V	3 x less	22 min	Minimal	Moderate	Male	Homozygous	N.D.
G679S	2 x less	30 min	Minimal	Moderate	Female	Heterozygous	Yes
R714Q	2 x less	80 min	2 x less	Severe	Female	Heterozygous	Yes
V729I	2 x less	120 min	4 x less	Severe	Male	Homozygous	No
F737L	2 x less	180 min	2 x less	Severe	Male	Heterozygous	Yes (time dependent)
I747M	2 x less	25 min	4 x less	Severe	Female	Heterozygous	Yes
L773P	2.6 x less	30 min	2 x less	Severe	Female	Heterozygous	Yes
2-bp deletion at nt 2318-9 aa 773	None	N.D.	None	Very severe	Male	Homozygous	N.D.

N.D. not determined.

No single correlate can predict the severity of PGGR as the combination of several factors (including type and location of the mutation) determines the symptoms observed. To date, all documented GR mutations have been found within the LBD of the receptor, with the exception of R469STOP and R477H which are both located in the GR DBD (Ruiz *et al.*, 2001;Bouligand *et al.*, 2010;Ruiz *et al.*, 2013). No natural mutations within the first 400 amino acids of the GR have ever been documented. This suggests that mutations in this region are either incompatible with life or do not impact receptor function. Mice that lack the AF1 found in the first 400 amino acids of the GR are viable, although their response to Gc is blunted (Miesfeld *et al.*, 1987;Mittelstadt *et al.*, 2003). This suggests that mutations within the NTD are not detrimental to receptor function.

GR substitution mutations are the most common and typically reduce GR affinity for Gc, where a more pronounced reduction in Gc affinity correlates with a more severe phenotype as seen in carriers of I559N and V571A (Karl *et al.*, 1996;Kino *et al.*, 2001;Mendonca *et al.*, 2002). The only reported mutation that does not affect Gc affinity is the R477H which lies within the DBD (Ruiz *et al.*, 2001). Another common feature of GR mutations are varied rates of nuclear translocation, much slower than the wild type GR, where the slowest mutants I559N, V729I and F737L are all associated with severe phenotypes (Malchoff *et al.*, 1993;Karl *et al.*, 1996;Kino *et al.*, 2001;Charmandari *et al.*, 2007). Transiently transfected GRE reporter plasmids permit quantification of the transactivation ability of the receptor mutants. All reported mutant receptors display a reduced ability to activate a GRE reporter. Interestingly the D641V mutation results in a receptor with decreased transactivation activity but increased transrepression compared to wildtype GR (de Lange P. *et al.*, 1997). The GR DBD facilitates dimerisation which is required for effective DNA binding. As such the R477H mutation located in the DBD impairs DNA binding and is unable to activate a GRE reporter, yet is able to effectively recruit coactivator GRIP1 through both its AF1 and AF2 domains. Also R477H GR retains the ability to repress TNFα induced NFκB reporter genes, indicating that the monomeric form of the GR is sufficient for this task (Ruiz *et al.*, 2001;Ruiz *et al.*, 2013).

Although the majority of reported GR mutations are substitutions, three deletion mutations have now been described. I have identified a novel deletion in GR exon 6 (Δ612 – bp C1835 – S612Y) which produces minor biochemical alterations without significant phenotypic presentation (Trebble *et al.*, 2010). The C1835 deletion results in a frame shift that introduces 15 novel amino acids and an early stop codon at amino acid 627, resulting in a truncated GR protein incapable of ligand binding or regulating gene expression. The rate of translocation and the transactivation ability of full length GR are both reduced when co expressed *in vitro* with Δ612GR. This is most likely due to dimerisation of Δ612 with wildtype GR as the transrepression activity of the full length GR (which acts as a monomer) was not impacted by co expression with Δ612.

A 2 bp deletion at nt 2318 (amino acid 773) in exon 9 has also been reported (McMahon *et al.*, 2010). This deletion also results in a frame shift which removes the stop codon at amino acid 778 and adds an additional 19 amino acids to the C-terminal of the GR. The resultant receptor has a greatly decreased affinity for Gc, slowed rate of nuclear translocation and reduced transactivation activity. Like Δ612, heterozygous carriers for this mutation lack any phenotypic or biochemical alterations. A 4 bp deletion at the 3' boundary of GR exon 6 has also been documented. This deletion results in the loss of a donor splice site at the exon 6 boundary, preventing correct mRNA processing. This results in a 50% loss in GR rendering carriers haploinsufficient, however the remaining protein has the functionality of wild type GR. The loss of this splice site was identified because female patients presented with manifestations of hyperandrogenism (Karl *et al.*, 1993).

The presence of homozygous GR mutations is highly indicative of a severe phenotype, with deletion mutations conferring the most negative clinical outcome. Four cases of individuals with homozygous GR mutations have been documented, V571A, D641V, V729I and 2-bp deletion at nucleotide 2318. A female homozygous for the V571A mutation had a severe phenotype presenting with pseudohermaphroditism (Mendonca *et al.*, 2002). The V571A mutation decreases Gc binding by 6 fold and reduces GR transcriptional activity by up to 50 fold resulting in a severe phenotype. A male homozygous for the D641V mutation presented with a moderate phenotype, with 7 times the normal circulating level of cortisol and

hypertension and hypokalemia; both due to mineralocorticoid activation (Chrousos *et al.*, 1982;Hurley *et al.*, 1991). The D641V mutation decreases Gc binding 3 fold and reduces GR transcriptional activity. A male homozygous for the V729I mutation presented with precocious puberty as a result of hyperandrogenism. The V729I mutation also dramatically reduces the rate of nuclear translocation which may explain why this phenotype is more extreme than the male homozygous for D641V (Malchoff *et al.*, 1993). It is possible that a female homozygous for either D641V or V729I would have a more extreme phenotype as any hyperandrogenism would be more apparent. A male homozygous for a 2-bp deletion at nucleotide 2318 had the most severe PGGR phenotype documented to date, demonstrating complete generalised Gc resistance. The homozygous mutation rendered the individual completely resistant to Gc, suggesting that some GR activity was retained in the V571A, D641V and V729I homozygote mutations (McMahon *et al.*, 2010). Interestingly in all cases one or more family members were heterozygous for the same mutation but presented with very mild or no phenotypic alterations. This is presumably due to negligible dominant negative action on the wildtype receptor.

Bouligand and co-workers published the first report of a GR substitution mutation that results in the insertion of a stop codon (CGA to TGA) at amino acid residue 469 (Bouligand *et al.*, 2010). The truncated R469STOP GR lacks the ligand binding domain and is therefore unable to bind ligand. Unsurprisingly the R469STOP GR completely lacks any transactivation activity in a GRE2 luciferase reporter gene assay. However, the group did not investigate whether R469STOP GR displays any dominant negative action upon wildtype GR. Although the mutation was demonstrated to be present in skin fibroblast DNA, no mRNA transcript bearing the early stop codon was found. Both the GR transcript and protein levels were reduced by 50% and there was a 50% reduction in Dex binding capacity. In accordance with this there was also lower induction of the endogenous Gc target gene FKBP5, when compared to cells from healthy controls. This mutation was found to activate a quality control mechanism called nonsense mediated messenger RNA decay (NMD) in lymphocytes and fibroblasts, resulting in GR haploinsufficiency. Restoration of R469STOP GR transcript level was evident following treatment with NMD inhibitors cycloheximide and emetine. Much like the family from our own study (Δ612 mutation), the R469STOP mutation produces only a mild clinical phenotype. This

mutation was discovered following the incidental discovery of bilateral adrenal hyperplasia during a computerized tomography (CT) scan performed to identify the cause of lumbago. Further investigation of close family revealed eight carriers from three generations. Each individual presented with sub-clinical hypercortisolism, yet had normal fertility and no virilisation was seen in the affected females. There was a progression up the generations in bilateral adrenal hyperplasia, most likely due to long term chronic exposure to elevated ACTH. The hypercortisolism in each patient resulted in illicit activation of the MR causing hypertension and hypokalemia which increased in severity with age. Interestingly this group highlighted that similar observations have been made in GR haploinsufficient mice.

Michailidou *et al* reported the design of a GR with a β-galactosidase-neomycin reporter cassette (GR-βgeo) integrated between exons 3 and 4, which led to the generation of a truncated receptor lacking part of the DBD and the entire LBD (Michailidou *et al.*, 2008). The truncated GR-βgeo displayed no activity in an *in vitro* reporter gene assay and had no dominant negative action when co-expressed with the full length GR. Heterozygous GR-βgeo mice ($GR^{\beta geo/+}$) produced 50% less GR compared to wildtype litter mates, although they did produce the larger non functional GR-βgeo, shown by western blot assay. The $GR^{\beta geo/+}$ mice had elevated basal plasma corticosterone (the rodent equivalent of cortisol) and significantly increased adrenal weight, due to hyperplasia (increased cell number), when compared to wildtype litter mates. The $GR^{\beta geo/+}$ mice displayed no differences in body composition or glucose homeostasis but had significantly higher blood pressure. The elevated corticosterone levels likely contributed to hypertension by overriding the HSD2 protective barrier and in turn activating MR.

As mentioned earlier, in some cases a GR mutation results in receptor haploinsufficiency due to quality control mechanism called nonsense mediated messenger RNA decay (NMD). The mutations R469STOP, S612Y (Δ612) and the 2-bp deletion at nucleotide 2318 all introduce a premature translation-termination codon (PTC) which acts as a substrate for NMD. This prevents the production of C-terminal truncated aberrant proteins that would potentially be dominant negative in action. The inclusion of a PTC could arise from either a mutation in the DNA or by faulty splicing of mRNA transcript (Stalder and Muhlemann, 2008;Nicholson and Muhlemann,

2010). In addition to quality control, NMD is responsible for homeostasis of approximately 1-10% of mammalian genes that contain a stop codon within a context that causes the transcript to be recognised as a NMD substrate (Mendell *et al.*, 2004;Chan *et al.*, 2007;Stalder *et al.*, 2008). The up-frameshift (UPF) proteins 1, 2 and 3 form the basis of the NMD machinery. During normal splicing of the nascent mRNA, an exon junction complex containing UPF3 is deposited on the mRNA in close proximity to the 5' site of the exon-exon boundary (Le *et al.*, 2000). Upon entry to the cytoplasm the exon junction complex plays a role in localisation, initiation of translation and targeting of mRNA for NMD (Le *et al.*, 2001;Lykke-Andersen *et al.*, 2001). During the first round of translation the ribosome displaces exon junction complexes as it moves along the mRNA until it reaches a translation-termination codon (Figure 4.1). Any transcripts that retain exon junction complexes after the first round of translation are targeted for NMD. UPF1 and 2 associate with UPF3 as part of the exon junction complex and signal recruitment of protein machinery that facilitates decay of the transcript, enabling recycling of the translational machinery (Kervestin and Jacobson, 2012).

NMD tightly regulates expression of transcripts in a cell specific manner. For example T cell receptor β transcript containing a PTC is efficiently targeted for NMD in T cells but not in HeLa cells (Carter *et al.*, 1996). Likewise collagen X transcript containing a PTC is rapidly degraded in cartilage cells but the same efficiency is not seen when expressed in non-cartilage cells (Bateman *et al.*, 2003). The efficiency of NMD also varies between cell types and even between different sub-lines of HeLa cells (Linde *et al.*, 2007a). This variation in NMD efficiency has been observed at the tissue level in mice (Zetoune *et al.*, 2008). Furthermore patients with cystic fibrosis (CF) show variation in NMD of transcripts for CF transmembrane conductance regulator (CFTR) that contain the same PTC (Linde *et al.*, 2007b). The patients displayed different levels of NMD substrate, which could be the result of genetic or epigenetic differences, and this suggests that NMD efficiency varies in humans (Huang and Wilkinson, 2012). Therefore patients heterozygous for GR mutations that act as substrates NMD may express mutant GR in some cells or tissues where NMD is less efficient.

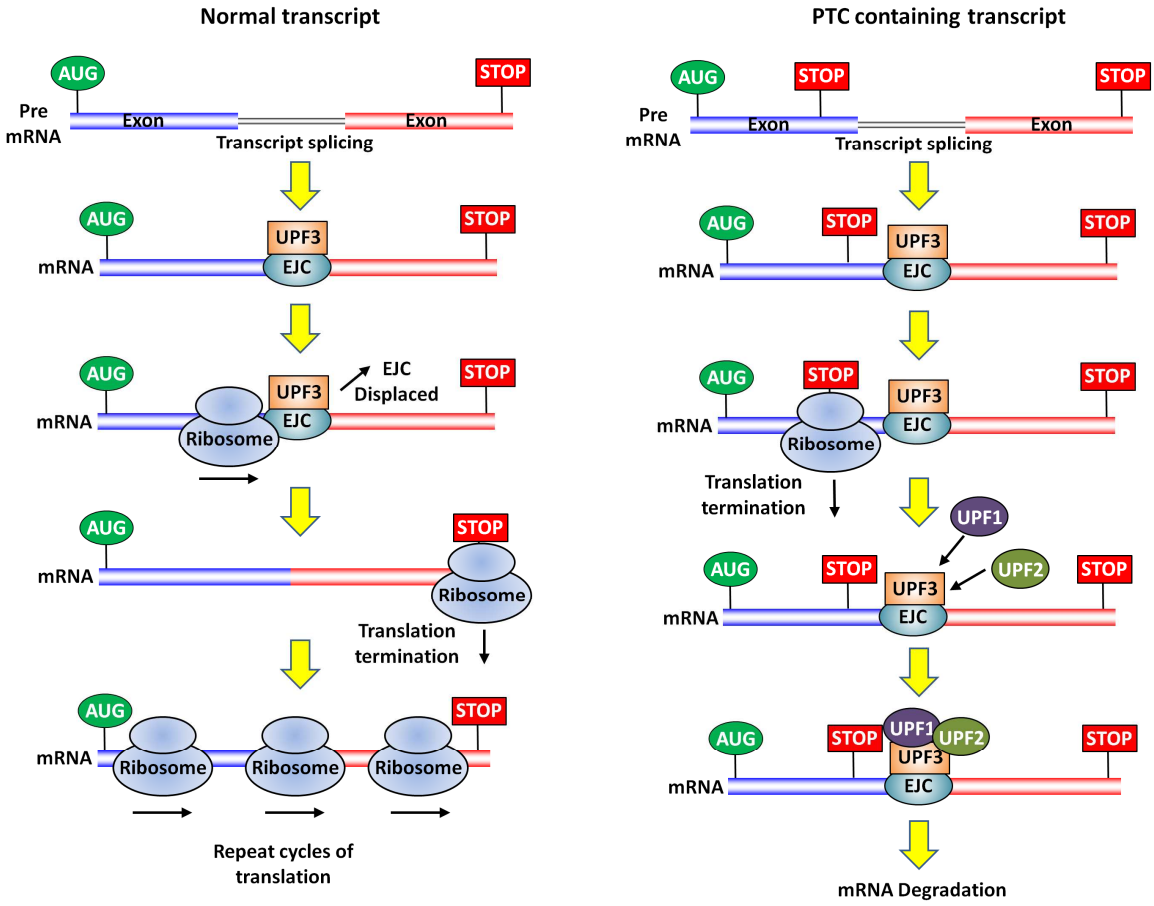

Figure 4.1: Nonsense mediate mRNA decay (NMD). Following splicing of pre mRNA the exon junction complex (EJC) is deposited at the exon exon boundary. During initial translation the ribosome displaces the EJC enabling repeated cycles of translation. Transcripts containing a premature translation-termination codon (PTC) retain EJC downstream of the PTC. This results in binding of UPF1 and UPF2 to the EJC targeting the transcript for degradation.

A 50% loss of GR protein results in mild phenotypes observed in patients with the Δ612 and R469X GR mutations and also from the asymptomatic heterozygous carriers of V571A, D641V and 2-bp deletion at nucleotide 2318 mutations. In these instances it is probable that activation of NMD is protective against any dominant negative activity observed experimentally. The 2-bp deletion mutation at nucleotide 2318 results in an additional 15 amino acids at the C terminal of GR followed by a stop codon, making this transcript a target for NMD. In this case, NMD is not protective as a homozygous carrier of this 2-bp deletion mutation would not express any GR leading to complete generalised Gc resistance. Some of the mild symptoms seen in the R469X and Δ612 GR heterozygous individuals could still be attributed to the dominant negative activity of receptor. As NMD efficiency varies between cell

types, cells and tissues that are targets of GR action may have high NMD efficiency for any aberrant GR transcript. This may explain why no R469STOP or Δ612 truncated GR was detected in lymphocytes, a major target cell for Gc actions. It is possible however that cells with lower GR expression could produce the aberrant GR with functional consequences.

Reviewing known GR mutations therefore provides insight into why PGGR symptoms are so varied. Individuals homozygous for GR mutations display more severe phenotypes, whereas heterozygous mutations give rise to varied PGGR severity dependent on whether the mutation activates NMD or exerts dominant negative activity. Typically, NMD acts as a protective mechanism resulting in very mild phenotype or asymptomatic presentation. Heterozygous mutations that are not targets of NMD produce moderate to severe phenotypes which can be explained by dominant negative activity of expressed mutant GR. There is one reported exception to these observations, the 4 bp deletion that leads to removal of a splice site. This mutation should effectively lead to GR haploinsufficiency which has a very mild clinical phenotype, yet the female proband in this study presented with hyperandrogenism. In this instance any potential dominant negative action of a receptor produced from the mutant allele was not determined. Given the severity of the symptoms this would suggest a dominant negative mechanism that prevents normal functioning of the wildtype GR. In addition to the functional consequences of a GR mutation some of the variation between PGGR patients could also be ascribed to differential expression levels and efficiency of enzymes that metabolise Gc such as 11β HSD. In this way an individual's genetic background would influence the disease severity through variation in tissue sensitivity to excess Gc, mineralocorticoids and androgens.

4.3 Using mutation analysis to direct targeted drug design

As well as the naturally occurring GR mutations, experimental mutations have been utilised to explore receptor function. This technique has been used to remove large sections of the GR but also to alter individual amino acids. These studies have revealed that clustering of hydrophobic amino acids in the NTD is important for GR transcriptional activity through recruitment of transcription factor II D (TFIID) and histone acetyl transferases (Ford *et al.*, 1997;Almlof *et al.*, 1997;Wallberg *et al.*,

1999). The essential role of monomeric GR in transrepression of NFκB and AP1 was also identified through mutation of specific residues in the GR DBD to block receptor dimerisation (Heck *et al.*, 1994;Liu *et al.*, 1995;Heck *et al.*, 1997;Wei *et al.*, 1998;Tao *et al.*, 2001). Studies in leukaemia cell lines identified two mutations within the GR LBD that reduce receptor activity (Hillmann *et al.*, 2000;Nagano *et al.*, 2002). Several targeted mutations of the GR LBD helped identify amino acids crucial for function. Using GR LBD fragments in a yeast phenotypic screening system C736, M560, M639, Q642, N564 and T739 were all identified as key amino acids for effective ligand binding (Lind *et al.*, 1996;Lind *et al.*, 2000). Further studies found that mutations G567A and Q642V decreased GR affinity for Gc whilst M565R, A573Q GR mutants show enhanced transcriptional activity (Warriar *et al.*, 1994;Schaaf and Cidlowski, 2003). Mutation of residue E755 markedly decreases GR transcriptional activity by abolishing recruitment of coactivators, due to its role with residue D590 in the formation of a primary charge clamp required for recruitment (Wu *et al.*, 2004). In addition residue Y735 in the ligand binding pocket has been identified as important for transactivation as it interacts with the D ring of steroid ligands. This interaction acts as a switch for the replacement of the corepressor NCoR with SRC1 upon binding of GR agonist (Ray *et al.*, 1999;Stevens *et al.*, 2003). The use of protein crystallisation techniques gives further insight into how mutations in the LBD directly alter binding to Gc.

Although the highly dynamic nature of the GR NTD has prevented crystallisation of the full length GR, the crystal structure of the GR LBD complexed with Dex and TIF2 fragment (GRIP1 homologue) was first described by Bledsoe and colleagues in 2002 (Bledsoe *et al.*, 2002). Crystallisation of the GR LBD required mutation of phenylalanine 602 to a serine residue in helix 5. This greatly improved the solubility of the resultant 521-777 GR fragment in the presence of 10μM Dex permitting formation of a crystal. The crystal was formed from packing together of symmetrical LBD dimers held together by hydrogen bonds formed between opposing beta sheets. This was confirmed through creation of an I628A GR mutant which much like the GR^{dim} mutant lacks transactivation activity but retains transrepression activity. The GR LBD consists of 12 α helices and 4 small β sheets that form a complex 3D structure that encapsulates a hydrophobic ligand binding pocket. Although similar to other steroid receptors the conformation of helices six and seven form a side pocket

that accommodates the C17α groups found on Gc. Formation of the ligand binding pocket and the AF2 interaction surface are reliant upon the positions of helices eleven (H11) and twelve (H12). Differential binding of GR agonists or antagonists determines the location of these helices in turn directing interaction with comodulatory proteins. Agonist binding induces a conformation whereby H12 closes the ligand binding pocket, creating a surface charge clamp that enables binding of coactivators to the surface hydrophobic cleft via their LXXLL motifs (Figure 4.2). In this way the TIF2 coactivator fragment containing a LXXLL motif binds the hydrophobic cleft on the GR LBD surface. The crystal structure of the GR LBD bound to the classic GR antagonist RU486 (mifepristone) revealed the mechanism for corepressor recruitment. In the opposite manner, antagonist binding leads to disruption of H11 and repositioning of H12 in the hydrophobic cleft blocking coactivator interaction and instead promoting recruitment of corepressors (Bledsoe *et al.*, 2002;Kauppi *et al.*, 2003).

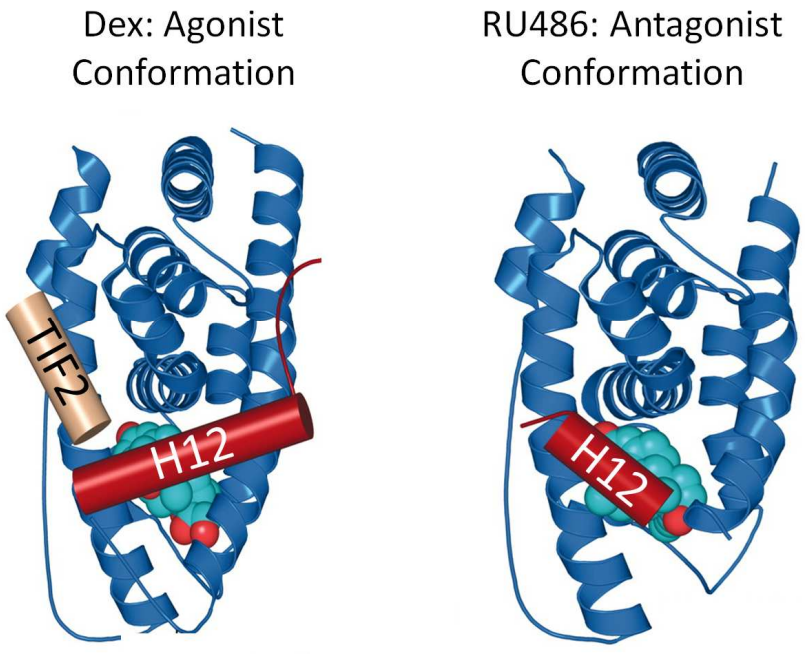

Figure 4.2: GR LBD crystal structure conformation. Comparison of GR LBD bound to Dex and RU486 reveals ligand directed movement of helix (H) 12. The position of H12 in Dex bound GR promotes recruitment of coactivator peptide (TIF2) a fragment from GRIP1. The position of H12 in RU486 prevents coactivator recruitment and promotes interaction with corepressors. Crystal structure images taken from (Kauppi *et al.*, 2003).

GR has a larger ligand binding pocket than AR, PR and ER with a volume of roughly 590Å^2 (Brzozowski *et al.*, 1997;Williams and Sigler, 1998;Sack *et al.*, 2001;Bledsoe *et al.*, 2004). The binding of the classic steroidal GR agonist Dex only fills two thirds of the available space, meaning that GR can bind a wide range of molecules (Bledsoe *et al.*, 2004). In order to accommodate hydrophobic ligands the binding pocket is predominantly lined with residues that have hydrophobic side chains, however there are specific polar interactions that direct ligand binding. Indeed all of the polar atoms in Dex are electrostatically bonded with the backbones and side chains of residues within the ligand binding pocket (Figure 4.3). The crystal structures of Dex and RU486 both demonstrated hydrogen bonding with the steroid A ring via residues M604, Q570 and R611. M604 in this region is able to adapt its conformation relative to the structure of the bound ligand demonstrating the flexibility of the GR LBD (Bledsoe *et al.*, 2004). The positioning of H12 is influenced by the conformation of N564 which forms a hydrogen bond with GR agonists such as cortisol and Dex but is displaced by the antagonist RU486 (Kauppi *et al.*, 2003). Dex has a large 17β substituent whereas RU486 has a much smaller group and as such lacks hydrogen bonding with T739, acting to further destabilise this region resulting in movement of the H12. The 17α hydroxyl group of Dex forms a hydrogen bond with Q642, but the flexible methionines M560, M639 and M646 in this region also allow binding of larger Gc such as FP (Bledsoe *et al.*, 2002;Biggadike *et al.*, 2008). By modelling Gc-GR LBD crystal structures, the functional consequences of natural (Table 4.2) and experimental mutations (Table 4.3) can be predicted.

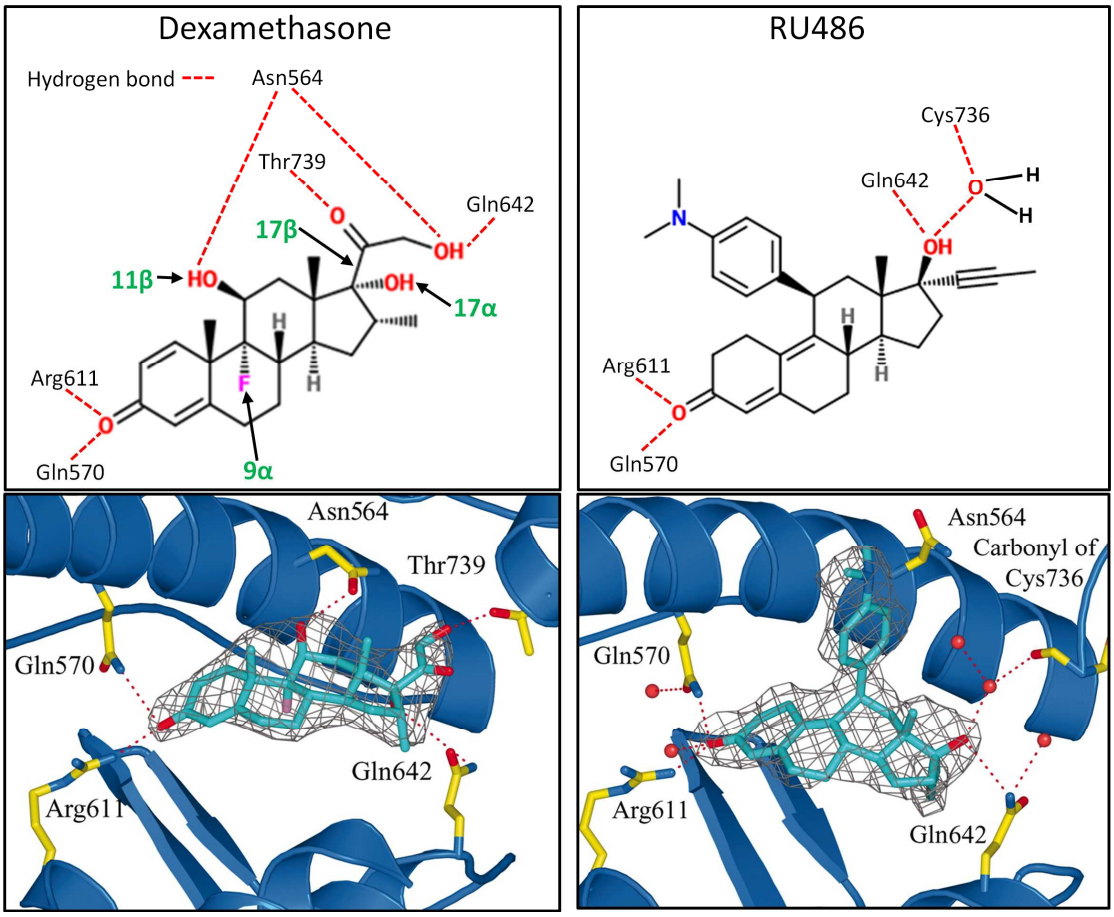

Figure 4.3: Comparison of Dex and RU486 in the GR ligand binding pocket. Hydrogen bonding to polar residues in both Dex and RU486 are shown by the red dashed lines. Crystal structure images taken from (Kauppi *et al.*, 2003).

Table 4.2: Natural GR mutations.

Mutation	Helix	Reason
I559N	H3	Influences the action of M560 in the 17alpha/beta binding region
V571A	H3	Alters binding of coactivators through alteration of LXXLL docking cleft
D641V	H7	Exposed surface residue close to 17alpha binding pocket
G679S	H8/9 Loop	Exposed surface residue interferes with ligand and coactivator binding
V729I	H10	Structural residue in the core of the LBD
I747M	H11/12 Loop	Could alter H12 position and interfere with 17beta interaction

Table 4.3: Experimental GR mutations.

Mutation	Helix	Effect	Cause
M560T	H3	Reduced affinity	Loss of Van der Waals
N564A	H3	Reduced affinity	Loss of H-bonding
M565	H3	Increased Dex affinity	Surface residue packing for loop before H12
G567A	H3	Reduced affinity	Van der Waals interference with A ring position
A573Q	H3	Increased Dex affinity	Partial surface residue
Q642A	H7	Reduced affinity	Loss of H-bonding
T739	H10	Reduced affinity	Loss of H-bonding and Van der Waals

The formation of electrostatic bonds between the ligand and the binding pocket of the steroid hormone receptor enables specific recognition. In this way the very closely related ER, PR, AR, MR and GR each selectively bind specific ligands (Figure 4.4). For example, GR does not effectively bind estradiol as it does not have the correct A ring orientation and lacks interaction with N564 and T739. AR ligands such as testosterone lack GR binding as they display steric hindrance through improper D ring orientation and have small 17α substituents. PR and GR are 61% identical and as such GR is able to weakly bind to progesterone. The affinity is lower due to lack of 17α hydrogen bond formation, decreased 17β interactions and loss of N564 binding (Bledsoe *et al.*, 2002;Bledsoe *et al.*, 2004). The greatest overlap in ligand recognition is seen between GR and MR which both bind to Gc and mineralocorticoids. MR ligands lack a 17α hydroxyl group generating some preference for binding to the MR, however as previously discussed tissue specific expression of conversion enzyme 11βHSD provides the primary mechanism for selectivity (Bledsoe *et al.*, 2004).

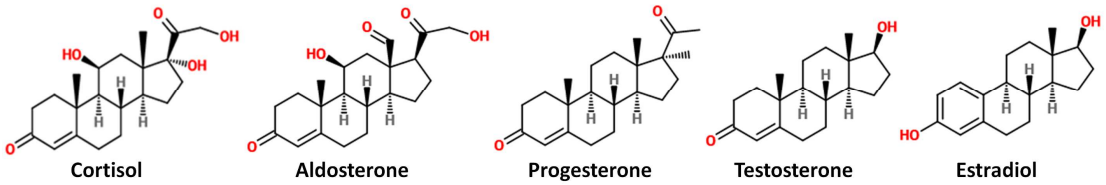

Figure 4.4: Comparison of steroid hormone ligands. Although highly similar the steroid hormone receptors are able to discriminate between ligands.

There are examples of synthetic Gc that lack any significant MR activity. The classic GR antagonist RU486 binds to GR with a Kd $\leq 10^{-9}$M but has no binding to MR (Cadepond *et al.*, 1997). Highly potent GR agonist cortivazol (CVZ) also binds to GR but lacks any affinity for the MR (Figure 4.5) (Yoshikawa *et al.*, 2002). Interestingly the L753F GR mutant can bind TIF2 when bound to CVZ but not when bound to Dex, confirming differential conformation of the ligand binding pocket directs comodulator recruitment. CVZ is a much larger steroid with a volume of 514Å compared to Dex 386Å (Yoshikawa *et al.*, 2005). Initially computer modelling was used to fit CVZ into the GR ligand binding pocket which revealed the requirement for displacement of R611 and Q570 side chains and an alteration in the conformation of residues N564, M604, L608, M646, and F749. A crystal structure of the GR LBD bound to the related steroid deacetylcortivazol demonstrated that the combined movement of these side chains creates a 'meta channel'(Suino-Powell *et al.*, 2008). The creation of the 'meta channel' helped to explain the high potency and selectivity of CVZ and deacetylcortivazol. These steroidal compounds only occupy 50% of the GR 'meta channel' and as such this sparked the drive for the creation of non-steroidal ligands that could exploit this region of the GR.

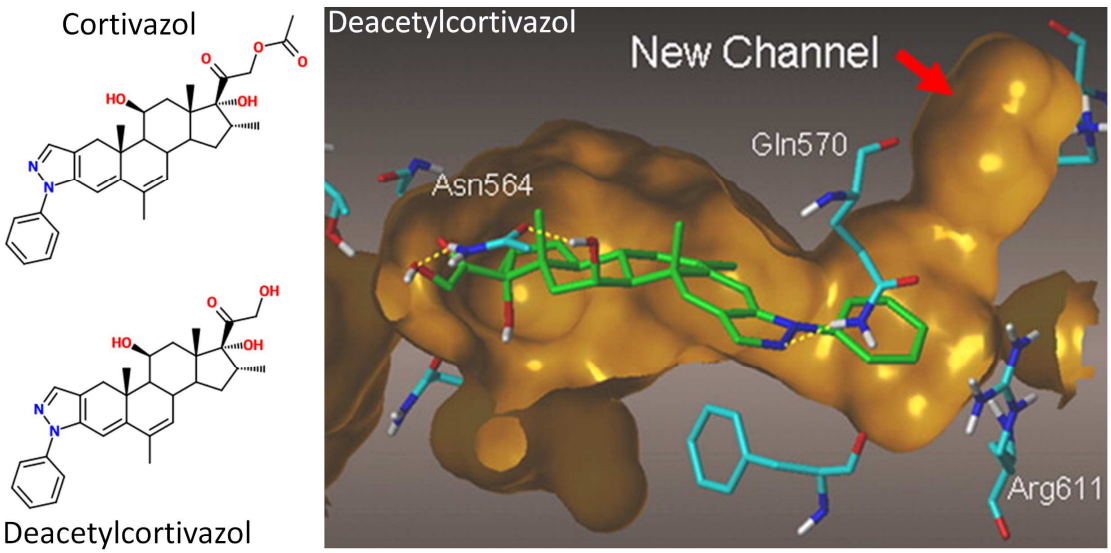

Figure 4.5: Crystal structure of Deacetylcortivazol bound to GR LBD. Binding of Deacetylcortivazol to the GR LBD allows access to the GR ligand pocket 'meta channel'. Crystal structure taken from (Biggadike *et al.*, 2009).

The first crystal structure of the GR bound to a non-steroidal Gc was demonstrated by Madauss *et al* in 2008 (Madauss *et al.*, 2008). Studies with non steroidal aminopyrazole compounds led to the discovery of several potent GR agonists (Clackers *et al.*, 2007;Barnett *et al.*, 2009). Further refinement of these compounds led to the identification of aminoindazole derivatives which are highly potent GR agonists. Computational modelling has directed the design of highly potent and selective GR ligands on the indazole and pyrazole templates that can explore the properties this meta channel (Biggadike *et al.*, 2009). Following computational modelling, a small series of compounds were initially tested for GR selectivity and potency. GR binding was measured in a competition assay against fluorescently labelled Dex with purified full length GR. The transactivation and transrepression activity were measured in a reporter gene assay using MMTV-Luc and NFkB reporter plasmids respectively. Selectivity was also demonstrated in reporter gene assays and fluorescent competition assays (Biggadike *et al.*, 2009). From this initial screening process GSK47867A and GSK47869A were identified as highly potent and specific GR agonists. Further study with these compounds revealed a greater degree of complexity in action than previously thought.

With the information gained from crystal structures of the GR LBD complexed with various steroidal and non steroidal Gc ligands, the goal of designing ligands that target specific aspects of GR function seems more attainable than ever. To this end synthetic Gc with attributes that enable topical application have been sought to circumvent the side effects seen with prolonged systemic Gc treatment. This approach yielded the highly potent GR agonists Budesonide, FP and Mometasone furoate that have been successfully used in the treatment of asthma, COPD, rhinitis and dermatitis (Uings *et al.*, 2013). Although these compounds provided some advancement in Gc therapy, further improvement was found through investigation of the 17α binding site in the crystal structure of GR bound to Dex (Figure 4.6). Enhanced Gc potency is found through alteration in the 17α substituent, seen with FP which carries a ethyl substituent at the 17α position and the highly potent fluticasone furoate which carries a 2-furoate in this position (Biggadike *et al.*, 2008). Further exploration of the 17α site led to the identification of the recently described steroidal agonist GW870086. This compound has potent anti-inflammatory action but only activates a subset of genes that are associated with the deleterious actions of the GR. GW870086 carries a tetramethyl cyclopropyl ester at the 17α position as well as cyanomethyl carboxylate derivative in the 17β position (Uings *et al.*, 2013). This compound has high affinity for the GR yet displays weak partial agonist activity on an MMTV reporter. This work further demonstrates that GR interactions can be manipulated by the structure of the bound ligand. As our understanding of GR biology has grown, a greater complexity in its action has been revealed. It is now known that for effective resolution of inflammation both the transrespressive and transactivation activities of GR are required. Therefore screening compounds based on simple GR reporters such as TAT3-Luc or inhibition of NFκB driven reporters are no longer sufficient to predict ligand-GR interactions. In the case of GW870086, multiple endogenous targets of Gc action were measured to confirm dissociative effects.

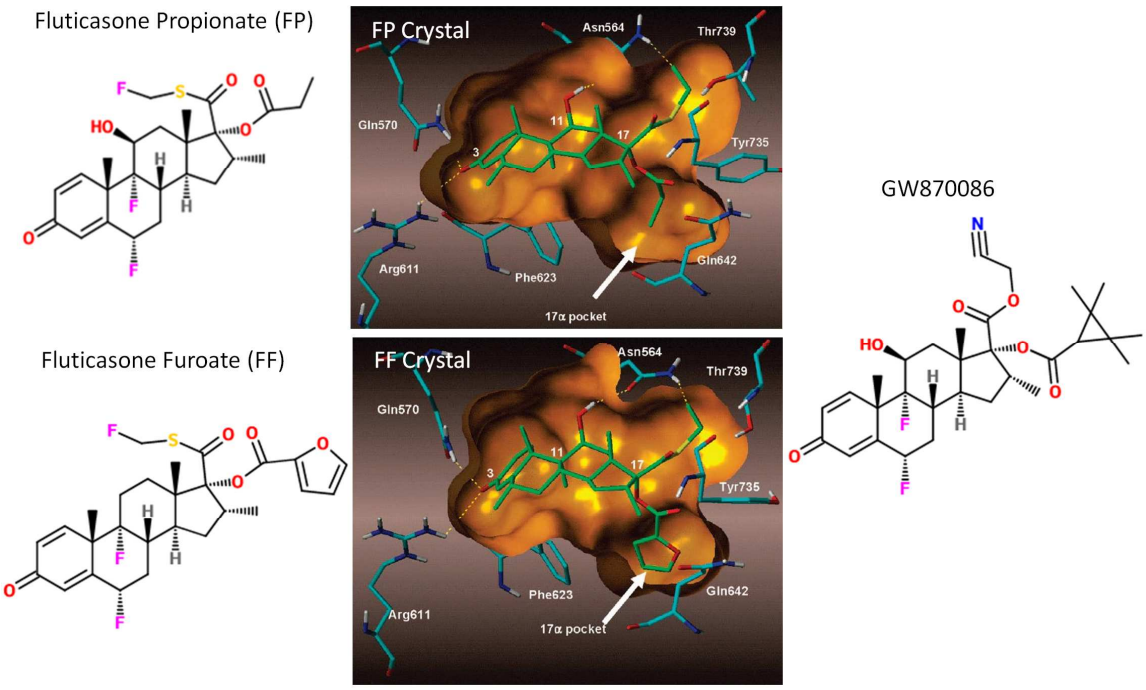

Figure 4.6: Investigation of GR 17α binding site: Crystal structure images taken from (Biggadike *et al.*, 2008). Structure of GW870086 shows large 17α substituent.

It is well established that association with Hsp90 is required for GR to bind to Gc. Mutation studies have demonstrated that there are large areas of interaction between Hsp90 and the GR LBD and DBD. Work with Hsp90 identified three regions located in the middle and C-terminal domains that interact with the GR (Bohen and Yamamoto, 1993;Nathan and Lindquist, 1995;Jibard *et al.*, 1999). Early studies demonstrated that amino acids 574 to 659 within GR contain two sites that are essential for Hsp90 binding. This data suggested that the primary site for Hsp90 association spans residues 632 to 659 whilst a secondary site lies between amino acids 574 to 632, both regions being highly conserved between nuclear hormone receptors (Dalman *et al.*, 1991). Further mutation studies have identified a region of seven GR amino acids (547-553) also vital for Hsp90 association. A single point mutation in this region was not sufficient to alter interaction but a triple mutation of P548A, T549A and V551A resulted in a 100-fold decrease in Gc binding (Kaul *et al.*, 2002b). Fang *et al* used a yeast functional screening assay to identify four mutations in rat GR (Y616N, F620S, M622T, and M770I) that reduce dependency upon Hsp90 interaction for Gc binding. Combining this mutation data with the crystal structures of the GR and Hsp90 led to the identification of an allosteric network within the GR LBD (Fang

et al., 2006). This network enables transmission of structural changes within the ligand binding pocket to the hydrophobic cleft that binds LXXLL motifs in comodulator proteins. The helix 2 in the Hsp90β CTD binds to the GR LBD forcing H12 of GR to dock with the GR surface hydrophobic cleft that binds to LXXLL motifs (Figure 4.7) (Fang *et al.*, 2006;Ali *et al.*, 2006). This conformation is very similar to that seen from the GR LBD complexed with RU486 and explains why the GR heterocomplex is stabilised by RU486 treatment (Distelhorst and Howard, 1990;Kauppi *et al.*, 2003). Furthermore Stevens *et al* found in GST pull down assays unliganded GR readily associates with the fragments of the corepressor NCoR, supporting a Hsp90 directed GR antagonist conformation (Stevens *et al.*, 2003). When GR binds agonist ligand H12 moves from the hydrophobic LXXLL binding cleft and closes the ligand binding pocket, facilitating recruitment of coactivators. This movement of GR H12 displaces interaction with Hsp90 helix 2 but allows association of Hsp90 helix 1 with the GR surface hydrophobic cleft. In this manner the Hsp90 is able to compete with the coactivators for binding of the GR LXXLL site, thereby fine tuning GR action (Kang *et al.*, 1999). Helices 1 and 2 of Hsp90 are in close proximity suggesting that the GR heterocomplex could remain in complex during this switch. GR ligand binding promotes a change in heterocomplex bound immunophilin, exchanging FKBP51 for FKBP52. The helix 1 of Hsp90 is very close to the immunophilin binding site and therefore a conformational change in this region could trigger FKBP5 switching (Fang *et al.*, 2006;Ali *et al.*, 2006). This alteration in the components of the GR heterocomplex facilitates retrograde transport to the nucleus where GR can modulate target gene activity.

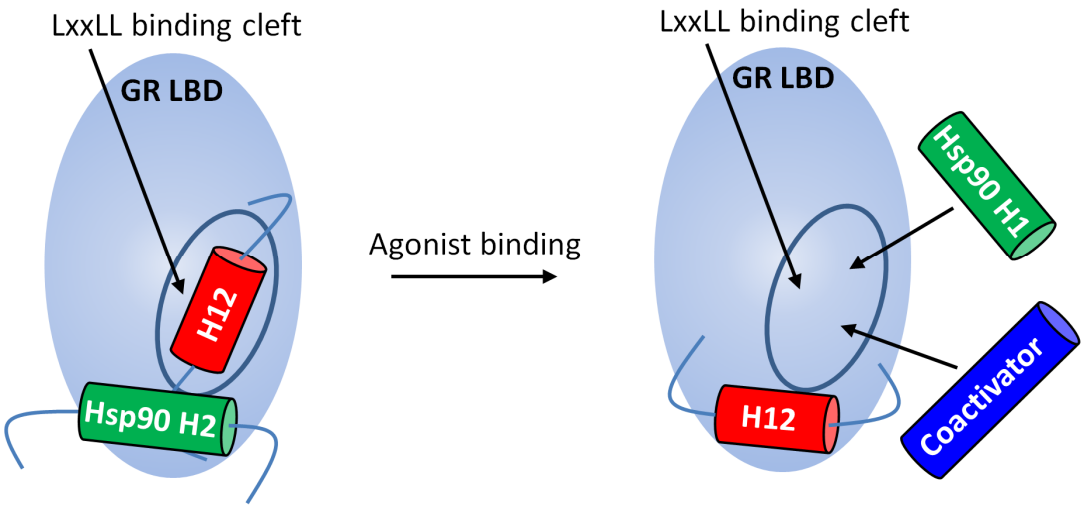

Figure 4.7: GR and Hsp90 interaction: In the absence of ligand Hsp90 helix (H) 2 forces H12 of GR to bind with the LxxLL binding cleft at the surface of the GR LBD. Following ligand binding the allosteric network within GR transmits structural changes in the ligand binding pocket that result in displacement of Hsp90 H2. The resultant movement of GR H12 allows for binding of either coactivators or Hsp90 H1 acting to fine tune GR function.

Ricketson *et al* identified mutations to rat GR that reduce the dependency on Hsp90 for receptor function. Of particular interest, the M622T mutation stabilises the GR agonist conformation and significantly increases responsiveness to Dex (Ricketson *et al.*, 2007). The M622T mutation required 10 times the concentration of the GR antagonist RU486 to competitively inhibit the response to Dex. Comparisons between Dex-GR and RU486-GR crystal structures revealed that the equivalent residue in human GR (M604) is directly involved in ligand binding. The crystal structure of deacetylcortivazol-GR also showed that M604 alters its conformation and as such it acts as a ligand dependent structural switch (Suino-Powell *et al.*, 2008). Superimposing the M604T mutation onto the crystal structure of the GR bound to GSK47866A revealed that this residue is in close proximity to the Arg611. The head region of the NSGs interacts with the GR meta channel resulting in significant movement of M604, R611 and Q570. Therefore the combination of the GR agonist conformation that results from movement of M604 and the positive charge patch created through R611 movement could provide the mechanism for dissociation of the Hsp90.

Early understanding of ligand-receptor interaction was based upon a lock and key mechanism, where ligand interacts with a rigid receptor binding site. More recently this mechanism has been superseded by an induced fit model which proposes that conformational changes in the receptor LBD stabilise interaction with ligand. The resultant conformation generates novel surfaces for receptor-protein interaction thereby dictating receptor function. Combined mutational analysis and chemical biology approaches have significantly advanced our understanding of how ligand-receptor interactions drive receptor-protein interactions and the consequences for the overall Gc response. This work has led to the identification of five distinct regions of the GR ligand binding pocket that can be modulated by ligand to direct GR function (Figure 4.8).

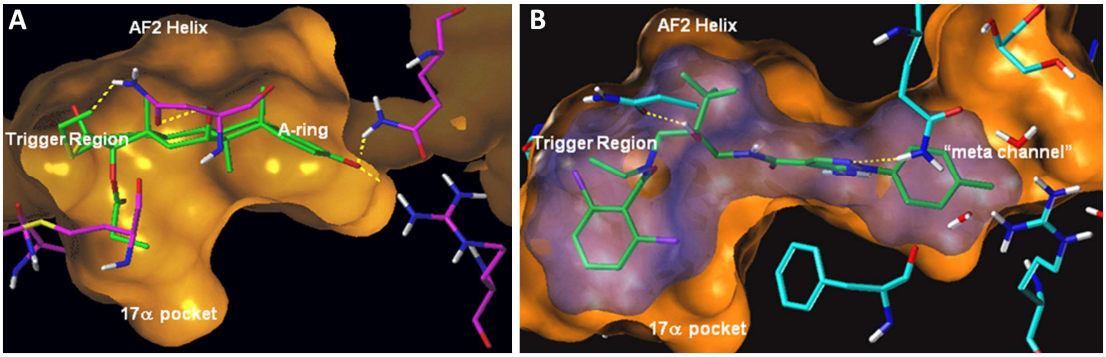

Figure 4.8: The five regions of the GR ligand binding pocket: Five distinct regions of the GR ligand binding pocket can be modulated by ligand structure to direct receptor function. (A) The GR LBD bound to a steroidal ligand indicates the location of the AF2 helix region, the trigger region, the A-ring region and the 17α pocket. (B) The GR LBD bound to a non steroidal ligand with a meta channel extension demonstrates the opening of this region of the GR ligand binding pocket.

Modulation of the AF-2 helix region influences cofactor recruitment by directing the position of H12, determining access to the hydrophobic groove at the GR surface responsible for binding LXXLL motifs (Figures 4.3 and 4.8). Using crystal structure guided design a non steroidal indazole that exploits the AF2 helix region demonstrated excellent antagonist properties similar to RU486 (Yates *et al.*, 2010). Occupation of the 17α pocket of the GR yields very potent Gc such as FP and FF and the dissociative Gc GW870086 (Biggadike *et al.*, 2008;Uings *et al.*, 2013). The trigger region of the GR ligand binding pocket is activated by bulky groups facilitating effective agonist activity, however full utilisation of this region is not vital for receptor activity (Barker *et al.*, 2006). Design of ligands with physiochemical

properties suitable for oral application led to the identification of potent non steroidal compounds with a smaller ethyl group occupying the trigger region (Barnett *et al.*, 2009). These compounds display some favourable characteristics but also contain a benzoxazinone which is metabolised by the liver therefore limiting therapeutic use. The benzoxazinone acts as a steroid A ring mimetic and replacement with an aryl pyrazole provides a much fuller agonist, highlighting the importance of the A ring region of the GR ligand binding pocket (Clackers *et al.*, 2007). Development of A ring mimetics provided a platform for the interrogation of the GR meta channel. Through my work with GSK47867A and GSK47869A I have demonstrated that manipulation of meta channel results in a prolongation of receptor activity likely due to impaired interaction with Hsp90 (Trebble *et al.*, 2013). In addition my study has highlighted the importance of using a variety of approaches to interrogate receptor activity when bound to different ligands. With the insight gained from mutational and crystal structure analysis the challenge now is to understand how the five regions of the GR ligand binding pocket work together to orchestrate receptor function.

Chapter 5: Future work

5.1 Introduction

The crystal structure of the GR LBD complexed with deacetylcortivazol revealed a new region of the ligand binding pocket termed the meta channel (Suino-Powell *et al.*, 2008). I have shown that GSK47867A and GSK47869A exploit this new region resulting in a prolongation of GR activation when compared to steroidal compounds Dex and FP. This is due to a reduced reliance on chaperone interaction for GR activity (Trebble *et al.*, 2013). This data suggests that GR ligands with meta channel extension have longer activity profiles. Biggadike *et al* described the design of an additional non steroidal compound that will allow further study of the GR meta channel (Biggadike *et al.*, 2009).

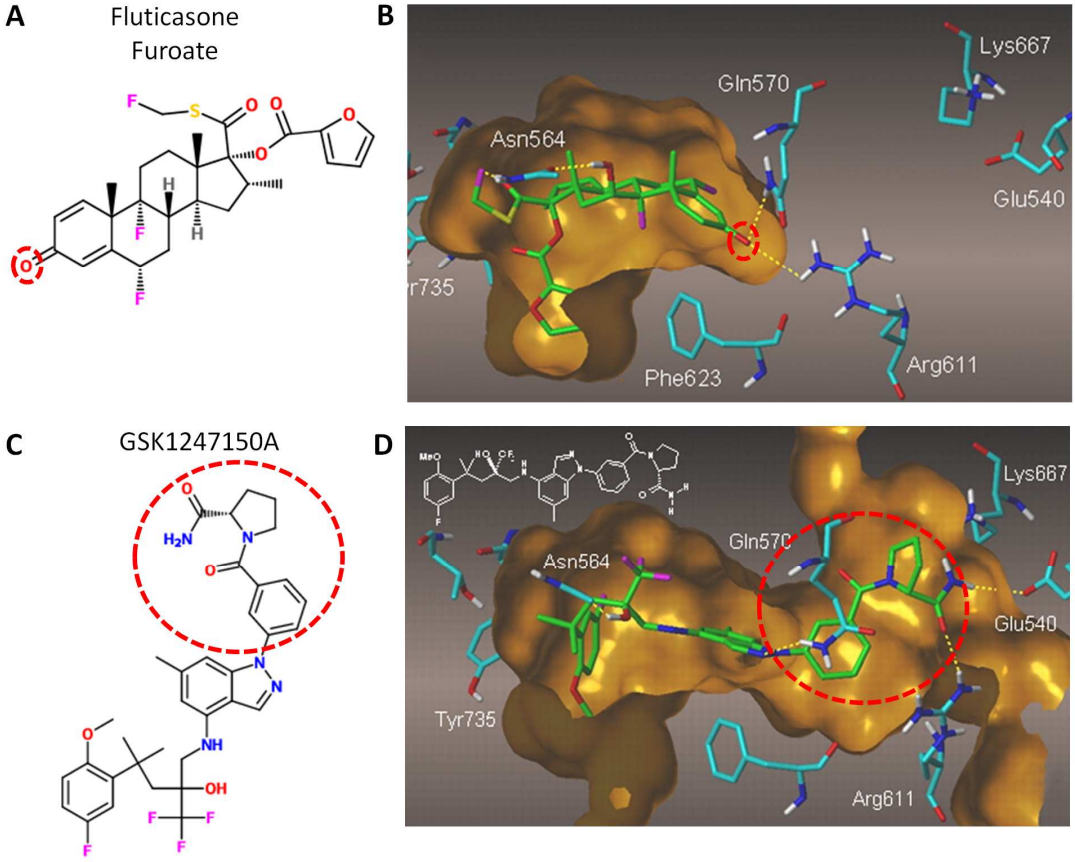

Figure 5.1: GR meta channel ligands. (A, C) Comparison between steroidal ligand Fluticasone Furoate and GR meta channel ligand GSK1247150A, meta channel region highlighted by dashed red circle. (B, D). Crystal structure of the GR LBD bound to Fluticasone Furoate and GSK1247150A demonstrates full occupation of the GR meta channel (D). Crystal structure image taken from (Biggadike *et al.*, 2009).

GSK1247150A is similar in structure to GSK47867A but has a much larger meta channel extension in order to fully occupy this region (Figure 5.1 D). The greater occupation of the meta channel is predicted to show greater functional effects than GSK47867A. Therefore this compound will be characterised in a cell line model in order to further understand the role of the GR meta channel.

5.2 Preliminary results

5.2.1 Potency of GSK1247150A as a GR agonist.

Transient GR transactivation models in HeLa cells were utilised in order to determine the potency of GSK1247150A compared to the steroidal Gc FP. In this assay GSK1247150A shows equivalent activity when compared to the highly potent Gc FP (Figure 5.2 A). A transient GR transrepression assay also demonstrated equal potency (Figure 5.2 B). For subsequent experiments an equal saturating concentration of 3 nM was used for comparison of the ligands.

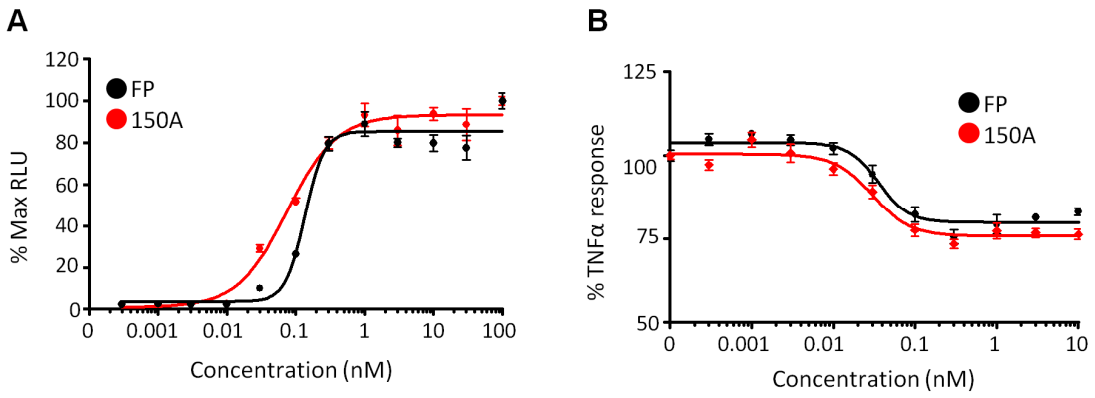

Figure 5.2: Potency of GSK1247150A as a GR agonist. Hela cells were transfected with a positive GR reporter gene (TAT3-luc) (A) or with a glucocorticoid repressed NFκB reporter gene (NRE-luc) (B). 24 hours post-transfection, NRE-Luc transfected cells were pre-treated with TNF α (0.5 ng/ml) for 30 minutes. Subsequently all transfected cells were treated with 0.001-100 nM FP or GSK1247150A (150A) for 18 hours then lysed and subjected to analysis by luciferase assay. Graph (mean ± SD) show the percentage of the maximum relative light units (RLU) (A) or percentage inhibition (B) from one of three representative experiments performed in triplicate.

5.2.2 Treatment with GSK1247150A delays GR nuclear translocation.

From the previous study with GSK47867A and GSK47869A binding of GR to ligands that exploit the meta channel results in slower nuclear translocation. Hela cells transfected with Halo tagged GR clearly demonstrate a delay in the rate of nuclear translocation following treatment with GSK1247150A, compared with FP (Figure 5.3).

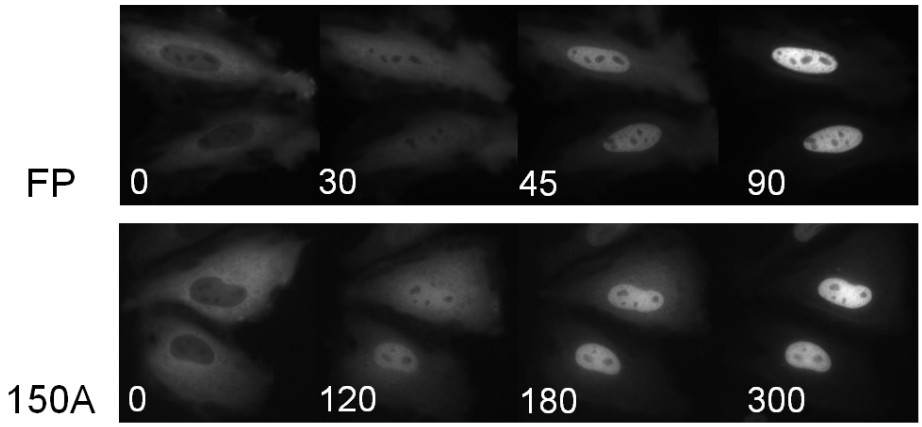

Figure 5.3: GSK1247150A delays GR translocation. Following transfection with HaloTag-GR HeLa cells were incubated with 3 nM FP or 3 nM GSK1247150A (150A). Cells were imaged in real time at 37 ^{o}C to determine the subcellular localisation of the GR (white) at the times (minutes) indicated. Images are representative of three independent experiments.

5.2.3 Treatment with GSK1247150A prolongs GR activity.

Previous work with GR meta channel ligands also demonstrated prolonged receptor activity following washout of ligand. To determine whether GSK1247150A displayed similar properties washout studies were carried out in HeLa cells. Cells transiently expressing a positive GR luciferase reporter (TAT3-Luc) revealed that GSK1247150A also prolongs GR activity when compared to the equally potent steroid ligand FP (Figure 5.4 A). To confirm these observations with endogenous genes a two hour ligand exposure was chased with a 24 hour washout before measurement of GILZ and FKBP5 transcripts (Figure 5.4 B-C). There was significantly enhanced preservation of transactivation seen with GSK1247150A compared to the potency matched control steroid FP.

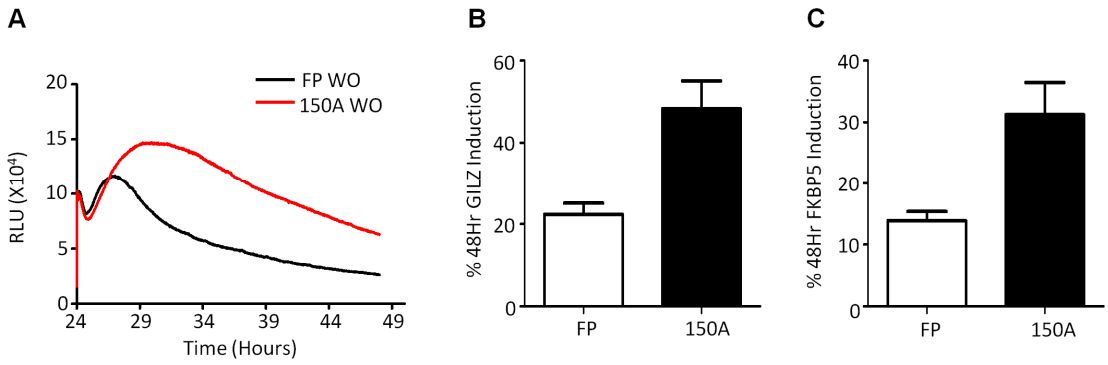

Figure 5.4: GSK1247150A prolongs GR activity. (A) HeLa cells transfected with a TAT3-Luc reporter plasmid were treated with 3 nM FP or 3 nM GSK1247150A (150A) for 24 hours. Subsequently cells were washed and placed in serum free recording media for a further 24 hours. The production of luciferase was tracked by measuring the relative light units (RLU) emitted from each sample. Graphs tracks RLU production for 24 hours following ligand removal. Graph is representative of three separate experiments. HeLa cells were treated with 3 nM FP or 3 nM 150A for 48 hours or 24 hours followed by washes and then cultured in ligand free media for a further 24 hours. Subsequently cells were lysed and RNA extracted using an RNeasy kit. RNA was reverse transcribed and subjected to qPCR of GILZ (B) and FKBP5 (C) using Sybr Green detection in an ABI q-PCR machine and data analysed by δδ CT method. Graphs (mean ± SEM) combine data from three separate experiments and display percentage induction compared to equivalent 48 hour constant treatment.

5.2.4 Potency of truncated GSK1740136A as a GR agonist.

In addition to GSK1247150A Biggadike *et* al also described a truncated non steroidal called GSK1740136A. This ligand resides fully in the GR meta channel but does not occupy a significant portion of the traditional steroid pocket due to its truncation (Figure 5.5 A-B). This compound will act as a useful tool in the study of the GR meta channel. As a preliminary step the potency of GSK1740136A was determined in a cell line model. The truncated GSK1740136A demonstrated a much lower potency than GSK1247150A, which is comparable to Pred (Figure 5.5 D). The ability of GSK1740136A to prolong GR activation through impaired Hsp90 interaction is yet to be determined.

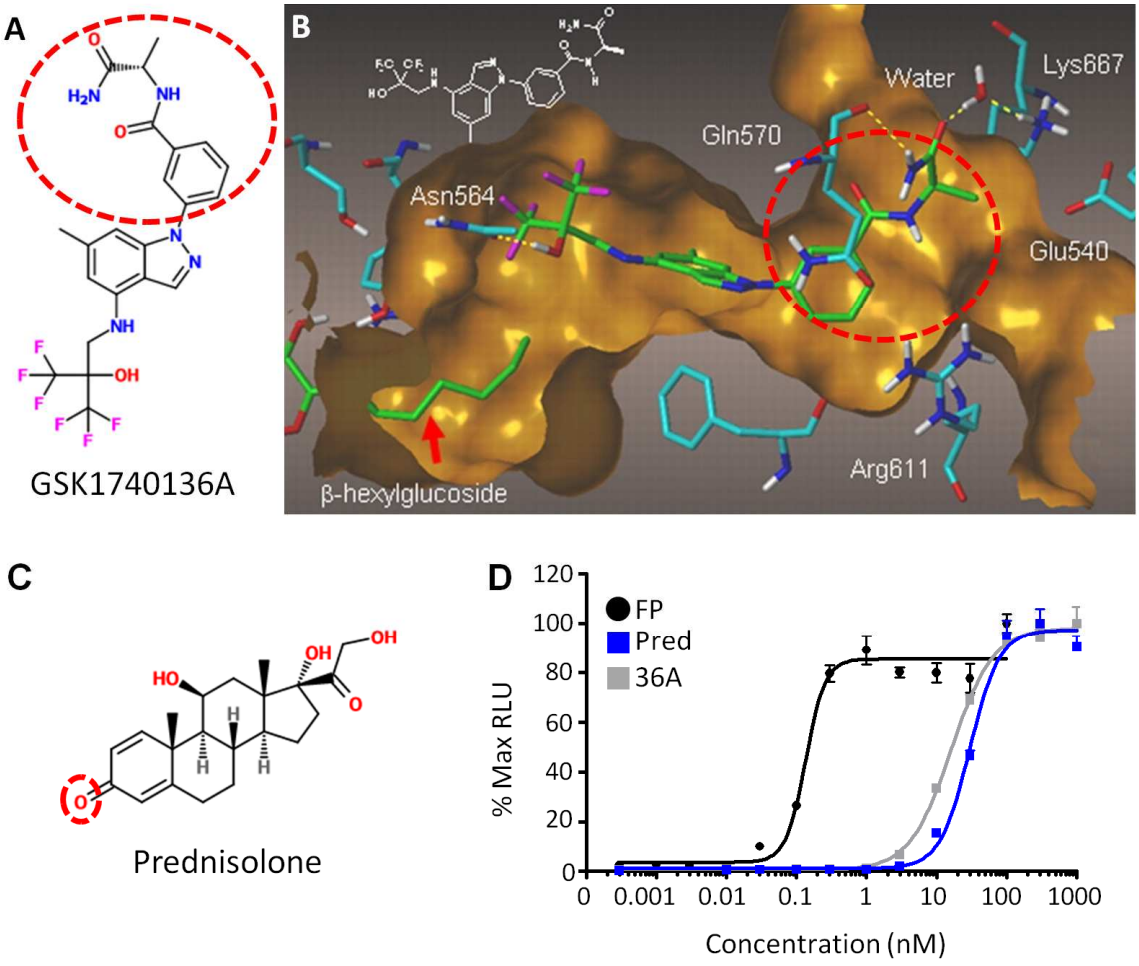

Figure 5.5: Potency of GSK1740136A as a GR agonist. (A, C) Structure of GSK1740136A and equipotent steroidal glucocorticoid Prednisolone, meta channel region highlighted by dashed red circle. (B) Crystal structure of the GR LBD bound to GSK1740136A demonstrates full occupation of the GR meta channel without filling the traditional steroid binding pocket. (D) Hela cells were transfected with a positive GR reporter gene (TAT3-luc). 24 hours post-transfection cells were treated with 0.01-1000 nM Prednisolone (Pred) or GSK1740136A (36A) for 18 hours then lysed and subjected to analysis by luciferase assay. Graph (mean ± SD) show the percentage of the maximum relative light units (RLU) (D) from one of three representative experiments performed in triplicate.

5.3 Discussion and future work.

Meta channel compounds prolong GR activity *in vitro*. Determining whether these compounds are able to promote longer GR activity *in vivo* would form an excellent next step. A mouse model of delayed type hypersensitivity (DTH) would act as an ideal system to investigate whether GR meta channel ligands prolong receptor activity. In this model ear thickness is used to measure the response to topical application of an inflammatory agent. This model is very responsive to systemic steroid treatment. GSK1247150A already been through the early phases of a clinical trial and therefore has a full pharmacokinetic profile. Comparison of GSK1247150A with a potency and pharmacokinetic matched steroid in the mouse model of DTH would reveal any prolonged activity.

The alteration in the temporal profile of GR activation with meta channel ligands could influence target gene induction. To investigate this genome wide sequencing of RNA transcript (RNA-SEQ) would be used to compare the action of GR meta channel ligands with classic steroidal compounds such as FP. Identification of any transcripts that differ would be useful for screening meta channel compounds. In addition GR ChIP SEQ could also be used to determine whether recruitment of transcription factors differs with the GR meta channel ligands. Finally quantitative proteomic analysis such as stable isotope labelling by amino acid in cell culture (SILAC), would enable determination of any differences in protein interaction upon GR binding meta channel ligands. The impaired Hsp90 interaction could also result in altered recruitment of comodulatory proteins which would alter GR function.

5.4 Concluding remarks

Glucocorticoid (Gc) action is complex and subject to regulation on many levels. Gc play critical roles in development, metabolic control and the stress response, and as such they modulate gene targets through a variety of mechanisms. Although potent anti-inflammatory agents in the majority of patients, clinical use of Gc is hindered by development of diverse side effect profiles. Additionally, administration of Gc is not always effective and the response can vary widely between individuals and also over time. In the preceding chapters I have presented work that defines a mutation causing generalised Gc resistance, and characterised novel therapeutics with the potential to open new avenues for more targeted Gc treatment.

I identified a novel GR mutation (Δ612) that generates a truncated GR protein, unable to bind Gc. This truncated protein is non-functional, and remains cytoplasmic. Importantly, it can dimerise with wildtype GR, acting as a dominant negative. Despite this effect, the three patients present with a surprisingly mild phenotype. It is likely therefore that similar mutations may be more prevalent than previously thought and may, in part explain the wide variation in Gc sensitivity commonly observed.

I also characterised two highly potent novel non-steroidal GR ligands, which have unique pharmacodynamic properties. This is proof of principal that compounds with different core structures can modify GR surface conformation to direct specific aspects of Gc biology. Importantly, this suggests that it is possible to design synthetic ligands that effectively favour the anti-inflammatory action of GR over the undesired metabolic actions. This is likely to be one of the major areas for potential advancement of Gc therapies.

Further study in these two important areas will no doubt broaden understanding of the complex nature of Gc biology and help define the mechanisms that lead to Gc resistance to allow better drug design.

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