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# EFFECTS OF SITE SPECIFIC PHOSPHORYLATION ON THE STRUCTURE AND FUNCTIONS OF THE GLUCOCORTICOID RECEPTOR

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# EFFECTS OF SITE SPECIFIC PHOSPHORYLATION ON THE STRUCTURE AND FUNCTIONS OF THE GLUCOCORTICOID RECEPTOR

**by**

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# Dedication

To my family and friends

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# EFFECTS OF SITE SPECIFIC PHOSPHORYLATION ON THE STRUCTURE AND FUNCTIONS OF THE GLUCOCORTICOID RECEPTOR

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Ligand dependant transcription factors, like nuclear hormone receptors (NHRs), are capable of exerting transcriptional regulation in the nucleus in response to various intra- and extracellular signals. Transcription factors contain segments that are intrinsically disordered (ID) under native conditions. Posttranslational modifications, such as phosphorylation, affect protein stability and activity of proteins. Conformational changes of such disordered domains have been shown to facilitate binding of one or more coregulatory proteins. The glucocorticoid receptor (GR) belongs to the NHR super family and contains such an ID domain in its N-terminal region, the AF1. This transactivation domain must interact with co-regulators for optimal activity and contains most of the conserved phosphorylation sites (S203, S211, and S226) in the human GR. Published data has linked site-specific phosphorylation of the GR to physiological functions of the GR in a leukemia cell line model (5). This project's aims were to study how site-specific phosphorylation affects the structure and function of the glucocorticoid receptor. The aims of the project were: 1) to test the effect of site-specific phosphorylation on the conformation of the recombinant AF1 domain of the human GR, 2) to test the effects of site-specific phosphorylation on the interactions of AF1 with specific coregulatory

proteins and the subsequent changes in transcriptional activity in CV-1 cells, and 3) to test if site-specific phosphorylation of the GR is controlled by MAPK activity and if this phosphorylation is sufficient to restore lost GR function in refractory hematological malignancies.

We show for the first time, that ID AF1 domain of glucocorticoid receptor (GR) adopts a functionally folded conformation due to site-specific (S211) phosphorylation by p38 MAPK that we have earlier shown to be involved in the apoptotic and gene-inductive events initiated by GR. These conformational changes are important for AF1s interaction with coregulatory proteins, and subsequent GRE mediated transcriptional activity of the GR.

Finally, these conformational changes are important for AF1s interaction with coregulatory proteins, and subsequent GRE mediated transcriptional activity of the GR. This activating phosphorylation, specifically S211, is controlled by balanced MAPK activity in *in vitro* cell line models providing and additional mechanism for resistance. Where phosphorylated p38 levels are high relative to low ERK and JNK activity levels. Further suggesting that p38 MAPK activity plays a role in structural and functional consequences of the GR.

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# Chapter 1: Introduction

Corticosteroids are currently frontline therapy in the treatments of several disease states, including certain types of hematological malignancies and respiratory malignancies. Despite their widespread use, their exact mechanism of action remains not fully understood. Lack of this knowledge also hinders the understanding and overcoming of steroid resistant disease. Therefore, the design of appropriate steroids that could lead to a desired clinical outcome has been greatly compromised. Understanding the exact biophysical structure and properties of steroid receptors, especially the functional structure of the N-terminal region of the receptor is necessary to accomplish the task.

## The Nuclear hormone receptor family

Nuclear hormone receptors (NHRs) were first identified more than 40 years ago; however, another 20 years had passed before it became apparent that these receptors are transcription factors. There are thus far 48 identified members of NHRs in the human genome (Berkenstam and Gustafsson, 2005), which include both receptors with identified ligands and "orphan receptors" with no identified ligands to date. All NHRs share a common structural organization consisting of separate DNA- and ligand-binding domains (DBD and LBD) (Evans, 1988). The superfamily includes receptors for steroids, thyroid hormones, retinoic acid, and fatty acids. The steroid hormone receptors include: glucocorticoid (GR), progesterone (PR), mineralcorticoid (MR), androgen (AR), and estrogen (ER) (Berkenstam and Gustafsson, 2005).

**The glucocorticoids and their receptor**

Glucocorticoids (GCs), like Cortisol, are small lipophillic steroid hormones. Cortisol is the major endogenous glucocorticoid in humans and is synthesized and secreted in the adrenal cortex. The function of GCs in the body include: regulation of carbohydrate, protein and lipid metabolism, suppression of inflammatory and immunological responses and suppression of the hypothalamus-pituitary-axis (HPA). GCs exert their effects in higher eukaryotes at the level of gene regulation via interaction with an intracellular, ligand-activated transcription factor, the GR. The GR has a domain structure arrangement typical of the superfamily of NHRs (Housley et al., 1985; Thompson and Kumar, 2003).

GCs regulate the expression of a large number of genes, arrest certain cells in G1 phase of the cell cycle, and cause apoptosis of certain cell types (Mangelsdorf et al., 1995). Because of their ability to arrest growth and induce apoptosis in a variety of malignant lymphocytes, GCs are frontline chemotherapy in many lymphoid derived malignancies. As shown in Illustration 1, the classical model of the mechanism of steroid action is that the GR in the cytosol is complexed with a group of heat shock (HSP) and other proteins (Housley et al., 1985; Silverstein et al., 1999; Pratt, 1993). Ligand binding causes a dissociation of the GR from these proteins, after which the ligand bound receptor gets hyperphosphorylated and undergoes conformational changes, following by translocation of the GR into the nucleus and subsequent binding to glucocorticoid response elements (GREs) (Mangelsdorf et al., 1995). This GR: GRE interaction can be associated with several coactivator/co-repressor proteins from basal transcription machineries and can also regulate transcription (Kumar and Thompson, 1999).

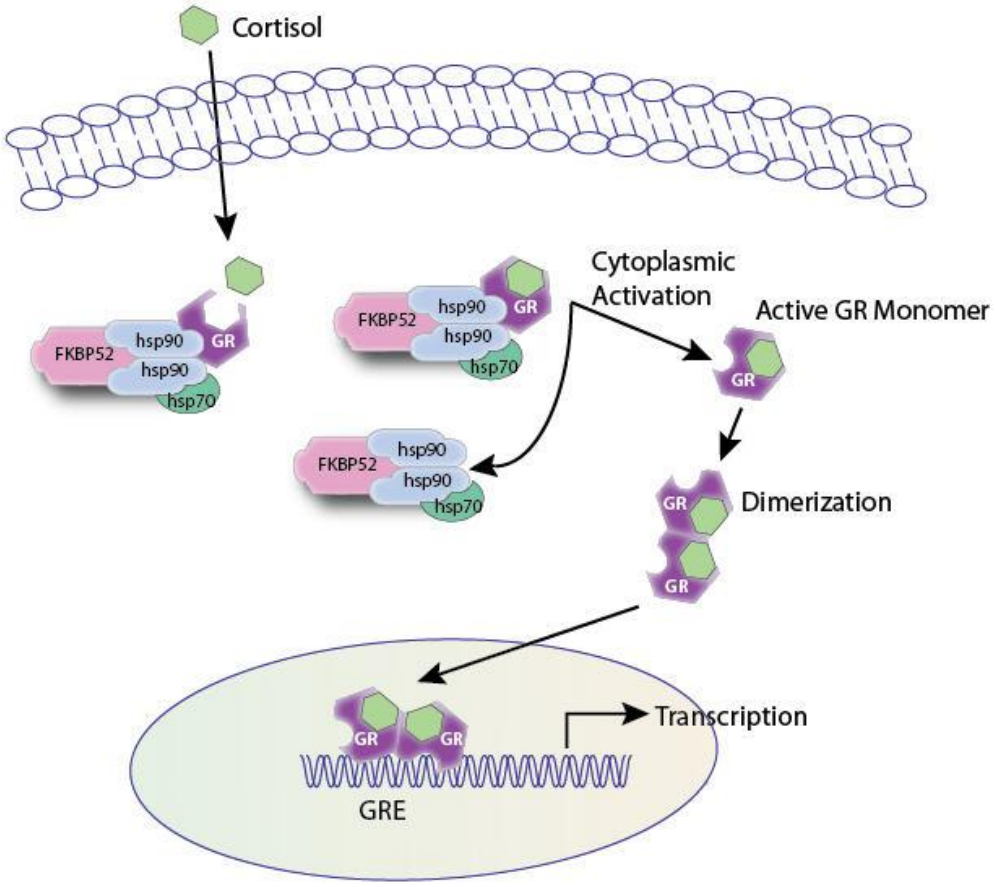



Illustration 1: Classical Mechanism of GC: GR action.

Small lipophilic GCs can cross the cell membrane bind the GR, which is complexed and stabilized in the cytosol with various chaperone proteins. Once the GC binds GR it will undergo cytoplasmic activation, release the chaperone proteins and dimmerize. Now it can translocate into the nucleus bind GRE's and transcribe GR regulated genes.

**Modular structure of the GR**

The modular structure of the GR consists of the N-terminal domain (NTD), the DNA binding domain (DBD) and the ligand-binding domain (LBD) and is shown in illustration 2. There are two transactivation regions: AF1 (in the NTD) and AF2 (in the LBD). The DBD contains two asymmetric zinc-finger motifs. Each has four conserved cysteine residues that coordinate the binding of a zinc atom to facilitate the formation of alpha helices that interact with GRE's (Luisi et al., 1991). AF2 is ligand dependant and AF1 is ligand independent. Both transactivation domains are thought to play an important role in communicating with co-regulators and the basal transcriptional machinery. Several reports suggest that AF1 is the major transactivation domain required for GR function. The 3-D structures of the GR's DBD and LBD are known (Godowski et al., 1987; Miesfeld et al., 1987; Bocquel et al., 1989). Our understanding of how AF2 functions has been greatly enhanced by the delineation of the basic LBD structure. Due to the intrinsic disordered nature of AF1 it has proven to be very difficult to solve its structure. When expressed independently as a recombinant peptide the GR AF1 appears to be present in a large collection of conformers that collectively appear to have limited dominant secondary/tertiary structure elements. Even when AF1 is present in a two-domain fragment of the GR containing its entire NTD and DBD, the AF1 component displays only slightly more structure, but is clearly not fully folded (Kumar et al., 1999). Similar findings have been reported for other SR AF1s, i.e. estrogen receptor (ER) (Henriksson et al., 1997). It is known that AF1 interacts with other TFs, and the available data by my mentors' laboratories as well as others strongly suggest that conditional folding of AF1 is the key for these interactions and the subsequent transcriptional activity (Almlöf et al., 1995; Almlöf et al., 1998; Ford et al., 1997; Baldwin and Rose, 1999; Wärnmark et al., 2001). How and what kind of functional conformation AF1 adopts under physiological conditions is an open question and this dissertation is aimed to answer the impact phosphorylation has on its functionally relevant conformation.

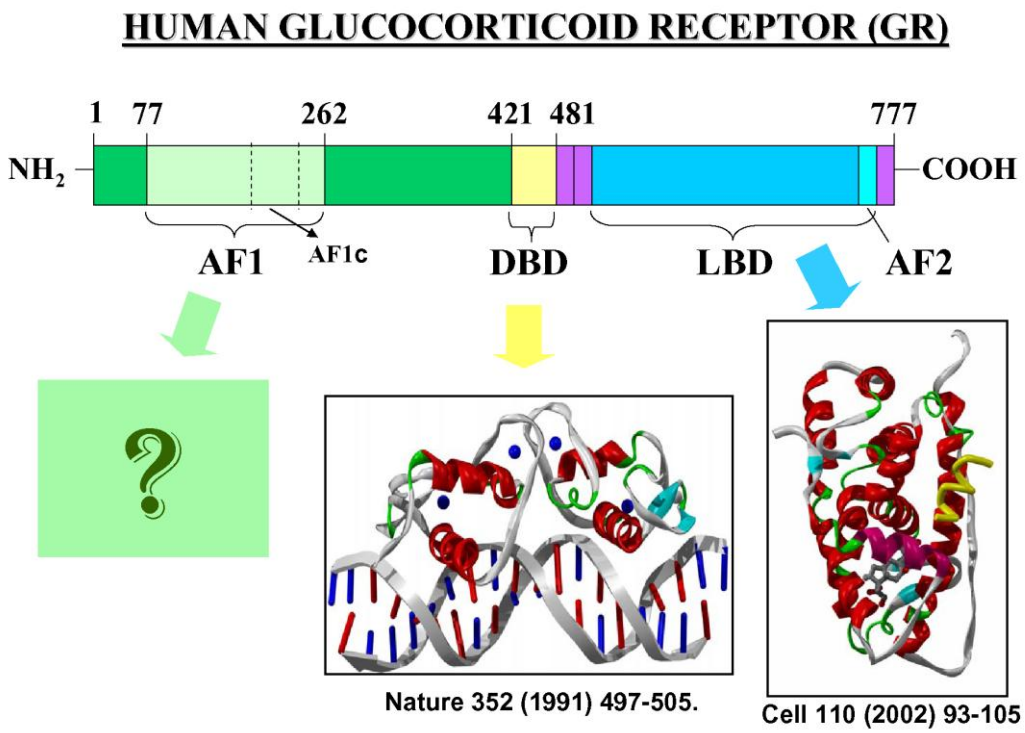



Illustration 2: The structure of the human glucocorticoid receptor protein.

The hGR protein is divided into three major domains, the N-terminal domain, the DNA binding domain (DBD) and the ligand binding domain (LBD).

**Intrinsically disordered proteins**

In recent years many biologically functional proteins or regions/domains of proteins have been identified that do not possess an orderly three-dimensional structure; rather they exist as dynamic ensembles of inter-converting conformers that do not automatically adopt a classical fully ordered and well defined, functional structures (Uversky, 2002; Dunker et al., 2002; Dyson and Wright, 2002; Fink, 2005; Kumar and Thompson, 2003). In other words these are known as intrinsically disordered (ID) proteins, and their structural flexibility and dynamic mobility has been reported to play significant functional roles while carrying out numerous important biological functions (Wright and Dyson, 1999; Dunker et al., 2001; Namba, 2001; Dunker et al., 2002; Dyson and Wright, 2002; Uversky, 2002; Kumar and Thompson, 2003; Fink, 2005; Dyson and Wright, 2005;versky, 2005; Eliezer, 2007). Though these ID segments, in most cases, do not possess any significant secondary/tertiary structures compared with well-ordered globular proteins, in some cases these ID sequences do not necessarily have to differ from well ordered ones in terms of secondary structural elements rather their dynamics play a crucial role in deciding their intrinsic disorder (Crivici and Ikura, 1995; Iakoucheva et al., 2002; Tompa, 2002; Romero et al., 2004; Ward et al., 2004; Liu et al., 2006). An important feature of the ID proteins is that they undergo disorder–order transition during or prior to their biological function, and in many cases this process has been reported to provide a logistic mechanism for their actions under physiological conditions (Kriwacki et al., 1996; Uversky et al., 2000; Sugase et al., 2007). These ID segments can vary in size from few amino acid sequences to the entire domain (ranging up to several hundred amino acids), to even the entire protein, as big as 200 kDa (Hernández et al., 1986; Uversky et al., 2000). Many of these ID regions significantly differ in terms of sequences homology except that in most cases they are found to be highly charged at neutral pH and show a low hydrophobicity (Uversky et al., 2000). The prevalence and regulatory functions of ID proteins have become increasingly recognized in last few years (Kriwacki et al., 1996; Campbell KM, 2000; Flaugh and Lumb, 2001; Uversky, 2002; Zor, 2004; De Guzman et al., 2006; Ng, 2007; Sugase et al., 2007; Ebert et al., 2008). The structural representation of these disordered states as ensembles of

inter-converting conformers can therefore provide insights for important biological functions.

There has been a growing interest in ID proteins and their biological actions under physiological conditions. This interest is due to several reasons, for one the classical text book assumption that well-ordered conformation in a protein may no longer be required for functioning, and second the conformational flexibility in ID proteins may serve specific functions (Dyson and Wright, 2005; Uversky et al., 2005). In support of this, some studies have suggested that the lack of an ordered conformation in ID proteins under physiological conditions might represent a considerable functional advantage by providing large conformational plasticity/flexibility/mobility that may allow them to interact efficiently with several different target molecules that are critical for specific functions (Wright and Dyson, 1999; Dunker et al., 2001; Dyson and Wright, 2005; Uversky et al., 2005). This might represent a simple mechanism for regulation of numerous cellular processes such as transcriptional and cell cycle control (Dunker and Uversky, 2008), which require their related transcription factor proteins to interact with several specific target molecules for efficient regulations (McEwan et al., 1996; Shen et. Al., 1996; Benjamin, 2000; Shoemaker et al., 2000; Williamson, 2001; Kumar et al., 2004a; Kumar et al., 2004c; Mark et al., 2005; Minezaki et al., 2006; Singh and Dash, 2007). ID proteins may have another functional advantage over well-ordered globular proteins in terms of their inherent conformational flexibility that could allow their local and/or global structures to be modified in response to inter- and intra- cellular environment by allowing one ID protein to function in several different biological systems (Kriwacki et al., 1996; Sugase et al., 2007). ID nature of proteins also allows precise control over the thermodynamics of the binding process and provides a possible mechanism for their actions through interaction with the components of the cellular machinery as well as post translational modifications such as kinase-dependent phosphorylation ( Dunker et al., 2002; Iakoucheva et al., 2002; Iakoucheva et al., 2004; Dunker and Uversky, 2008). In fact, ID activation domains of several transcription factor proteins have been shown to support this phenomenon (Dyson and Wright, 2005; Sugase et al., 2007). Thus, it is logical to think that nature utilizes these ID ensembles of

conformations to perform the myriad functions of the living cell in the most efficient way possible.

Using statistical analysis, it has been shown that amino acid sequences encoding for ID proteins or regions/domains significantly differ from those of ordered proteins at least in terms of amino acid composition, flexibility index, hydrophobicity, charge, coordination number among others (Romero, 1997a, 1997b; Romero et al., 1998; Dunker AK, 1998; Dunker et al., 2000). Unlike an ordered protein, the backbone bonds and angles of an ID protein may vary significantly over time, with no specific equilibrium values while undergoing conformational transitions (Uversky et al., 2005). ID proteins are known to play a critical role in a variety of biological processes related to cell-signaling pathways including cell cycle control, transcriptional and translational regulation, and signal transduction (Dyson and Wright, 2002; Dyson and Wright, 2005; Uversky et al., 2005). It has been predicted that high number of signaling proteins possess long ID regions, suggesting the involvement of ID sequences in signaling and regulation (Iakoucheva et al., 2002). These ID regions/domains are also involved in molecular recognition and post transcriptional protein modifications (Dunker et al., 2002).

**Properties of AF1, the major transactivation domain of the GR**

The AF1 of the human GR is located between amino acids 77-262 in the NTD. Studies have shown that 60-80% of GR's transcriptional activity is controlled by AF1 alone (Härd et al., 1990; Luisi et al., 1991). AF1 appears to be intrinsically disordered when expressed independently as a recombinant peptide. However, there are suggestions that AF1 must acquire a folded functional structure for its interaction with critical co-regulatory proteins and subsequently have transcriptional activation activity (Godowski et al., 1987; Dalman et al., 1988). Published data have shown that under certain conditions AF1 can adopt a functional folded structure. These conditions to achieve this folded structure include: i) presence of naturally occurring osmolytes, ii) binding of DNA to a two domain fragment consisting of the entire NTD plus DBD (GR500, iii) and direct binding to TBP (Kumar et al., 1999; Kumar et al., 2001).

**Phosphorylation regulates structure and functions of SHRs**

Like many other transcription factors, the GR is a phosphoprotein, and it has been suggested that phosphorylation plays an important role in the regulation of steroid receptor activity (Webster et al., 1997). Protein phosphorylation is generally an important phenomenon in regulation of protein function in eukaryotic cells, and is often concerned with switching of a cellular activity from one state to another. For transcription factors, three main mechanisms of regulation by phosphorylation can be identified; 1) the DNA binding affinity of TFs can be modulated negatively or positively; 2) the interaction of transactivation domains of transcription factors with components of the transcription initiation complex can be affected; and 3) the shuttling of transcription factors between the cytoplasmic compartments can be influenced.

All of the SHRs contain multiple phosphorylation sites. Most of the sites are serines and threonines in the amino terminal regions of the receptors; they are positioned in Ser/Thr-Pro motifs, suggesting that the receptors are direct targets of proline-directed kinases including the cyclin-dependent kinases and the MAPK family, which is consistent with studies showing that these kinases modulate receptor activity. Some of the phosphorylation sites in steroid receptors are conserved across species, whereas others are unique to specific species. There is a GSK3α phosphorylation site in rat GR that is absent in human GR (Rogatsky et al., 1998b). Chicken PR contains four Ser-Pro phosphorylation sites (Denner et al., 1990; Poletti A, 1993) and all are in the regions common to the PR-B and PR-A isoforms. In general the receptors are partially phosphorylated in the absence of hormone and are more phosphorylated after hormone treatment.

Each receptor has multiple phosphorylation sites, which made identifying the kinases that phosphorylate the individual sites *in vivo* very challenging. Studies with site-specific antibodies which have been made for a number of the sites reveal that some sites are targets for multiple kinases, allowing the receptors to respond to a variety of stimuli, e. g., p42/p44 MAPK phosphorylates Ser294 in PR-B in response to EGF, but hormone-

dependent phosphorylation of the site occurs despite blocking p42/p44 MAPK activation (Narayanan et al., 2005).

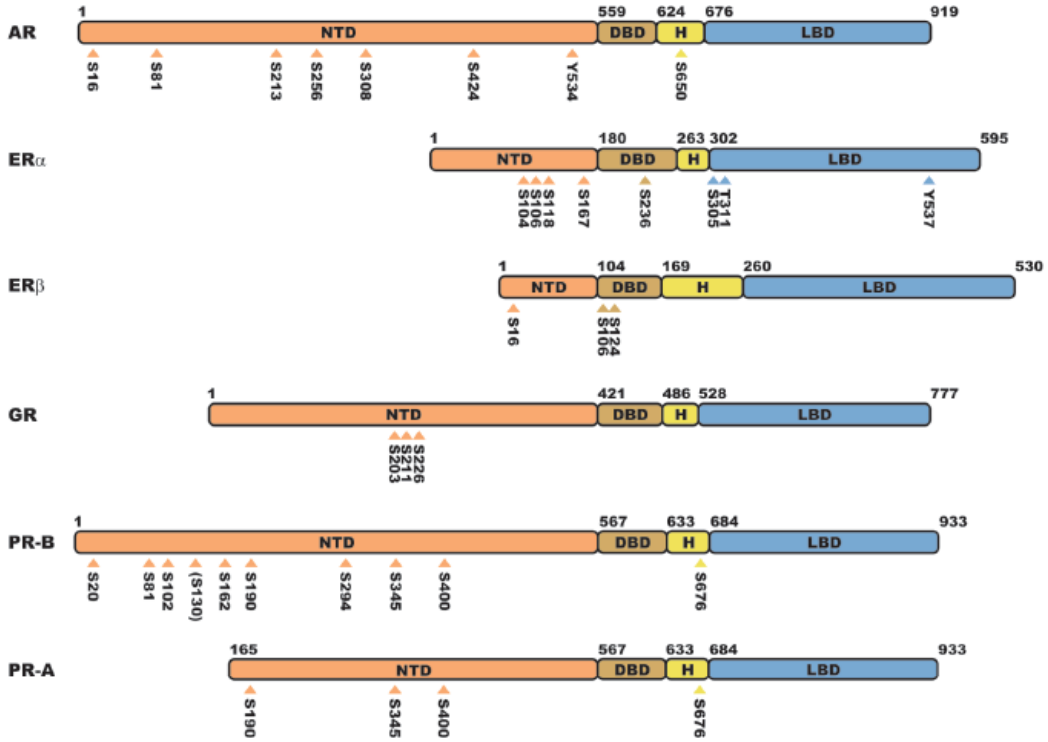
Application of phosphorylation site-specific antibodies has also been useful in resolving whether candidate sites are authentic *in vivo* sites. Studies of the role of phosphorylation in receptor function have revealed that receptor phosphorylation contributes to a variety of functions including receptor stability, interaction with coregulators, transcriptional activity, nuclear localization, and splicing, as well as subcellular distribution.

Furthermore, analyses of individual receptor functions have revealed more specific roles for phosphorylation. Substitution with an Ala for Ser294 in human PR increases receptor stability. It was found that mutation of the phosphorylation sites in GR increases receptor stability (Webster et al., 1997). TSG101, GR-interacting protein, was found to preferentially bind to hypophosphorylated GR and is able to protect it from degradation (Ismaili et al., 2005). In another receptor, phosphorylation of Thr311 in ERα increases nuclear localization (Lee, 2002). Stress kinase-induced phosphorylation of Ser650 in AR enhances cytoplasmic localization (Gioeli et al., 2006). Phosphorylation of Ser118 It has been implicated with the interaction of splicing factor (SF)3a p120 and increased splicing (Masuhiro et al., 2005). On the other hand, phosphorylation of this site enhances interaction with the estrogen receptor repressor SPBP (stromelysin-1 platelet-derived growth factor-responsive element-binding protein) (Gburcik et al., 2005).

It is known that steroids can activate a variety of cell signaling pathways that influence transcription and enzyme activities independent of the genomic activities of the receptors (Watson and Lange, 2005) and these pathways also modulate receptor function. This is mediated by SHRs, as well as by other steroid-respond proteins. AR and ER has been shown to interact with Src and MNAR (modulator of nongenomic actions of the estrogen receptor) (Wong, 2002; Haas, 2005). Hormone binding results in activation of Src and the downstream activation of p42/p44 MAPK, kinases that regulate receptor function through phosphorylation of coactivators and, in some cases, phosphorylation of the receptors themselves. Furthermore, PR is able to directly interact with Src family tyrosine kinases through a proline-rich motif in the NTD of PR, which also activates a kinase cascade (Boonyaratanakornkit et al., 2001). PR also induces long term activation

of p42/p44 MAPK through induction of Wnt-1 and the resulting activation of the EGF receptor (Faivre and Lange, 2007).

Overall it has been established that cell signaling pathways that regulate phosphorylation of SHRs and their coactivators are critical factors in determining the activities of steroid receptors under different physiological conditions. Specifically many phosphorylation sites can respond only to a specific signaling pathway and a transient phosphorylation could be very important for biological activity of the receptor. Illustration 3 below shows all the thus far known functionally important phosphorylation sites in each of the SHR's.




Adapted from *Weigel et al., 2007*

Illustration 3: Domain structures and Phosphorylation sites of steroid receptors.

**Site-specific phosphorylation and GR action**

Phosphorylation is a well-studied posttranslational modification known to alter the structure and function of many proteins (Gille et al., 1996; Jans and Hübner, 1996; Zhou et al., 2003; Ismaili and Garabedian, 2004). NHRs are greatly regulated through the action of cellular kinases; reports involving several steroid receptors have shown the ability of kinases to enhance transactivation activity in a ligand dependant (Nielsen et al., 1977; Pratt et al., 1979) and independent manner ( Kurl and Jacob, 1984; Dalman et al., 1988). Many cellular processes such as ligand binding, nuclear translocation, receptor dimerization as well as the interaction with general transcription factors have all been linked to phosphorylation (Dalman et al., 1988; Bodwell et al., 1998; Wang et al., 2002). My mentors' laboratories have shown that site-specific phosphorylation in the AF1 (S211) of the human GR by p38 MAPK is involved in the functional events initiated by GCs (Miller et al., 2005).

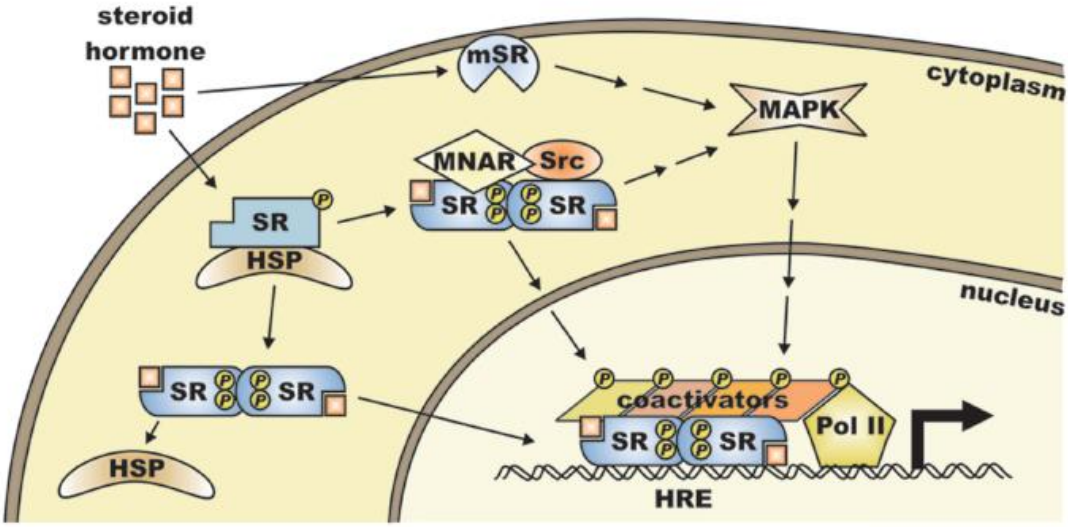
The GR is a phosphoprotein with several known phosphorylation sites located in the AF1 domain (Serine 113, 141,203,211,226) (Dalman et al., 1988; Ortí et al., 1992; Webster et al., 1997; Rogatsky et al., 1998a; Ismaili and Garabedian, 2004). Only site S308 is located in the NTD outside AF1. Reports have shown the role of phosphorylation in stabilizing protein structure. Since most physiologically relevant phosphorylation sites in the GR are in or near the AF1 domain, I hypothesize that phosphorylation plays a crucial role in allowing AF1 to adopt conformation(s) in vivo, which is/(are) important for its interaction with other coregulatory proteins, and subsequently play a role in gene regulation. Knowledge of the conformational changes in AF 1 due to phosphorylation and its role in interaction with other coregulatory proteins will lead to an understanding of the role of this region in the transcription process, this information essential to understanding how glucocorticoids affect gene regulation.

**Activation of SHRs through crosstalk with cell signaling pathways**

It has been shown that some steroid receptors can be activated in the absence of measurable levels of ligand but through conditions that increase kinase activity and inhibit phosphatase activity. Pioneer work by Denner et al. proved that 8-Br cAMP treatment of cells transfected with a chicken progesterone receptor (cPR) expression vector and a PR-responsive reporter caused hormone-independent, but cPR-dependent, activation of the reporter (Denner et al., 1990). Later Bai et al. found that this activation was independent of the four characterized phosphorylation sites in cPR and 8-Br cAMP did not induce phosphorylation of PR (Bai et al., 1997). Further studies lead to the discovery that 8-Br cAMP activated p42/p44 MAPK (mitogen-activated kinase) and increased phosphorylation of the p160 coactivator, SRC-1 (steroid receptor coactivator-1); these SRC-1 phosphorylation sites contributed to the ligand-independent activation (Rowan et al., 2000).

Another member of the SHR family, ERα, has multiple known pathways for hormone-independent activation. EGF (epidermal growth factor)- dependant activation of ERα is one of the best characterized of the hormone-independent pathways. EGF-dependent activation induces phosphorylation of Ser118 in the amino terminus of ER (Kato et al., 1995). EGF-dependent activation requires phosphorylation of ERα at Ser118, as well as phosphorylation of at least one other target (Kato et al., 1995). Further studies found that EGF treatment typically results in activation of Rsk downstream of p42/p44 MAPK; Ser167 is a substrate for Rsk (Joel et al., 1998; Clark et al., 2001). Other pathways are independent of Ser118 phosphorylation, demonstrating that there are multiple means of activating ER independent of its ligand (Patrone et al., 1998).

These are just a few examples of how signaling pathways can crosstalk with SHR's and modulate their function and activity. Illustration 4, shows a general scheme of these processes.




Adapted from *Weigel et al., 2007*

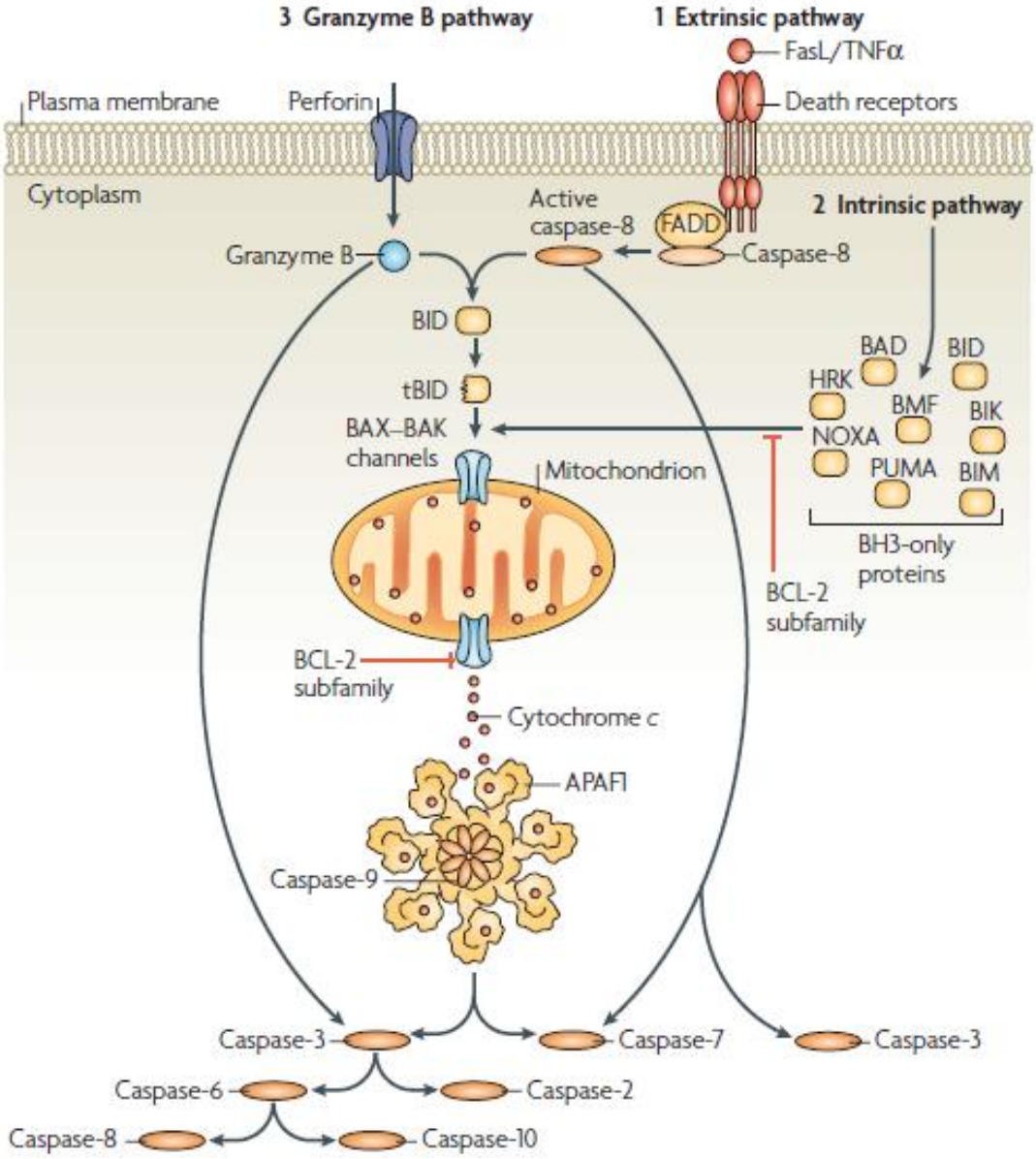
Illustration 4: Crosstalk of cell signaling pathways with SHR action.

**Kinases cross talk with the glucocorticoid receptor to regulate hormone action**

Cell signaling pathways that regulate phosphorylation of steroid receptors and their coactivators are critical factors in determining the activities of steroid receptors under different physiological conditions. In the absence of hormone, GR is predominantly cytoplasmic. Dexamethasone (Dex) treatment causes nuclear localization, and wash out of Dex causes a gradual relocalization to the cytoplasm over the course of several hours (Itoh et al., 2002). Research has shown that if JNK is activated by UV irradiation and combined with Dex depletion, GR rapidly relocates to the cytoplasm. JNK has been shown to phosphorylate Ser226 in the amino terminus of human GR, and the S226A mutant exhibits both reduced basal relocalization as well as reduced JNK-stimulated relocalization (Itoh et al., 2002). An earlier study of rat GR showed that treatment with the phosphatase inhibitor, okadaic acid, enhanced GR phosphorylation and prevented entry into the nucleus in response to hormone (DeFranco et al., 1991). Although many of the phosphorylations enhance receptor activity, phosphorylation of Ser246 in rat GR reduces its activity (Rogatsky et al., 1998a). Recent published data from my mentors' laboratories, however, show that p38 is a potent kinase for in vitro phosphorylation of S211 on the human GR (Miller et al., 2005). Thus, the cellular levels of active kinases and phosphatases, as well as the milieu of coregulators and other receptor-interacting proteins, will determine the specific response of a receptor to its cognate ligand for phosphorylation in regulating known biological functions of receptors. Therefore the hypothesis of this dissertation was that we could restore GC induced apoptosis in steroid resistant lymphoid leukemia cell lines by targeting pathways that affect the phosphorylation status of the GR. This knowledge applied and confirmed on patient samples can lead to new rational approaches for combined targeted therapy with steroids to stabilize or even cure the resistant disease.

**Apoptosis**

Apoptosis, also called Type 1 cell death is a form of programmed cell death essential for the development and maintenance of multicellular organisms. Characteristic features of apoptosis, classically speaking, are activation of caspases, chromatin condensation, and display of phagocytotic markers on the cell surface. There are two mechanistic pathways that can lead to apoptosis, namely the extrinsic and intrinsic pathways. The extrinsic pathway is initiated through the activation of death receptor proteins on the cell membrane (Fas/CD95) as well as adaptor protein FADD. These will interact with cysteine aspartate protease pro-caspase 8, a zymogen at this point. This interaction will lead to the cleavage of the pro-caspase and activate it to become caspase 8, which will in turn activate downstream caspases in the cytoplasm of the cell, which include caspases –3, -6, -7, known as effector caspases. Alternatively, activated caspase 8 can trigger the activation of caspase 3 in an indirect fashion, through a pathway that involves Bcl family members and release of mitochondrial cytochrome c. The intrinsic pathway involves the direct release of soluble proteins that are contained in the mitochondrial membrane space, these include cytochrome c, apoptosis inducing factor (AIF), endonuclease G, Omi/HtrA2 and Smac/DIABLO. So, for example, if cytochrome c is released into the cytosol it will interact with Apaf-1 (apoptosis protease activating factor-1) and pro-caspase-9 leading to the formation of caspase-9 activation complex. Active caspase-9 now triggers activation of procaspase-3, and so on. In addition, apoptosis can also be induced in a caspase independent manner, for example through the action of death effectors like AIF, endonuclease G, and Omi/HtrA2 (Taylor et al., 2008).




Adapted from *Taylor et al., 2008*

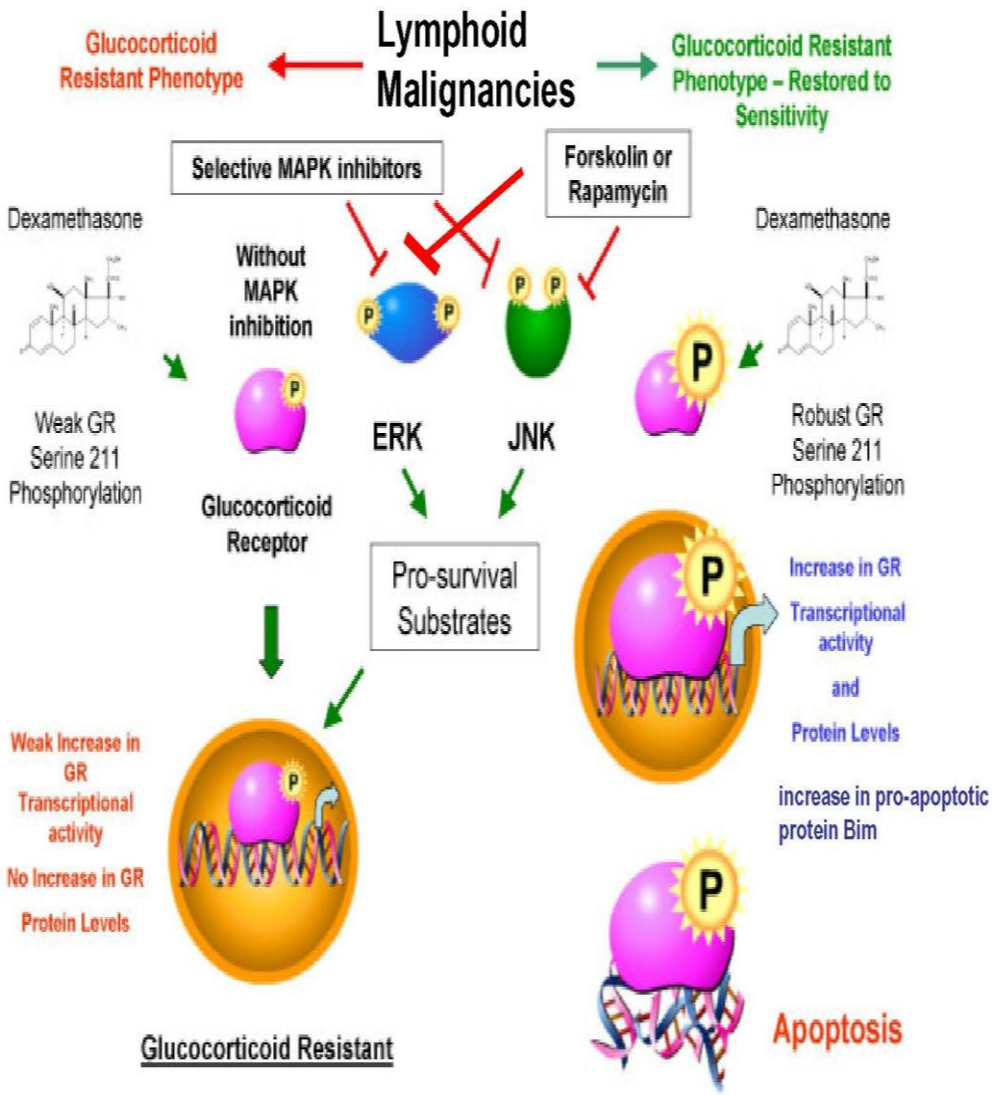
Illustration 5: Caspase activation pathways.

Shown are caspase activation by extrinsic, intrinsic and granzyme B pathways.

**Cell Signaling Cascades and the effect on GR induced apoptosis**

Steroids have been frontline therapy in the treatment of several malignancies for decades; however the mechanism by which steroid receptors pass signals from ligand to specific gene is not fully understood. In recent years it has become clear that the resistance to steroids of GR+ lymphoid and myeloid cell lines may well involve cross-talk between the GR and cell signaling cascades (Rogatsky et al., 1998a; Medh et al., 1998; Bruna et al., 2003; Kyriakis, 2000; Wada and Penninger, 2004). Signaling molecules, such as MAPKs, ERK (extra cellular signal-regulated kinase), p38 and JNK (c-Jun N¬-terminal kinase), as well as the cAMP-driven PKA and mTOR pathways direct such diverse cellular processes as cell growth and death. Previous studies from our laboratory had shown that ERK and JNK serve to inhibit the apoptotic effects of GCs in resistant CEM clones derived from a human acute lymphoblastic leukemia (Miller et al., 2005). Furthermore my mentor's laboratory has shown that p38 is a key mediator in GC-evoked apoptosis in lymphoid cells (Miller et al., 2007). Rapamycin, an inhibitor of mTOR, has been shown to stimulate G1 arrest in cycling B-CLL cells and to interfere with many important molecules for cell cycle regulation in these cells (Wang and Garabedian, 2003). Yet another study has linked Rapamycin to Dex-induced apoptosis in the multiple myeloma cell lines Karpas 707 and U-1958 (Wei et al., 2006). FSK activates cAMP production, which in turn will cause the activation of PKA (Medh et al., 1998). PKA and GC's have been found to act synergistically to overcome resistance in human leukemic CEM-C1 cells, and this finding has been correlated with c-myc suppression (Medh et al., 1998). C-myc is a known human oncogene over-expressed in a large number of human cancers. Loss of PKA activity has been previously demonstrated to cause a significant decrease in GC sensitivity (Miller et al., 2007). Based on these findings in a T-lymphocyte derived leukemia model as well as other published results, I found that the deregulation of signal transduction pathways is a general mechanism that prevents the GC induced apoptotic effect in more than one type of GC-resistant lymphoid malignancies. Furthermore, I theorized that ERK and JNK act to oppose the apoptotic effect of GCs, while p38 MAPK promotes it, and that these pathways converge on the amount of total GR as well as the phosphorylation state of GR (S211 and S226). I tested

this hypothesis by screening a variety of types of GC resistant malignant lymphoid and myeloid cells for such interactions.




Adapted from *Miller et al., 2007*

Illustration 6: Model of signaling pathways that converge on GR to increase GRE driven GR transcriptional activity.

Dex-resistant cells in their natural state harbor high levels of pro-survival, anti-apoptotic active JNK (green symbol) and low levels of active ERK (blue symbol) which is Dex-inducible. The cells also contain GR (pink symbol). The sequence on the left side shows the result in CEM cells which resist Dex-dependent apoptosis. In this case, added Dex mediates a weak increase in GR phospho-Ser 211 as well as GRE reporter driven activity, but no increase in GR protein levels, and the cells remain resistant. The sequence on the right depicts the results when CEM-C1-15 cells are treated with combinations of Dex and MAPK inhibitors, FSK, or rapamycin. These treated cells convert to a GC-sensitive phenotype. All treatments converge at inhibition of the JNK MAPK pathway. Upon restoration of the Dex-sensitive phenotype, a robust increase in GR phospho-Ser 211, GR protein, and transcriptional activity is observed. These effects culminate in an apoptotic response.

**Project Aims and significance**

Protein phosphorylation represents an important regulatory mechanism in eukaryotic cells. At least one-third of all eukaryotic proteins are estimated to undergo reversible phosphorylation (Rennecke et al., 1996). Phosphorylation modulates the activity of numerous proteins involved in signal transduction, and regulates the binding affinity of transcription factors to their coactivators and DNA thereby altering gene expression, cell growth and differentiation (Zor et al., 2002). Ligand dependant transcription factors, like nuclear hormone receptors (NHRs), are capable of exerting transcriptional regulation in the nucleus in response to various intra- and extracellular signals. Transcription factors contain segments that are intrinsically disordered (ID) under native conditions. Posttranslational modifications, such as phosphorylation, affect protein stability and activity of proteins. Conformational changes of such disordered domains have been shown to facilitate binding of one or more coregulatory proteins. This

has been evidenced in recently published literature (Kumar et al., 2007). Conformational changes upon phosphorylation often affect protein function. For example, serine phosphorylation of the peptide corresponding to the calmodulin binding domain of human protein p4.1 influences the ability of the peptide to adopt an alpha-helical conformation and thereby impairs the calmodulin-peptide interaction (Vetter and Leclerc, 2001). The glucocorticoid receptor (GR) belongs to the NHR super family and contains such an ID domain in its N-terminal region, the AF1. This transactivation domain must interact with co-regulators for optimal activity and contains most of the conserved phosphorylation sites (S203, S211, and S226) in the human GR. Published data has linked site-specific phosphorylation of the GR to physiological functions of the GR in a leukemia cell line model (Miller et al., 2007). This dissertation focused on how site-specific phosphorylation affects the structure and function of the glucocorticoid receptor. I found that: Site-specific phosphorylation of the N-terminal transactivation domain, AF1, leads to changes in its conformation that are important for AF1's interaction with coregulatory proteins; and subsequent transcriptional activity of the GR. This activating phosphorylation is controlled by the balance between ERK, JNK and p38 (MAP) kinase activities in several cell line models. The hypothesis was tested through the following specific aims:

Aim 1 tested the effect of site-specific phosphorylation on the conformation of the recombinant AF1 domain of the human GR. Phosphorylation has been shown to affect conformations and protein-protein interactions for several transcription factors (TFs) including steroid receptors (Chen et al., 1993; Cheng and Lee, 1998). Work that resulted from the dissertation shows that site-specific phosphorylation (S211) induced more alpha helical content in AF1. I determined the secondary and tertiary structural changes in AF1 due to in vitro phosphorylation of S203, S211, and S226 residues by using specific kinases. The conformational changes were monitored by: circular dichroism- (CD), fluorescence emission spectroscopies and restricted proteolytic digestion experiments.

Aim 2 tested the effects of site-specific phosphorylation on the interactions of AF1 with specific coregulatory proteins and the subsequent changes in transcriptional activity in CV-1 cells. Data suggests that *in vitro* phosphorylation of AF1 induced more ordered conformations, which facilitate the interaction of AF1 with TBP.

Aim 3 tested if site-specific phosphorylation of the GR is controlled by MAPK activity and if this phosphorylation is sufficient to restore lost GR function in refractory hematological malignancies. Under this aim I: **a)** selected cell lines derived from patients with Burkitt's lymphoma, multiple myeloma, T-cell leukemia, B-cell leukemia, and myeloid lineages and measured the extend of restored sensitivity by combining inhibitors of JNK, ERK, mTOR and/or activator of PKA plus Dex; **b)** confirmed apoptosis; **c)** measured the active and total protein levels of the glucocorticoid receptor ($GR^{P}$ S211+ GR) and MAPK's, and the effect of ERK and/or JNK, mTOR inhibition and PKA activation as well as the induction of Bim in response to the sensitizing treatments.

AF1 is quantitatively the major transactivation domain of the GR. Thus far, all conserved and functional phosphorylation sites have been mapped to or around the AF1 domain; however not much is known about how phosphorylation affects structure and functions of AF1. Lack of this information has undermined our understanding of how the GR transmits the transcriptional signal from ligand to specific gene(s). This includes the ability of lymphoid hematological malignancies to become resistant to steroid chemotherapy. The studies were based on published work as well as my preliminary data. The work from this dissertation will provide pivotal knowledge about the role of phosphorylation on the structure and functions of AF1, and will also give important new insights into how the GR and related transcription factors; transmit the transcriptional signal from ligand to specific target gene(s). This information will lay the groundwork for future structure-based drug discovery.

# Chapter 2: General Methods

## Protein Expression and Purification

Recombinant AF1 was expressed in *E. Coli* (BL21 DE3) by use of the recombinant vector pGEX-4T1-AF1 (Amersham Biosciences, Piscataway, NJ). AF1-S203,211, 226A and –S203,211,226E mutants were generated using the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) using pGEX-4T1-AF1.

Primers were generated using Stratagene's primer design software. The primers for the AF1-S211A mutant were forward GGTAAAGAGACGAATGAGgcTCCTTGGAGATCAGACCTG and reverse CAGGTCTGATCTCCAAGGAgcCTCATTCGTCTCTTTACC; and for the S211E mutant the forward primer used was GGTAAAGAGACGAATGAGGagCCTTGGAGATCAGACCTG and the reverse primer used was CAGGTCTGATCTCCAAGGctCCTCATTCGTCTCTTTACC. Primers used to generate mutant from pGEX4T-AF1: S226A: 5'-gatagatgaaaactgtttgcttgctcctctggcggg-3'S226A_antisense:5'cccgccagaggagcaagcaaacagttttcatctatc-3‘; S226E: 5'-cctgttgatagatgaaaactgtttgcttgagcctctggcgggagaag-3‘; S226E_antisense: 5'cttctcccgccagaggctcaagcaaacagttttcatctatcaacagg-3'. Quick ChangeR Lightning (Stratagene) site-directedmutagenesis kit was used to generate mutant recombinant AF1 protein, expressed in *E. Coli* (BL-21). Primers used to generate mutant from pGEX4T-AF1: S203A: 5'-ggagttttcttctggggccccaggtaaagagac-3'; S203A_antisense: 5'-gtctctttacctggggccccagaagaaaactcc-3‘; S203E: 5'-gatttggagttttcttctggggagccaggtaaagagacgaatgag-3'; S203E_antisense: 5'-ctcattcgtctctttacctggctccccagaagaaaactccaaatc-3'. Quick ChangeR Lightning (Stratagene) site-directed mutagenesis kit was used to generate mutant recombinant AF1 protein, expressed in *E. Coli* (BL-21).

Plasmid DNA was sequenced to verify mutation site. pGEX4T-AF1S226A; pGEX4T-AF1S203A and –E expressed in *E. Coli* were then loaded onto a GST-Sepharose 4b column. Purified protein was verified with MS+ MS/MS. Recombinant protein expressed in *E. Coli* was induced with isopropyl-b-D thiogalactopyranoside (1 mM) for 4 h, lysed, and extracted. The bacterial extracts were loaded onto a glutathione–Sepharose column at 4 ºC as described (Kumar et al., 2001). Final protein purity of both proteins was greater than 98% as verified by presence of a single band on SDS-PAGE.

**Plasmids**

The pGRE_SEAP vector (BD Biosciences, Palo Alto, CA) contains three copies of a GRE consensus sequence in tandem, fused to a TATA-like promoter ($P_{TAL}$) upstream from the reporter gene for secreted alkaline phosphatase (SEAP). GR500 encodes amino acids 1–500 of the hGR, plus a five-residue nonspecific extension (Chen et al., 1997). The GR500 variants (GR500S211A and –E) were generated via PCR using phCMV2-GR500 as the starting template and inserting the PCR fragments into pECFP-C1 (BD Biosciences) using *Xho*I/ *Sma*I cloning sites. TBP was cloned into the pcDNA3.1(+) expression vector (Invitrogen, Carlsbad, CA) and into pEYFP-C1 (BD Biosciences). DNA sequencing was performed on all clones to confirm correct sequence. The CFP-YFP fusion protein was generated as described elsewhere (Bai and Giguére, 2003).

***In Vitro* Phosphorylation Assays**

*In vitro* phosphorylation of AF1 at S211 using p38α/ SAPKa Assay Kit (Upstate, Cell Signaling solutions, Lake Placid, NY). In each case 10 µg of AF1 or AF1-S211A and 10 ηg active p38 MAPK was incubated for 2h at 37°C in a shaking water bath, followed by immunoblot analysis with specific antibodies against GR-P S211 (Cell Signalling Technologies, Danvers, MA ) and GR (Affinity Bioreagents, Golden, CO).

**Mass Spectroscopy**

Samples were prepared for Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). One uL of the sample was deposited onto the MALDI plate and allowed to dry. One uL of matrix (sinapinic acid, or 3,5-dimethoxy-4-hydroxycinnamic acid; Aldrich Chemical Co.) was then applied on the sample spot and also allowed to dry. External calibration was performed on each sample spot using a nearby spot consisting of Cytochrome C (Sigma-Aldrich, St. Louis, MO) mixed with matrix in the same manner as described previously. MALDI-TOF MS was performed using an Applied Biosystems 4800 MALDI TOF/TOF Proteomics Analyzer in positive ion and linear modes. For MS data, 2000-4000 laser shots were acquired and averaged from each sample spot.

**Cell culture and transient transfection**

CV-1 (monkey kidney epithelial) cells (American Type Culture Collection, Manassas, VA) were grown at $37^0$ C in MEM with Earle's salts (Invitrogen) supplemented with 10% (vol/vol) fetal bovine serum (Atlanta Biologicals, Norcross, GA). Cells were subcultured every 2–3 days. CV-1 cells were plated on a 24-well plate (1000 µl/well) 1 day before the transfection and transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Transfected cells were maintained at $37^0$ C in 5% $CO_2$/95% air for the duration of the experiment (24–48 h). The level of transfection was estimated to be greater than 60% by use of fluorescence microscopy of the cells receiving CFP. Transfection efficiency was normalized using immunoblot analysis with specific antibodies against AF1, GFP, TBP, CBP and SRC-1 (Figure 3). Protein extracts from whole cell lysates were loaded on SDS-PAGE gels and transferred to PVDF-membrane, blocked with 5% milk at room temperature and incubated with 5%BSA containing the appropriate antibody at 4°C overnight. Membranes were washed with TBS/Tween, incubated with HRP conjugated secondary antibody for 2 hours at room temperature and visualized with ECL (Amersham Biosciences).

**Fluorescence Spectroscopy and FRET analysis**

CV-1 cells grown on a tissue culture dish with integrated slide (Matec) 1 day before transfection were cotransfected with 1 μg of pGRE-SEAP reporter and 3 μg of pECFP-YFP (positive control), 1.5 μg of pECFP-C1, and/or 1.5 μg of pEYFP-C1 (negative control). To test the dependence of FRET on AF1 in the GR, cells were cotransfected with 1.5 μg of pEYFP-TBP, -CBP, or –SRC-1 and 1 μg of pGRE-SEAP. Pairs of pGRE-SEAP received 1.5 μg of either pECFP-GR500, pECFP- GR500S211A, or pECFP- GR500S211E. Cells were washed 24 h later twice with isotonic PBS pH 7.4 , fixed with 4% paraformaldehyde/PBS for 10 min and washed twice with PBS. Cells were visualized using a Zeiss LSM-510 META confocal microscope (Carl Zeiss, Thornwood, NY) with a Plan-Apochromat 63 x 1.4 oil-immersion objective and 6.1 Amp Argon laser. Pre- and postbleach (PB) images were collected at 12-bits resolution on two channels: 458 nm for CFP and 514 nm for YFP. Five images were taken, two before and three after the PB, with 20-sec intervals. To assure more than 90% PB, an arbitrarily selected region of interest, containing examples of both nuclear and cytoplasmic compartments, was irradiated with the 100% intensity laser line at 514 nm at 200-2000 iteration. Increased CFP (donor) fluorescence intensity upon YFP (acceptor) PB was indicative of positive FRET, and its efficiency (FE) was calculated by the equation:

$$FE\ \% = [(I_{DA} - I_{DB}) / I_{DA}] \times 100$$

Where, $I_{DA}$ is donor intensity after PB (extracted from image 2 of time series) corrected for background and fractional PB; $I_{DB}$ is donor intensity before PB background corrected (estimated from image 3 of the PB time series). Images that showed any focal plane drift were eliminated. In addition, we tested CFP, CFP-GR500, YFP, and YFP-TBP alone each time to account for any bleed-through and background FRET as recommended (data not shown) (Karpova et al., 2003).

**Gene reporter assays**

We employed the SEAP reporter system due to its high signal-to-noise ratio and quantifiable transcriptional activity without the need for cell disruption. In the experiments with holo-GR, CV-1 cells were cotransfected as described above with 0.13

μg of pGRE_SEAP reporter vector, 0.13 μg of pECFP-GR500, -S211A, or –S211E and 0.5μg of pcDNA3.1-TBP, pRSLV-CBP, or SRC-1. The total amount of DNA added was kept fixed at 0.8 μg by addition of empty pECFP vector. Medium (25 μl) was collected 24 h later and tested for the presence of SEAP (Great EscAPe SEAP Detection Kit; BD Biosciences) according to the manufacturer's protocol. Experiments were performed at least three times, in triplicate. Data from different experiments were normalized to GR500 activity.

**Fluorescence emission spectroscopy**

Fluorescence emission spectra of purified recombinant AF1 in solution were recorded in the absence or presence of varying concentrations of osmolyte using a Spex FluoroMax spectrometer at excitation wavelengths of 278 or 295 nm as described (Baskakov et al., 1999; Kumar et al., 2001). Measurements were taken in a 1 cm rectangular cuvette at 22 °C, and all data were corrected for the contribution of the buffer. Similar spectra were recorded.

**Circular dichroism (CD) spectroscopy**

CD spectra of protein (at 200 μg/ml with or without p38MAPK in 5 mM Tris, pH 7.9, 25 mM NaCl) were recorded on an Aviv 62 spectropolarimeter using a 0.1 cm quartz cell, with the bandwidth of 1.0 nm and a scan step of 0.5 nm, as described (Baskakov et al., 1999). Each spectrum is representative of at least three independent experiments, corrected for the contribution of the buffer, and smoothed.

**Limited proteolytic digestion**

Digestion of 10 μg purified AF1, AF1-S211A, or AF1-S211E was carried out using sequencing grade trypsin (Sigma–Aldrich), chymotrypsin (Sigma–Aldrich) or Endopeptidase-Glu-C (Sigma-Aldrich) at 4 °C for 15 min in 20 mM Tris, 150 mM NaCl, pH 8.3, at a protein: enzyme mass ratio of 100:1. Reactions were terminated by adding SDS loading buffer and boiling for 5 min. Digested samples were run on SDS–PAGE gel and stained by Coomassie Blue R-250.

**Immunoprecipitation**

HeLa nuclear extract containing 1 mg total protein, 5 μl of antibody (TBP, CBP, or SRC-1), and 50 μL Protein A–agarose conjugate were incubated for 4 h at 4 °C. 10 μg of purified AF1 ± p38 MAPK, AF1-S211A ± p38 MAPK were added and incubated for another 2 h at 4 °C. Beads were centrifuged, washed thoroughly, re-suspended in SDS loading buffer, and boiled for 5 min. to release bound proteins. The released proteins were resolved on SDS–PAGE, and immunoblotted using a GR antibody after transferring onto a PVDF membrane as described (Kumar et al., 2004).

**Statistical analyses**

Results are expressed as means ± S.E. Levels of significance were evaluated by a two-tailed paired Student's t-test, and $P<0.05$ was considered significant.

**Cell Lines**

The following cell lines were obtained from ATCC: HL-60, IM-9, K-562, Mo, Molt-4, and Ramos. OPM-I (Katagiri et al., 1985) and RPMI 8226 (Matsuoka et al., 1967) were established as previously reported.

**Cell culture and drug treatments**

RPMI 8226, IM-9, Ramos and Molt 4 cells were grown in RPMI 1640 (Cellgro Media Tech, Herndon, VA) at pH 7.4 supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA). OPM-I cells were cultured in RPMI 1640 at pH 7.4 supplemented with 10% defined FBS (HyClone, Logan, UT); HL-60, K-562 and Mo cells were grown in Iscove's modification of DMEM (Cellgro Media Tech, Herndon, VA) at pH 7.4, supplemented with 10% FBS. Cells were cultured at 37ºC in a humidified atmosphere of 95% air / 5% $CO_2$ and subcultured regularly to ensure logarithmic growth. In preliminary experiments, the concentrations of forskolin (Fsk) and rapamycin that provided optimal effect with minimal toxicity were determined. RPMI 8226 and Ramos cells were treated as appropriate for each experiment with vehicle (ethanol/DMSO/and/or sterile HPLC-grade water < 0.1% final concentration), 1 μM Dex, 1 μg/ml SP600125, 1 μg/ml U0126, 10 μM Fsk, 1 μM cell permeable JNK inhibitory peptide (ip) (Calciochem, San Diego, CA), 15 nM rapamycin, or various combinations of each. Molt 4 cells were treated identically except using 5 nM rapamycin. IM-9 cells were treated the same as RPMI 8226 except for substituting 3 μg/ml U0126 and 100 nM rapamycin. OPM I cells were treated as RPMI 8226 except 5 μM Fsk and 5 nM rapamycin. All reagents were from Sigma-Aldrich (St. Louis, MO), Burdick and Jackson (Muskegon, MI), or Calbiochem.

**Viable cell determination**

Cell culture density ranged from $1x10^5$ to $1x10^6$ viable cells/ml. Cell viability was determined by Trypan blue vital dye exclusion (Sigma-Aldrich) using automated cell counting (Vi-Cell, Beckman Coulter, Miami, FL). Doubling times, in hours, were generated using GENTIME: OPM-I 32.8, HL-60 30.0, Mo 29.6, Ramos 24.5, CEM-C7-14 22.0, Molt-4 19.5, RPMI 8226 16.5, IM-9 14.5, and K-562 12.0. For flow cytometry, cells were collected by centrifugation and stained with propidium iodide (PI) as described (Miller et al., 2007). At least 25,000 cells per assay were analyzed.

**Detection of active caspase 3**

To evaluate caspase involvement when cell death was observed, cells were treated with the pan-caspase inhibitor Z-VAD (15μM) (R&D Systems, Inc., Minneapolis, MN) for 2 hours, followed by the above mentioned concentrations of Fsk, rapamycin and Sp600125 + U0126 with and without Dex. APO ACTIVE 3 antibody-FITC detection kit was used per manufacturer's instructions (Cell Technology, Mountain View, CA.). Approximately 20,000 cells were analyzed using single color flow cytometry at 488 nm.

**Immunochemical analysis**

Cells in mid-logarithmic growth ($3x10^5$ cells/ml) were pretreated for varying times with Dex +/- appropriate pharmacological agents, after which whole cell lysates were immunoblotted. Antibodies used: phospho-specific GR at S211 (Cell Signaling Technology, Danvers, MA); anti-GR (Affinity Bioreagents, Golden, CO); β-actin (Santa Cruz Biotechnology, Santa Cruz, CA); and Bim (Sigma-Aldrich).

MAPK Immunoblots were performed with antibodies specific for: phospho-(Threonine$^{202}$ and Tyrosine$^{204}$) – ERK; phospho (Threonine$^{183}$ and Tyrosine$^{182}$) – p38

(Cell Signaling Technology); or phosphorylation state independent ERK (Calbiochem), or JNK (Cell Signaling Technology), or p38 MAPK (Calbiochem).

**MAPK proportion calculation**

Preliminary experiments in each cell line established the linear range for detection of immunochemical reaction for ERK, JNK, and p38 (data not shown). Working within this range, total and phosphorylated ERK, JNK, and p38 were estimated quantitatively by image analysis. In 3 independent experiments, none of the MAPK's showed variation in the basal state or after Dex treatment. Therefore, the amount of each immunochemically detected MAPK could be expressed in terms of total protein extracted (adjusted to X/ml in every experiment). The relative phosphorylated forms of each MAPK, estimated immunochemically, could be calculated:

$$\frac{\text{Phospho MAPK X}}{\text{Phospho-(ERK+JNK+p38)} \div \text{(total µg protein)}} = \frac{\text{Fraction of X}}{\text{total phospho MAPK}}$$

For more details on this analysis refer to Miller et al., 2007.

# Chapter 3: Site-specific Phosphorylation of an Intrinsically Disordered Activation Domain of the glucocorticoid receptor Leads to Structural and Functional Consequences


## ABSTRACT

Numerous proteins or protein regions/domains exist in an intrinsically disordered (ID) conformation(s), and these ID regions are disproportionately higher in cell signaling proteins and transcription factors, suggesting an important role in their regulatory capacity (Dyson and Wright, 2005). The large flexible regions in this class of proteins have an advantage over fully folded proteins that can allow them to make more efficient physical functional interactions with their target partners, which represents a mechanism for regulation of cellular processes (Dyson and Wright, 2005; Sugase et al., 2007). Published results from a variety of systems have shown that ID domains of the transcription factors are platforms on which large multi-protein complexes are built (Dyson and Wright, 2005). Recently it has been suggested that signaling via phosphorylation-regulated protein: protein interaction often involves ID regions (Sugase et al., 2007). The prediction of protein phosphorylation sites has shown that IDs have a much higher frequency of known phosphorylation sites than ordered regions, suggesting a strong preference for locating phosphorylation sites in the ID regions (Iakoucheva et al., 2004). Site-specific Phosphorylation represents an important regulatory mechanism in the activities of signaling proteins (Zor et al., 2002; Iakoucheva et al., 2004). Steroid receptors that belong to the super-family of intra-cellular transcription factors possess many of the functionally important known phosphorylation sites within the ID activation domain AF1 (Ismaili and Garabedian, 2004 ; Lavery and McEwan, 2005). In this study for the first time, we have shown that AF1, an ID activation domain of the glucocorticoid receptor (GR) adopts a functionally folded conformation due to site-specific phosphorylation of S211 by p38 MAPK that we have earlier shown to be involved in the apoptotic and gene-inductive events initiated by GR. Using biophysical and proteomic approaches, we have shown that site-specific phosphorylation induces secondary/tertiary

structure in the GR AF1 ID region that specifically facilitates its interaction with critical coregulatory proteins and subsequently its transcriptional activity. These data provide a mechanism through which ID activation domain of the steroid receptors and related transcription factors may adopt a functionally active conformation under physiological conditions.

## INTRODUCTION

Protein phosphorylation is generally an important phenomenon in regulation of protein function in eukaryotic cells, and is often concerned with switching of a cellular activity from one state to another (Marks and F., 1996). For transcription factors (TFs), three main mechanisms of regulation by phosphorylation can be identified; i) the DNA binding affinity of TFs can be modulated negatively or positively; ii) the interaction of transactivation domains of TFs with components of the transcription initiation complex can be regulated; and iii) the shuttling of TFs between the cytoplasmic compartments can be influenced (Rechsteiner and Rogers, 1996; Hay and Meek, 2000; Zor et al., 2002). Like many other TFs, the glucocorticoid receptor (GR) is a phosphoprotein, and it has been suggested that phosphorylation plays an important role in the regulation of GR activity (Munck and Náray-Fejes-Tóth, 1994; Wang and Garabedian, 2003; Wang et al., 2007). GR is phosphorylated in the absence of glucocorticoid, with enhanced phosphorylation occurring when it is bound to agonist but not antagonist steroids (Bodwell et al., 1993; Kumar and Thompson, 2005). There are also reports suggesting that phosphorylation may affect GR stability and thus alter receptor activity (Bodwell et al., 1993; Webster et al., 1997). All seven phosphorylation sites identified in mouse GR are found in the N-terminal domain (NTD), in or near the AF1 domain (Dalman et al., 1988). Except for one threonine, all of these sites are serine residues (Dalman et al., 1988; Blind and Garabedian, 2008). All the GR phosphorylation sites that are conserved among human, mouse, and rat are located within the AF1 domain (Mason and Housley, 1993; Wang et al., 2002). In the human GR AF1, major functionally important known phosphorylated residues are S203, S211 and S226 (Figure 1a). At least two of these

(S211 and S226) are thought to be important for transcriptional activity of the GR (Mason and Housley, 1993; Wang et al., 2003; Miller et al., 2005; Blind and Garabedian, 2008). Our recent studies show that GR S211 is a substrate for p38 MAPK, and that a mutation of Ser211 to Ala residue reduced GR-mediated transcriptional activation and apoptosis in a human leukemia cell line, suggesting a role for p38 MAPK signaling in glucocorticoid-induced apoptosis of lymphoid cells (Miller et al., 2005). With respect to the consequences of p38 MAPK phosphorylation, it has further been shown that S211 residue of AF1 is a specific substrate site for p38 MAPK, and that mutation S211A, which prevents phosphorylation at this position, diminishes apoptosis driven by the constitutively active GR lacking the ligand binding domain, suggesting a possible role of AF1-mediated GR transcriptional activity (Miller et al., 2005; Kumar et al., 2007; Miller et al., 2007). Recently, Garabedian and co-workers have also demonstrated that site-specific phosphorylation in GR, particularly S211 and S226 play an important role in gene regulation by the GR, and AF1 appears to be a main player in this process (Blind and Garabedian, 2008).

However, it is not yet known exactly how phosphorylation influences the structure and functions of the GR AF1. Though the importance of the AF1 domain as a major activation region was established long ago (Godowski et al., 1987; Dieken and Miesfeld, 1992), we are only beginning to understand its structure/function relationship. To understand how the GR transmits the transcriptional signal from ligand to specific gene(s), it is essential to gain this information. However, the structure of AF1 has been difficult to determine because in solution it seems to exist as a random ensemble of conformers (Kumar et al., 2001; Lavery and McEwan, 2005). The GR AF1 appears to be an example of an intrinsically disordered (ID) domain, frequently found in TFs (Kumar et al., 2001; Minezaki et al., 2006). It is believed that ID sequences usually achieve structure to carry out their functions. These disordered protein regions/domains promote molecular recognition primarily by creating propensity to form large interaction surfaces suitable for interactions with their specific binding partners (Härd and Kearns, 1990; Baldwin and Rose, 1999; Baskakov et al., 1999; Kumar et al., 2001). It is generally accepted that the structural uniqueness of most proteins determines their biological function. This raises the question: what is the structural basis of the functional activity of

such ID proteins/domains? Recent studies have suggested that signaling via phosphorylation-regulated protein: protein interaction often involves ID regions, and ID regions have a much higher frequency of known phosphorylation sites than ordered regions, suggesting a strong preference for locating phosphorylation sites in the ID regions (Zenke et al., 1999; King et al., 2000; Iakoucheva et al., 2004).

Site-specific phosphorylation represents an important regulatory mechanism in the activities of signaling proteins including steroid receptors (Rogatsky et al., 1998b; DeFranco et al., 1991; Wang et al., 2002). Since all the known phosphorylation sites in the GR are located in the ID AF1/NTD, we hypothesize that site-specific phosphorylation of GR AF1 leads to changes in its conformations that are important for AF1's interaction with other critical coregulatory proteins, and subsequent transcriptional activity. We report that GR's ID AF1 domain adopts a functionally folded conformation due to site-specific (S211) phosphorylation by p38 MAPK that we have earlier shown to be involved in the apoptotic and gene-inductive events initiated by GR (Miller et al., 2005).

## RESULTS

### *Site-specific phosphorylation induces secondary/tertiary structure in otherwise ID GR AF1 domain.*

Similar to other steroid receptors, the GR contains several major functional domains. These are shown diagrammatically for the human GR in Fig. 1a. The AF1 transactivation region, amino acids 77-262 is highlighted, with vertical lines above the bar indicating the location of the two Tyr and one Trp residues, and three potential phosphorylation sites within AF1. We have earlier shown that AF1 can be phosphorylated at Ser residue by p38 MAPK (Miller et al., 2005). Previously published data indicated the importance of the S211 site on the functional outcome of the GR mediated pathways (Miller et al., 2007). Therefore we generated S211 to A and S211 to E mutant AF1 recombinant protein to compare structural and thereof resulting functional changes due to phosphorylation. Recombinant wild-type and two AF1 mutants S211A or

S211E were expressed in a bacterial system and purified to homogeneity (Fig. 1b). To test the effects of phosphorylation on the conformational changes in AF1, first we establishing conditions that can phosphorylate AF1 in vitro. Using GR S211-phospho site-specific antibody (Fig. 1c) and MALDI mass spectroscopy, we confirmed p38 MAPK-mediated *in vitro* phosphorylation of AF1 (Fig. 1d).

We recorded far-UV circular dichroism (CD) spectra of unphosphorylated and phosphorylated AF1. As expected, unphosphorylated AF1 shows characteristics of an ID protein whereas phosphorylated AF1 adopts significantly higher secondary structural elements in it with helical content increased by ~50% at the expense of random coil compared with unphosphorylated AF1 (Fig. 2a). Under similar conditions, mutation of S211A does not show any significant structural changes in AF1 (Fig. 2b), suggesting that p38 MAPK-mediated phosphorylation of S211 is important for inducing a compact structure in otherwise ID AF1 domain. We also compared the far-UV CD spectra of unphosphorylated AF1, AF1-S211A and AF1-S211E to determine whether addition of negative charge by way of substituting Ser to Glu results in similar effects as seen due to phosphorylation of S211. Our results indicate that S211A mutant does not alter AF1 conformation whereas S211E mutation showed only a modest increase in helical content compared with wild-type AF1 (Fig. 2c), suggesting that site-specific phosphorylation of ID AF1 leads to induction of secondary structural elements in it and negative charge per se is not sufficient to mimic these effects.

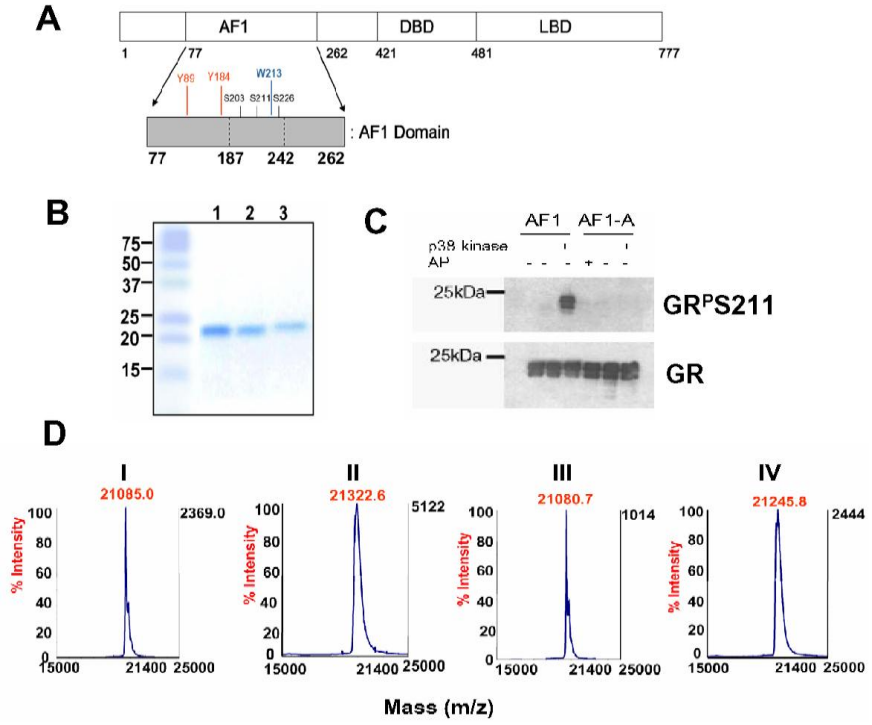



Figure 1: p38 phosphorylates conserved Ser residues in GR's AF1.

A, Topological diagram of the GR with expended AF1 domain containing conserved phosphorylation sites. *B*, Coomassie stained SDS-PAGE gel of purified recombinant AF1 (= lane 1), AF1-S211A (= lane 2) and AF1-S211E (= lane 3). *C, In vitro* phosphorylation of AF1 and AF1-S211A using p38 MAPK. AF1 or AF1-S211A and active p38 MAPK were incubated for 2h at 30° C, followed SDS-PAGE and by immunoblot analysis with specific antibodies against GR-P S211 and GR. Recombinant proteins were also incubated with calf alkaline phosphatase (AP) and assayed like above to account for non-specific antibody interactions. AF1 but not AF1-S211A is phosphorylated at site 211. *D,* Recombinant AF1 and AF1-S211A were *in vitro* phosphorylated as above and phosphorylation was confirmed with MALDI mass spectroscopy. **I**) AF1 without p38 MAPK; **II)** AF1 phosphorylated with p38 MAPK; **III)**

AF1-S211A without p38 MAPK; **IV)** AF1-S211A with p38 MAPK. The difference in mass between I and II and between III and IV is equivalent to three and two phospho groups respectively.

We also recorded fluorescence emission spectra of AF1 (Fig. 3) after excitation at 278 or 295nm, which reflect the changes coming from Tyr (Y89, Y184) and Trp (W213) residues or from of the single Trp (W213), respectively. In both sets of spectra, the quantum yield of the fluorescence is significantly increased in the phosphorylated AF1 compared with unphosphorylated AF1, and corresponding blue shifts in the emission maxima (from 351 nm to 346 nm and from 355 nm to 341 nm at 278 nm and 295 nm excitation wavelengths respectively; Fig. 3a). Under similar conditions, AF1-S211A failed to show any significant changes both in terms of fluorescence intensity and shift in wavelength maxima (Fig. 3b). A comparison of spectra from AF1 and S211E showed only moderate changes in case of S211E (Fig. 3c). These fluorescence changes due to site-specific phosphorylation of AF1 are typical of those accompanying the removal of aromatic residues from polar, aqueous solution into a more hydrophobic environment within the protein. Because three amino acids excited are located well apart in AF1 (Fig. 1a), the conformational changes reflected in the fluorescence emission changes should be happening throughout the AF1 peptide. We further carried out partial proteolytic digestion experiments using three different proteases (Trypsin, Chymotrypsin, and Endo Gluc-C) to determine tertiary structural changes in AF1 with or without p38 MAPK-mediated phosphorylation. Partial proteolysis data indicate protection of peptides in phosphorylated AF1 (indicated by an arrow), suggesting that site-specific phosphorylation of AF1 results in a compact structure formation in it (Fig. 4). Taken together, these results demonstrate formation of secondary/tertiary structure in the ID AF1 domain due to site-specific phosphorylation. Thus, our data clearly show that site-specific (S211) phosphorylation of the GR AF1 results in the formation of an ordered conformation in AF1, which is otherwise an ID protein.

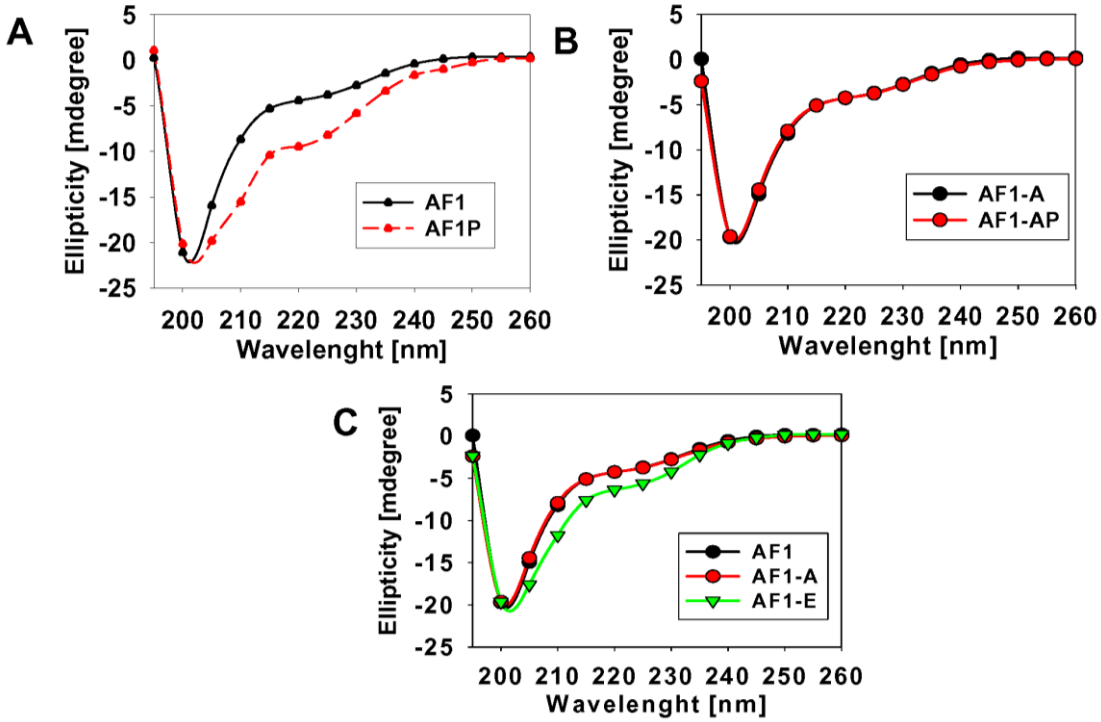



Figure 2: Far-UV CD spectra showing increased secondary structure in phosphorylated AF1 domain.

A, Far- UV spectra of AF1 unphosphorylated (black) and phosphorylated (red). B, Far- UV spectra of AF1-S211A untreated (black) and treated (red) with p38 MAPK. C, Far- UV spectra of AF1 (black), AF1-S211A (red) and AF1-S211E (green). Each spectrum is representative of at least three independent experiments, corrected for the contribution of buffer and smoothed.

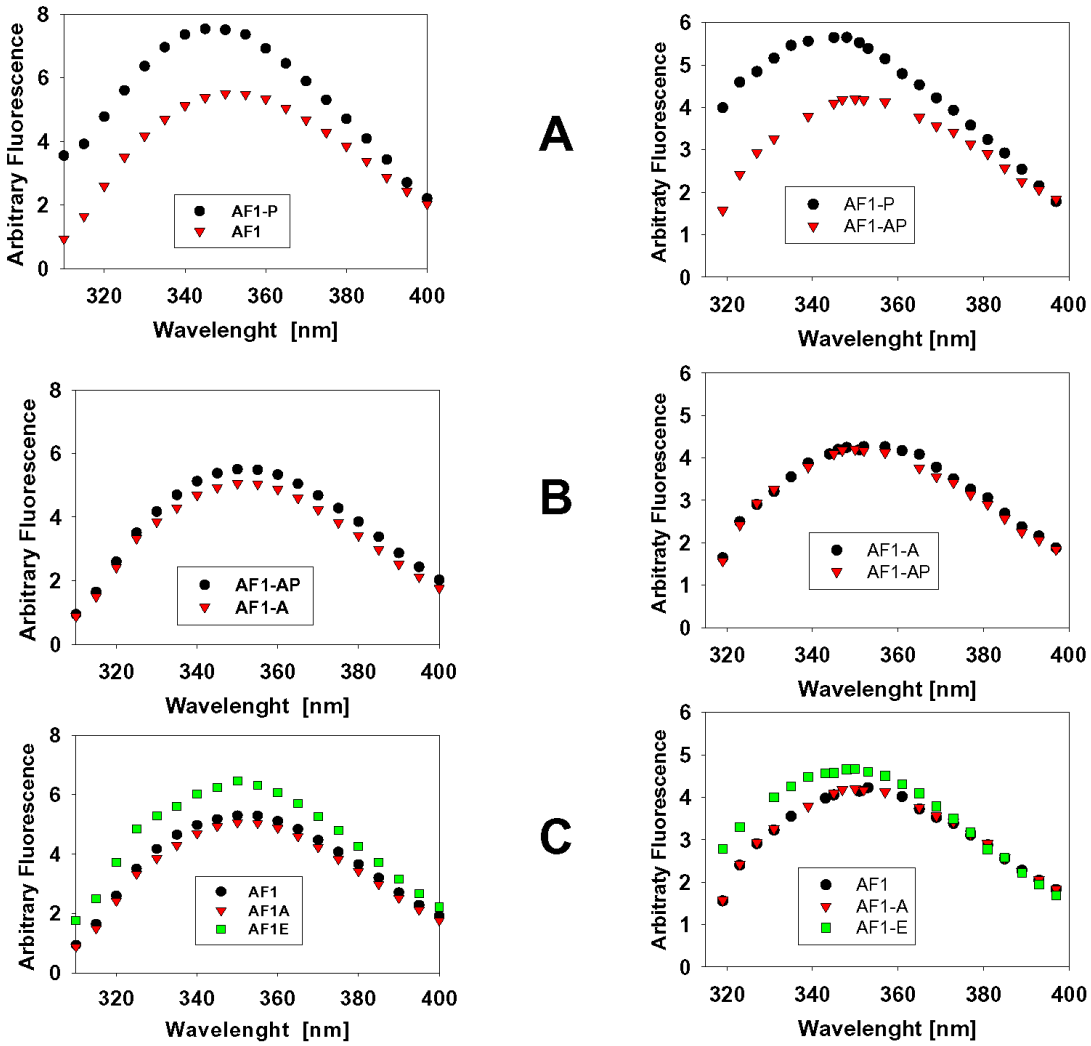



Figure 3: Fluorescence emission spectra shows perturbation of Tyr and Trp residues in the phosphorylated AF1.

A, Fluorescence emission spectra of unphosphorylated (red) and phosphorylated AF1 (black). B, Fluorescence emission spectra of AF1 untreated (black) and treated (red) with p38 MAPK. C, Fluorescence emission spectra of AF1 (black), AF1-S211A (red) and AF1-S211E (green). Left panel shows spectra recorded at an excitation wavelength of 278 nm (Tyr & Trp) and right panel at 295 nm (Trp). Each spectrum is an average of three spectra recorded and corrected for the contribution of buffer.

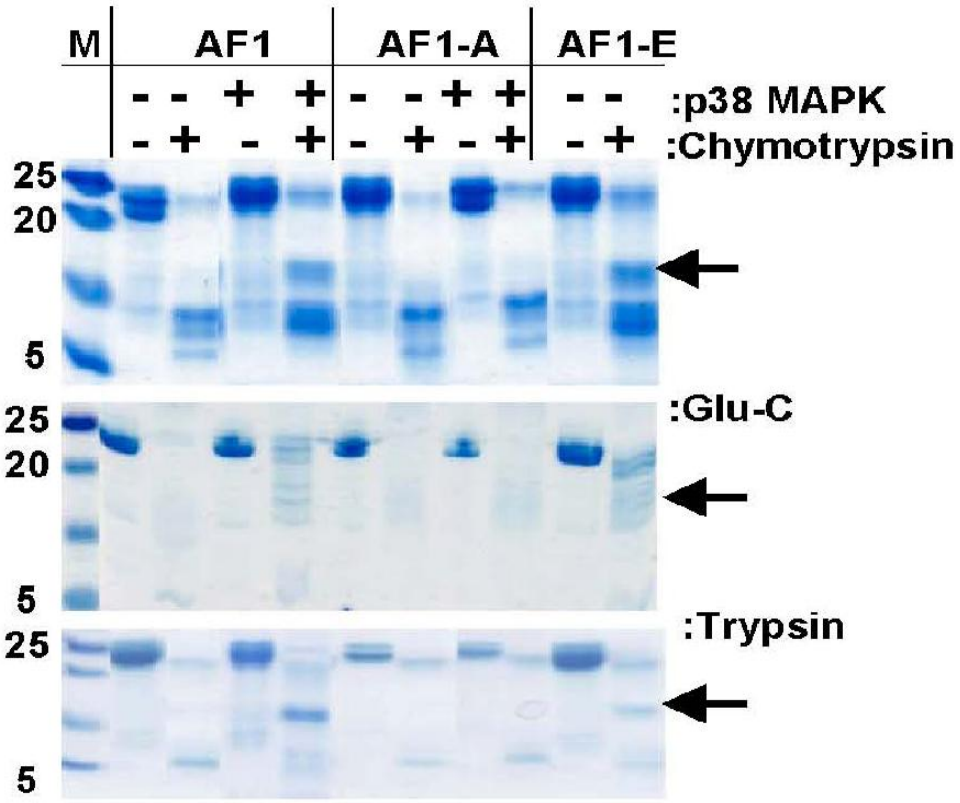



Figure 4: Site-specific phosphorylation of the GR AF1 protects it from partial proteolysis.

Coomassie-stained SDS-PAGE gel showing products of partial proteolysis of purified AF1, AF1-S211A (AF1-A), and AF1-S211E (AF1-E) proteins. Experiments were carried out using sequencing grade chymotrypsin (upper panel), Endoproteinase Glu-C (middle panel), and trypsin (bottom panel) at 4°C for a protein: enzyme mass ratio of 100:1. Black arrow shows a major protected band in each case. Experiments were repeated at least 5 times with similar results.

***Phosphorylation-induced structure formation in the GR AF1 facilitates its interaction with specific coregulatory proteins.***

Conformational changes upon phosphorylation often affect protein function. We have shown that conditional folding of the ID AF1 is a pre-requisite for its interactions with specific co-regulatory proteins (Kumar et al., 2004a; Kumar et al., 2004b; Kumar et al., 2004c). It is presumed that AF1 makes physical interactions with other factors in order to transactivate gene(s) and that conditional folding is important for these interactions (Kumar et al., 2007). It has recently been hypothesized that one of the reasons why ID proteins particularly TFs are rich in phosphorylation sites is that kinase-mediated phosphorylation can induce conformation(s) in ID region, which can facilitate its interaction with other binding partners. We therefore evaluated whether the conformation induced in ID AF1 domain by site-specific phosphorylation is important for specific protein-protein interactions. Using immunoprecipitation assay, we tested these AF1 interactions with specific co-regulatory proteins (TATA box binding protein: TBP; CREB binding protein: CBP; and Steroid receptor coactivator-1: SRC-1) from HeLa nuclear extracts. Separate HeLa nuclear extracts supplemented with purified AF1/AF1-S211A protein with or without p38 MAPK pretreatment were made. The extracts were then incubated with antibody-linked beads specific to each of the partner proteins. The antibody-linked beads were recovered, washed extensively, and the bound proteins were released and resolved by SDS–PAGE. An antiserum to amino acids 150–175 of the GR was then used to identify AF1 on the gels. Consistent with previous reports (Kumar et al., 2007), in case of unphosphorylated AF1, we detected a very weak interaction with each of these coregulatory proteins. These interactions were increased significantly when AF1 was phosphorylated, suggesting that phosphorylation of AF1 facilitates its interaction with all the three coregulatory proteins tested (Fig. 5). Mutation of S211A does not show any significant differences in AF1's interaction with these coregulatory proteins under similar conditions (Fig. 5).

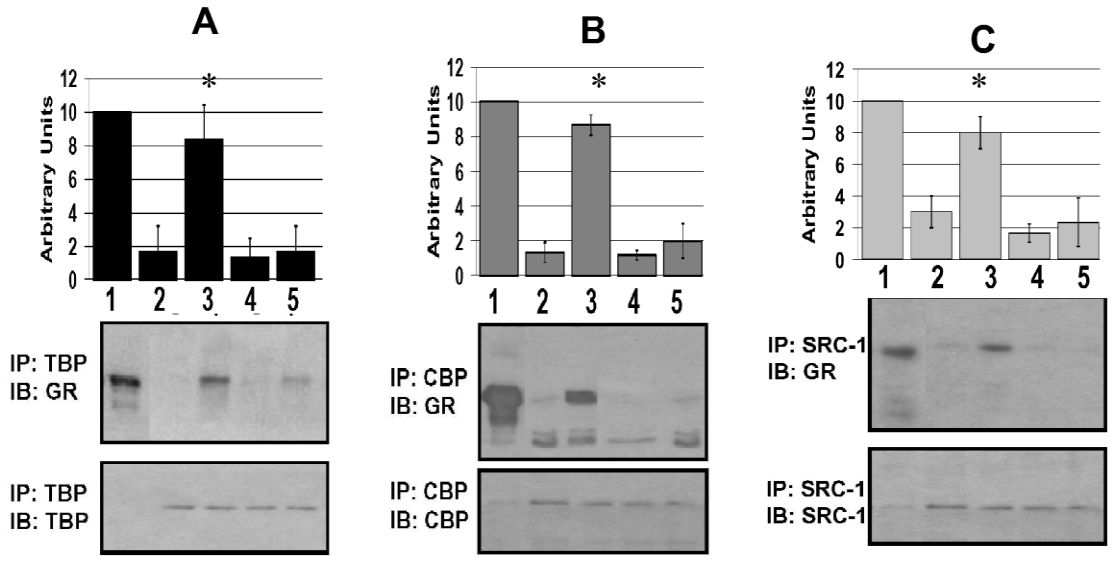



Figure 5: Phosphorylation-induced conformational changes significantly enhance interactions of AF1 with critical coregulatory proteins from HeLa nuclear extracts.

Immunoprecipitation (IP) with indicated antibodies was carried out using HeLa cell nuclear extracts with AF1. Shown are representative immunoblots (IB) of at least three independent experiments and graphs showing densitometric analysis of three experiments. Results are expressed as means ± SE; p values based on two-tailed students t-test using Excel (*) signifies $p < 0.05$. Lanes 1**,** control AF1; 2, unphosphorylated AF1; 3, phosphorylated AF1; 4, unphosphorylated AF1-S211A; 5, phosphorylated AF1-S211A. (A**)** TBP; (B) CBP; (C) SRC-1.

We further determined whether these interactions are taking place within a cellular environment. We applied the fluorescence resonance energy transfer (FRET) method. Plasmids expressing cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) were obtained, and from that we generated constructs that express the fluorophores linked to GR500 (CFP-GR500), (CFP-GR500-S211A), or (CFP-GR500-S211E), and TBP (YFP-TBP), CBP (YFP-CBP), or SRC-1 (YFP-SRC-1). The GR500 construct is constitutively active as a transcription factor while avoiding the possibility of any contribution from AF2. The constructs were co-transfected into GR-deficient CV-1 cells with co-transfection of a promoter-reporter construct containing GRE sites [GRE-SEAP (secreted alkaline phosphatase)]. Several control experiments were included. Independent CFP and YFP-expressing constructs were co-transfected with GRE-SEAP and tested for FRET as negative control. As a positive control, a CFP-YFP construct that linked CFP-YFP by eight amino acids was co-expressed with GRE-SEAP. Fig. 6a shows examples of results from such controls. Our results show that AF1 interacts directly with TBP (Fig. 6b), CBP (Fig. 6c), and SRC-1 (Fig. 3d) in the nuclei of GR-deficient CV-1 cells co-transfected with GR500 ± each coregulator. Interaction of the AF1-S211A mutant with each binding partner is greatly diminished, whereas the S211E mutant indicates only a modest interaction (Fig. 6b-d). These results suggest that phosphorylation-induced conformational changes in ID AF1 allow its protein surfaces to facilitate AF1's interactions with specific coregulatory proteins, an essential requirement for activation domains of TFs to regulate transcriptional activity of the target gene (Lavery and McEwan, 2005; McEwan et al., 1996).

Figure 6

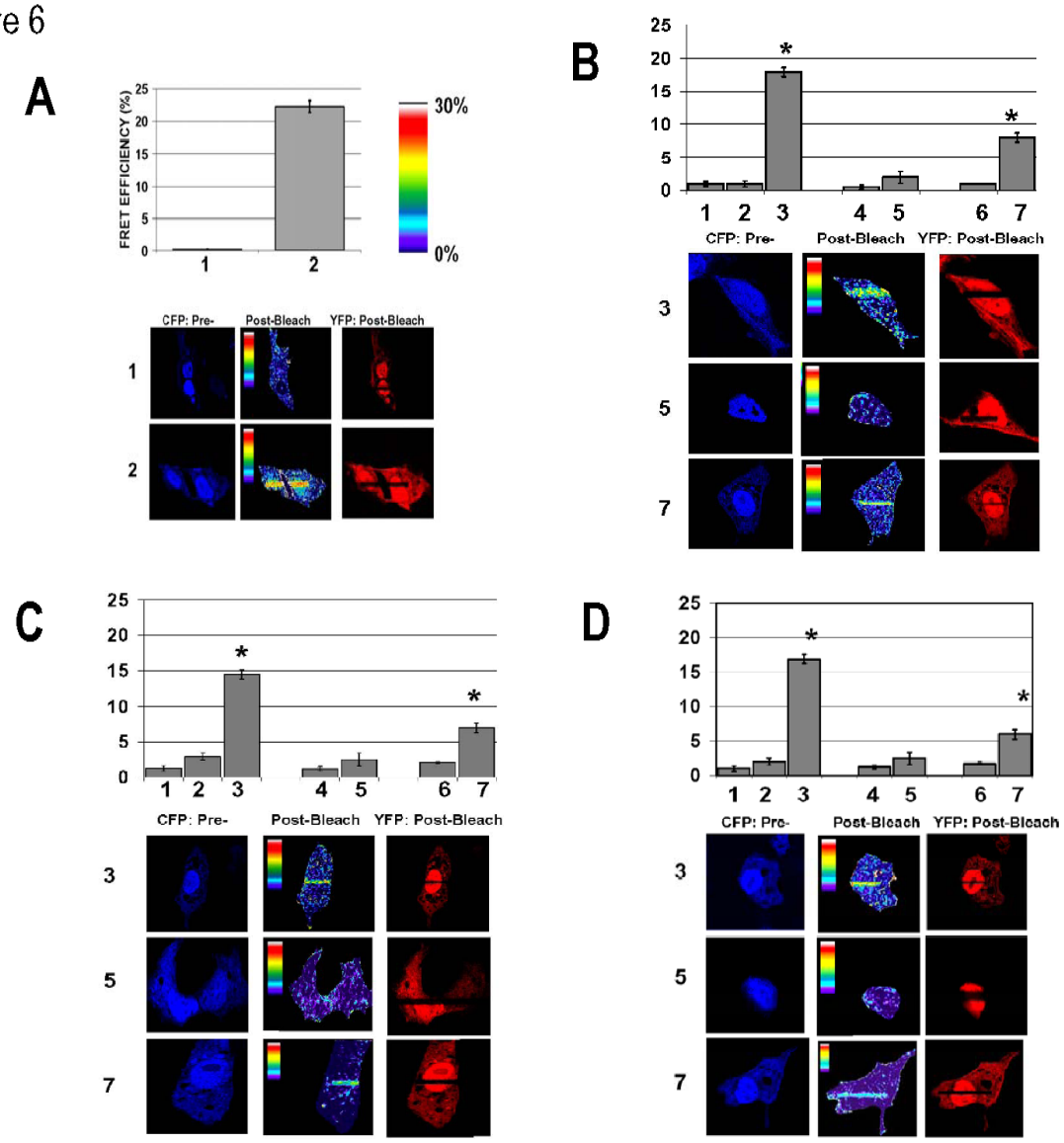



Figure 6: Protein:protein interactions involving the GR AF1 and TBP, CBP, or SRC-1 measured by FRET analyses.

The abscissa numbers correspond to the numbered conditions. Below representative image same-cell images in the donor (CFP) channel before and after PB; and YFP channel image is shown post bleach to demonstrate bleach

efficiency. A, Controls receiving fluorescent proteins without TBP or GR500 to establish basal (no interaction) and maximal FRET. Graph Lanes 1: CFP-empty + YFP- empty negative control. 2: CFP-YFP fusion constructs positive control. B, 1: CFP-empty + TBP-YFP; 2: GR500-CFP+ YFP-empty; 3:GR500-CFP+ TBP-YFP; 4: GR500-S211A+ YFP-empty; 5: GR500-S211A+ TBP-YFP ; 6: GR500-S211E+ YFP-empty; 7: GR500-S211E+ TBP-YFP. C, 1: CFP-empty + CBP-YFP; 2: GR500-CFP+ YFP-empty; 3:GR500-CFP+ CBP-YFP; 4: GR500-S211A+ YFP-empty; 5: GR500-S211A+ CBP-YFP ; 6: GR500-S211E+ YFP-empty; 7: GR500-S211E+ CBP-YFP. D, 1: CFP-empty + SRC-1-YFP; 2: GR500-CFP+ YFP-empty; 3:GR500-CFP+ SRC-1-YFP; 4: GR500-S211A+ YFP-empty; 5: GR500-S211A+ SRC-1-YFP ; 6: GR500-S211E+ YFP-empty; 7: GR500-S211E+ SRC-1-YFP. Experiments were carried out three independent times and were analyzed and calculated average FRET efficiencies ±SE of 15 cells were graphed for each of the conditions. Results are expressed as means ± SE; p values based on two-tailed students t-test using Excel (*) signifies $p< 0.05$.

***Effects of phosphorylation-induced interactions of TBP, SRC-1 or CBP on AF1-driven transcription.***

We tested the effects of phosphorylation-induced folding/binding events on AF1-driven transcription using GR-responsive promoters, in transient transfection-based reporter assays in GR-deficient CV-1 cells. The promoter-reporter plasmid (GRE-SEAP) contains three GREs upstream from a TATA-box and a reporter gene that encodes alkaline phosphatase secreted into the medium. To test the effects of these coregulators on transcription driven by human GR AF1, we co-transfected CV-1 cells with a GRE-dependent reporter gene, and constant amount of GR500 expression vector alone or with added vectors expressing TBP, SRC-1 or CBP. Lacking the LBD, GR500 is transcriptionally active without steroid and can induce genes and/or apoptosis in cells to nearly the same extent as steroid-bound holo-GR. GR500 alone significantly increased reporter activity compared to empty CFP vector alone (Fig. 7; upper panel), and input of the plasmids expressing TBP, SRC-1 or CBP gene enhanced the GR500 induction of the GRE–SEAP reporter several fold (Fig. 7; upper panel). These reporter activities were significantly reduced when the GR500-S211A mutant was used, whereas GR500-S211E mutant showed only a modest activity compared with GR500 (Fig. 7; upper panel). The levels of expression of GR500 was assessed using two different antibodies (GR and GFP), and that of each coregulatory protein using respective antibody. The results show expected levels of expression of each protein. One such experiment is shown here (Fig. 7; lower panel). These results strongly suggest that the enhancement of GR-induced transcription by TBP, SRC-1 or CBP is achieved through the AF1 region, and that p38-mediated site-specific phosphorylation plays an important role in it by inducing a compact structure in the otherwise ID AF1 region, confirming that phosphorylation-induced structure formation in ID regions aid in facilitating protein: protein interactions, and subsequently GRE-mediated AF1 transcriptional activity.

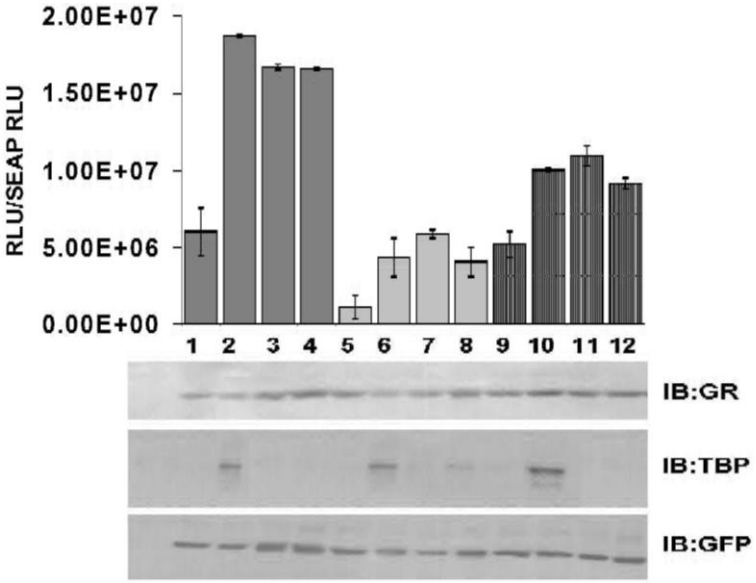



Figure 7: Phosphorylation-dependent cofactor-binding increases AF1-mediated transcriptional activity of a promoter containing 3xGRE.

*A*, CV-1 cells constitutively expressing AF1 in a two domain GR fragment containing entire N-terminal and DNA-binding domains (GR500), or GR500S211A, or GR500S211E were cotransfected with DNA of the pGRE-SEAP plasmid alone or plus DNA for TBP, SRC-1 or CBP. Lanes 1: GR500-GFP; 2: GR500-GFP + TBP; 3: GR500-GFP + CBP; 4: GR500-GFP+ SRC-1; 5: GR500S211A-GFP; 6: GR500S211A-GFP+TBP; 7:GR500S211A-GFP+ CBP; 8: GR500S211A-GFP+ SRC-1; 9: GR500S211E-GFP; 10: GR500S211E-GFP+ TBP; 11: GR500S211E-GFP+ CBP; 12: GR500S211E-GFP+ SRC-1. Results are expressed as means ±SE. Experiments were repeated five times. Levels of significance were evaluated by a two-tailed paired Student's t test and $P<0.05$ was considered significant. Graphs were normalized to transfection efficiency of each construct assayed by immunoblot with specific antibodies GR, TBP, CBP, SRC-1 and GFP of which three are shown.

## DISCUSSION

Compared to prokaryotes, eukaryotic genomes contain a much higher number of ID proteins, indicating a greater need for signaling and regulation in nucleated cells (Dunker et al., 2002; Iakoucheva et al., 2002). ID regions commonly exist within TFs and are often located in their transactivation domain (Minezaki et al., 2006). The mechanisms by which TFs control gene expression poses a central problem in molecular biology and the role of their activation domains in this complex is of immense importance. TFs remodel chromatin structure in an extremely dynamic situation such that they have the capacity to rapidly form and reform multi-protein complexes involving coactivators/corepressors and/or proteins from the fundamental initiation complex (Liu et al., 2006). In this context the role of ID domain(s) with flexible conformations becomes extremely important by providing platforms for inclusion/exclusion of specific protein complexes; thus ultimate composition of the complex assembly may dictate final outcome responsible for specific gene regulation (Liu et al., 2006). Therefore, the notion that ID domains of TFs must have significantly ordered conformation in their normal cellular milieu pose a paradox that must be solved before we can fully understand their role in gene regulation (Iakoucheva et al., 2004). It has been predicted that IDs have a much higher frequency of known phosphorylation sites than ordered regions, and that signaling via phosphorylation-regulated protein: protein interactions often involves ID regions (Kumar and Thompson, 2005; Parker et al., 1998; Vetter and Leclerc, 2001; Zor et al., 2002).

In recent years it has become clear that the cross-talk exists between GR signaling and other receptor cascades including inflammatory kinases (MAPKs, ERK, p38 and JNK) as well as the cAMP-driven PKA pathways (Bruna et al., 2003; Kyriakis, 2000; Medh et al., 1998; Rogatsky et al., 1998a; Rogatsky et al., 1998b; Wada and Penninger, 2004). There is evidence that that differential phosphorylation is a potential regulator of species-specific actions of the GR (Bodwell et al., 1998; Dalman et al., 1988; Kurl and Jacob, 1984; Wang et al., 2002). Miller et al. showed that p38 MAPK is a component of the glucocorticoid-evoked pathway to lymphoid cell apoptosis in human and mouse cell systems and that the p38-specific upstream kinase kinase MKK3 appears to be involved.

Further, site-specific phosphorylation of the human GR AF1 by p38 MAPK was found to be involved in the apoptotic and gene-inductive events initiated by glucocorticoid (Miller et al., 2005). It is clear that GR-mediated glucocorticoid signaling is a multi-facetted process involving crosstalk with various regulatory kinase pathways (Medh et al., 1998; Miller et al., 2007). Thus, signaling cascades that induce phosphorylation of the GR and its coactivator proteins are critical factors in determining the physiological actions of the GR. However, the underlying mechanism that governs this important yet complex phenomenon is not well understood. Our results from this study provide a mechanism of how site-specific phosphorylation leads to GR-mediated signaling. Further, phosphorylation-induced functional conformation in AF1 may also shed light on why all the major known phosphorylation sites are located in the ID activation domain of the GR and several other steroid receptors. Based upon the available literature and our findings from this study, we propose that p38 MAPK-mediated site-specific phosphorylation of the GR results in the recruitment of critical coregulatory proteins (by providing AF1 surfaces available for these interactions) that subsequently leads to transcription of target gene(s) (Fig. 8a).

Site-specific phosphorylation represents an important regulatory mechanism in the activities of signaling proteins including TFs, and regulates the binding affinity of TFs to their coactivators and DNA thereby altering gene expression, cell growth and differentiation (Zor et al., 2002). It has been hypothesized that protein phosphorylation of Ser residue predominantly occurs within ID regions of signaling molecules rather than merely on surface residues. Our data clearly support this idea and provide a physical proof of it. Relatively few ID regions have been structurally characterized, yet a significant fraction of them contain phosphorylation sites (Dunker and Uversky, 2008; Iakoucheva et al., 2004). One of the main reasons for such propensity is to facilitate extensive formation of hydrogen binding between the backbones and/or side chains that can occur through disorder-order transition within the ID region. The formation of these hydrogen bonds would be difficult if the sites of phosphorylation were located within ordered regions.

ID activation domain of many TFs have been shown to function by recruiting the transcriptional apparatus to the promoter (Minezaki et al., 2006), and ID regions do

indeed fold into a more ordered helical structure under physiological conditions (Zor et al., 2002). The characterization of phosphorylation–induced structure formation in facilitating protein: protein interactions should be of particular importance in understanding the mechanism by which kinase(s) regulate the transcriptional regulation of the target genes in the nucleus. In sum, we propose that under physiological conditions, site-specific phosphorylation plays a crucial role in allowing the ID activation domain of the GR to adopt functionally active conformation(s) in vivo (Fig. 8b). The resulting structurally modified forms of ID region suit it for its varied interactions with other critical coregulatory proteins, and possibly additional modulations in its structure essential for gene regulation by the signaling molecules. These interactions give a set of functionally active folded structure to ID AF1 region and form the basis for the multi-protein assemblies involved in GR-mediated regulation of transcription. This information will lead to an understanding of the role of this important phenomenon in the transcription process and provide a biological mechanism, information essential to understanding how GR affects gene regulation. Since several protein kinase pathways are involved in cell signaling leading to diverse cellular responses through TFs, phosphorylation-induced conformational changes observed in this study should provide a potential mechanism thorough which ID activation domains of signaling molecules exert their effects in gene regulation.

Figure 8

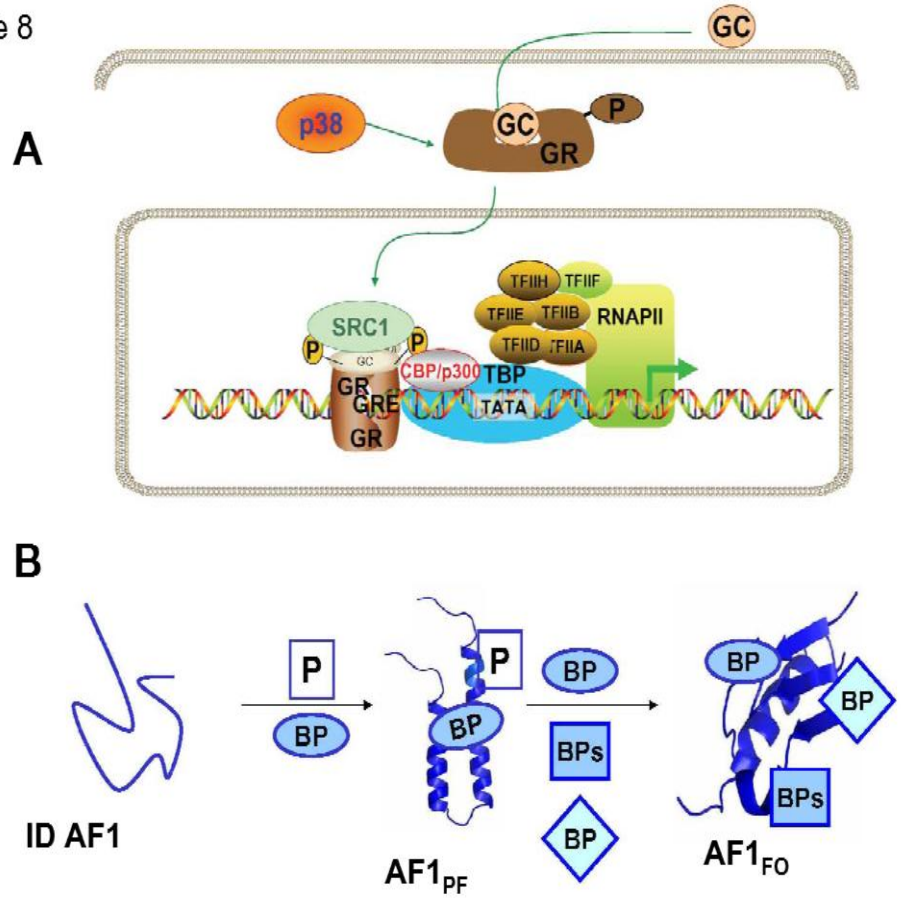



Figure 8: Models showing proposed effect of phosphorylation by p38 on GR mediated transcription (based on our data and available literature).

A, Working model of the GR undergoing phosphorylation at S211 mediated by p38 MAPK. The cofactor(s) bound may differ by particular cell types and by the state of the folded structures of the GR, particularly AF1. The complex alters local chromatin structure, e.g. by catalyzing histone acetylation or deacetylation, and affects the stabilization of the transcription pre-initiation complex including TBP, TAFs, Pol II among others. The receptor complex, bound to DNA enhancer sites, thus recruits and regulates Pol II via accumulations of specific proteins, which make a functional bridge

between the receptor and Pol II. The activity of kinases and phosphatases regulating signaling pathways also contribute to this process by altering the state of phosphorylation of both receptor and cofactors (not shown). The GR:coregulactor assembly may also interact directly with the basal transcription machinery at TBP/TATA box to regulate transcription. B, A hypothetical model of the role of site-specific phosphorylation on the structure and functions of the AF1 domain. The GR AF1 domain exists in an intrinsically disordered (ID) conformation i.e. as an ensemble of conformers that collectively appear to lack any significant secondary/tertiary structure. Phosphorylation of one or more sites within the AF1 alters its conformation in such a way that AF1 adopts a partially folded (PF) conformation that facilitates AF1's interaction with one or more of the coregulatory proteins (BP). This protein:protein interaction allows structurally modified forms of AF1 to suit for its varied interactions with other critical coregulatory proteins, and possibly additional modulations in receptor structure essential for gene regulation by the GR. This AF1;coregulators assembly provides optimal ordered conformation in the AF1 for subsequent transactivation activity. Alternatively, one or more of the coregulator proteins directly interact with one or more unphosphorylated AF1 conformers that may trigger phosphorylation of AF1. The resultant assembly of proteins may depend upon the cellular environment, specific promoters used, and the kinase pathway involved. All the available phosphorylation sites in the GR could be involved simultaneously or there may be a coordinated synergy between each site. In the full length GR, AF1: coregulators assembly may also be influenced by other factors such as DNA binding to its DBD, ligand binding and the cross talk between AF1 and AF2. In addition to the phosphorylation level of AF1, phosphorylation of different coregulators may also influence the outcome, and certain coregulators may be included or excluded from the assembly depending upon the interacting surfaces of the AF1 and/or coregulator proteins.

# Chapter 4: Converting cell lines representing hematological malignancies from glucocorticoid-resistant to glucocorticoid-sensitive: signaling pathway interactions


## ABSTRACT

Mitogen-activated protein kinases (MAPKs), cAMP-driven protein kinase A (PKA) and mTOR pathways modulate the apoptotic effects of glucocorticoids in human lymphoblastic leukemia CEM cells. We hypothesized that these signal transduction pathways act to oppose the apoptotic effect of glucocorticoids in cell lines representative of other hematological malignancies. Eight glucocorticoid-resistant cell lines representing various types of lymphoid and myeloid malignancies were chosen: RPMI 8226, IM-9, OPM I (myeloma), Ramos (Burkitt's lymphoma), Molt-4 (T-cell line acute leukemia), HL-60, K-562 (myeloid leukemia) and Mo (hairy cell leukemia). Cells were treated with U0126 and SP600125/or i.p. to inhibit the ERK and JNK MAPK pathways respectively, or Rapamycin to inhibit mTOR; or Forskolin to activate the cAMP-dependant PKA pathway. Then cells were challenged with the glucocorticoid, dexamethasone (Dex). Immunoblot analysis demonstrated that the basal phosphorylation levels of the anti-apoptotic MAPKs, JNK and ERK were greatly elevated in the Dex-resistant cell lines compared to a sensitive CEM cell clone. Cell viability after apoptosis determined by: (1) Trypan blue vital dye exclusion; (2) flow cytometry propidium iodide DNA staining; and (3) detection of caspase 3 activation. Dex sensitivity was restored in all of the resistant lymphoid-derived cell lines except Mo and the myeloid linage. The sensitized cells were characterized by a decrease in phosphorylated JNK and ERK, an increase in phosphorylated and total glucocorticoid receptor, and an induction of Bim protein levels. The pharmacologic targeting of the ERK, JNK, PKA or mTOR pathways may be a valuable therapeutic approach for restoring glucocorticoid sensitivity in certain lymphoid malignancies.

## INTRODUCTION

Many hematological malignancies are treated with glucocorticoids (GCs), and recent work has begun to reveal the network of genes leading to GC-dependent apoptosis. From such studies it has become clear that the action of GCs involves cross-talk between the glucocorticoid receptor (GR) and several cell signaling cascades (Bruna et al., 2003; Kyriakis, 2000; Miller et al., 2007; Miller et al., 2005; Rogatsky et al., 1998b; Wada and Penninger, 2004). Pathways involving the MAPKs ERK (Miller et al., 2007; Wada and Penninger, 2004), JNK (Miller et al., 2007; Wada and Penninger, 2004) and p38 (Miller et al., 2007; Miller et al., 2005) as well as cAMP-driven PKA (Medh et al., 1998; Miller et al., 2007) and mTOR (Miller et al., 2007; Strömberg et al., 2004) can influence GC-GR effects on cell growth and differentiation. Resistance to GC may therefore derive not from lack of GR but altered pathway interactions.

Previous studies from our laboratory had shown that ERK and JNK serve to inhibit the apoptotic effects of GC in CEM clones (Miller et al., 2007). CEM cells are CD4+/CD8+ early lymphoblasts in the T-lineage derived from a patient with acute lymphoblastic leukemia (ALL). Studies of these and other malignant lymphoid cells have shown a correlation between GC-driven induction of the GR and apoptotic outcome (9-14). We have shown in GC-sensitive CEM clones that the GR is phosphorylated at serine 211 ($GR^P$ S211) through p38 MAPK (and certainly additional protein kinases). This activating phosphorylation heightens GR-driven transcription and apoptosis (6,15). PKA and GC synergize to overcome resistance in human leukemic CEM-C1 cells, and this finding was correlated with *c-myc* suppression (Ji et al., 2007; Medh et al., 1998; Medh et al., 2001). In lymphoid T-cells, GR autoinduction also has been correlated with sensitivity to GC (Ramdas et al., 1999; Tonko et al., 2001). Gene array analysis of Dex-sensitive CEM cells destined for apoptosis 24 hours after GC-activated the GR showed a striking increase in mRNA of the pro-apoptotic Bcl2 family member Bim, just at the onset of the cell death process (Wang et al., 2003; Webb et al., 2003). We hypothesized

that the disregulation of signal transduction pathways is a general mechanism in GC-resistant hematological malignancies.

We tested predictions of our hypothesis: that ERK and JNK and mTOR activation act to oppose the apoptotic effect of GCs, while activation of PKA and p38 MAPK promotes it. The outcome and quantity of pro-apoptotic pathway adjustments, we reasoned, would converge on the induction and phosphorylation state of the GR. A final pro-apoptotic consequence should be the induction of Bim. Eight cell lines from various types of human hematological malignancies were selected. All contain functional GR; and all are resistant to GC-dependant apoptosis. RPMI 8226, IM-9, and OPM I are B-lymphoblastic myelomas derived from peripheral blood. Ramos represents Burkitt's lymphoma, a B-lymphocyte, as well as an EBV negative cell line. Molt-4, like CEM, is a T-cell lineage ALL. Mo (MoT) is a hairy cell (specialized T-cell) leukemia line. We chose two myeloid lineage cell lines: HL-60 (acute myelogenous leukemia, AML) and K-562 (chronic myelogenous leukemia, CML).

Our results are consistent with our hypothesis. Inhibition of ERK and JNK or of mTOR, and activation of PKA restored GC sensitivity to all but one of the resistant lymphoid malignancies. Increased total GR and/or $GR^P$ at S211 and increased levels of Bim were accompanied with restored sensitivity. Appropriate pharmacological modulations of the ERK, JNK, PKA and/or mTOR pathways in combination GC may offer potential for treatment of certain refractory hematological malignancies.

## RESULTS

### *Characterization of resistant cell lines*

All chosen cell lines had been reported, with varying degrees of detail, to be GC-resistant. We confirmed that they were resistant to 1μM Dex by following growth and viability for 96 hours. Three lines (Ramos, RPMI 8226 and IM-9) showed minimal to

modest slowing of doubling time with Dex, without loss in viability. (Data not shown; see Dex controls, Fig. 11A, Figure 9).

Most lymphoid cells in tissue culture selected for GR resistance have been shown to lose active GR (Helmberg et al., 1995; Thompson et al., 1992). Cells from GC-resistant patients, however, are often GR+ (Leventhal, 1981). We therefore selected lines previously reported to be GR positive: RPMI 8226 37,000 sites/cells (Gomi et al., 1990), IM-9 23,000 (Harmon et al., 1984), K-562 12,300 (Koeffler et al., 1980), HL-60 10,300 (Koeffler et al., 1980), Ramos 8,870 (Sasaki et al., 1982), MOLT-4 8,600 (Harmon et al., 1984); and the Dex-sensitive cell line CEM-C7-14 8,400 (EBT, unpublished data). The GR content of OPM-I has been reported as 64.3± 11.0 fmol GR protein/$10^6$ cells (Gomi et al., 1990). To our best knowledge, only GR presence and function in Mo (MoT) has been reported, as discussed later (Kontula et al., 1982). The cell lines all had similar GR binding affinities for Dex, with Kds ranging from $1.9x10^{-9}$M for CEM-C7-14 cells to $12.5x10^{-9}$M for Ramos. We have confirmed the presence of GR in each cell line by immunoblot analysis (data not shown).

Functionality of the GR in these cell lines has been reported. IM-9 cells showed some biochemical responses to GCs but were not growth inhibited (Lozzio and Lozzio, 1975; Rousseau et al., 1980). HL-60 cells were growth inhibited when GCs were administered together with interferon-α (Sica et al., 1990). Functional GR was documented for RPMI 8226 and OPM-1 (Harmon et al., 1984), Molt-4, K-526 (Lozzio and Lozzio, 1975) and Ramos (Sinclair et al., 1994). Transient transfection experiments of the Hairy cell leukemia cell line MoT with reporter plasmid driven by a GC response element (GRE) resulted in an increase in GRE-dependent transcriptional activity indicating endogenous functional GR (Srivastava and Anderson, 2007).

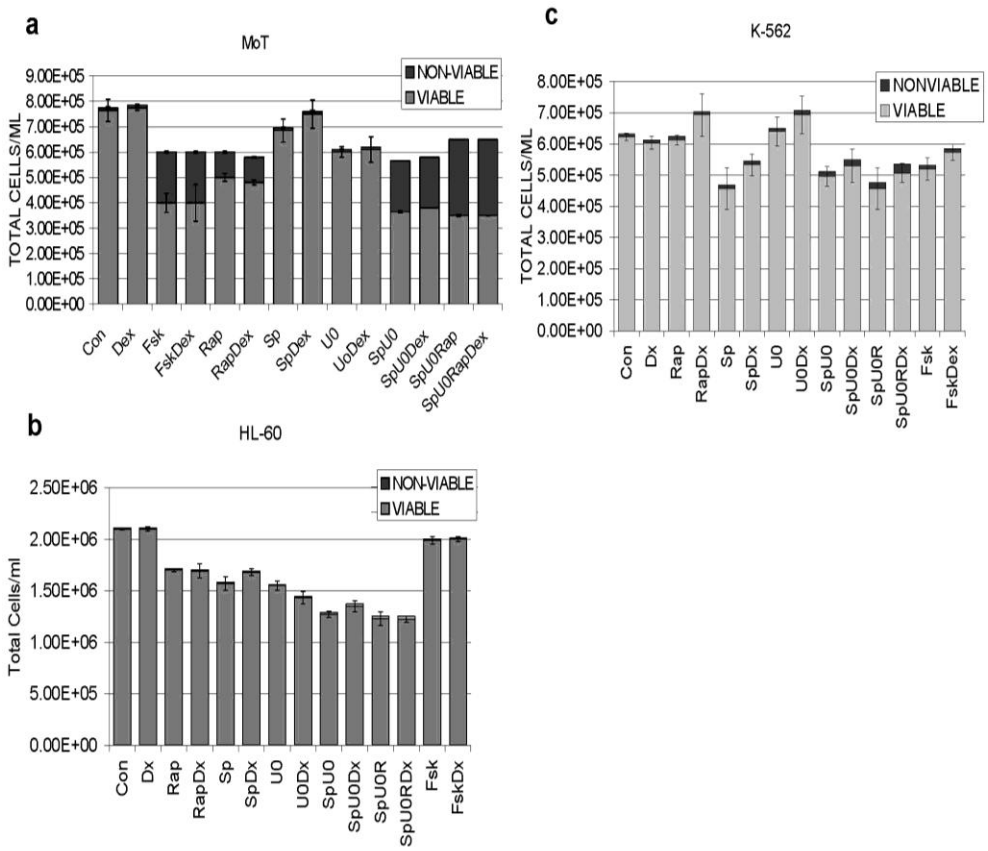



Figure 9 Manipulation of three signaling pathways does not sensitize MoT cells or myeloid cells to Dex.

Equal numbers of growing cells were plated in triplicate in 12-well tissue culture plates. In each experiment, the pathway-altering agents at empirically determined concentrations were added first, and after a suitable interval, Dex was added to 1 μM. Control wells (Con) received <0.1% by volume ethanol vehicle. Total, viable and non-viable (Trypan-blue stained) cells were counted (Vi-cell Counter, Beckman) 96 h after addition of Dex. Results are shown as averages from three such experiments. Error bars correspond to 1 standard

deviation: upward, for non-viable cells; downward, for viable cells. Where no error bars are shown, the standard deviation fell within the margins of the bar. Error bars= 1 standard deviation of average experiments, n=3 each performed in triplicates. Treatments are labeled on the graph. (**a**) Mo (**b**) HL-60 (**c**) K-562.

***Dex-resistant cell lines have higher basal levels of phosphorylated (active) JNK and ERK than a Dex-sensitive cell line.***

Data from CCRF-CEM clones indicates that JNK and ERK protect against, whereas p38 enhances, GC-dependent apoptosis (Miller et al., 2007). The GC-resistant clone CEM-C1-15 contains high basal levels of phosphorylated (activated) JNK as well as phosphorylated ERK, compared to sensitive clones CEM-C1-6 and CEM-C7-14. Inhibition of ERK and JNK converted C1-15 cells to GC-sensitive (Miller et al., 2007). We therefore compared the phosphorylation levels of JNK and ERK in the eight malignant Dex-resistant cell lines with those of the Dex-sensitive clone CEM-C7-14 without or with Dex treatment. Within each cell line neither total nor phosphorylated JNK nor ERK varied significantly with or without Dex (Figure 10). However the levels of phosphorylated JNK and ERK were much higher in each of the resistant lines than in GC-sensitive clone CEM-C7-14 (Fig. 10). The bar graph shows the average phosphorylated JNK and ERK levels, with and without Dex treatment; In the same scale, in CEM-C7-14 cells they are too low to be seen, though they are detectable with longer film exposure. Phosphorylation of MAPKs is associated with their activation; hence these results are consistent with our hypothesis that ERK and JNK activities are protective and may play a crucial role in resistance to GCs. We concluded that: 1. total JNK and ERK protein levels were not affected by the addition of Dex; 2. total JNK and ERK protein levels were somewhat higher in the resistant cell lines when compared to the sensitive cell line; and 3. All the GC-resistant cell lines expressed higher levels of phosphorylated JNK and ERK than GC-sensitive CEM cells.

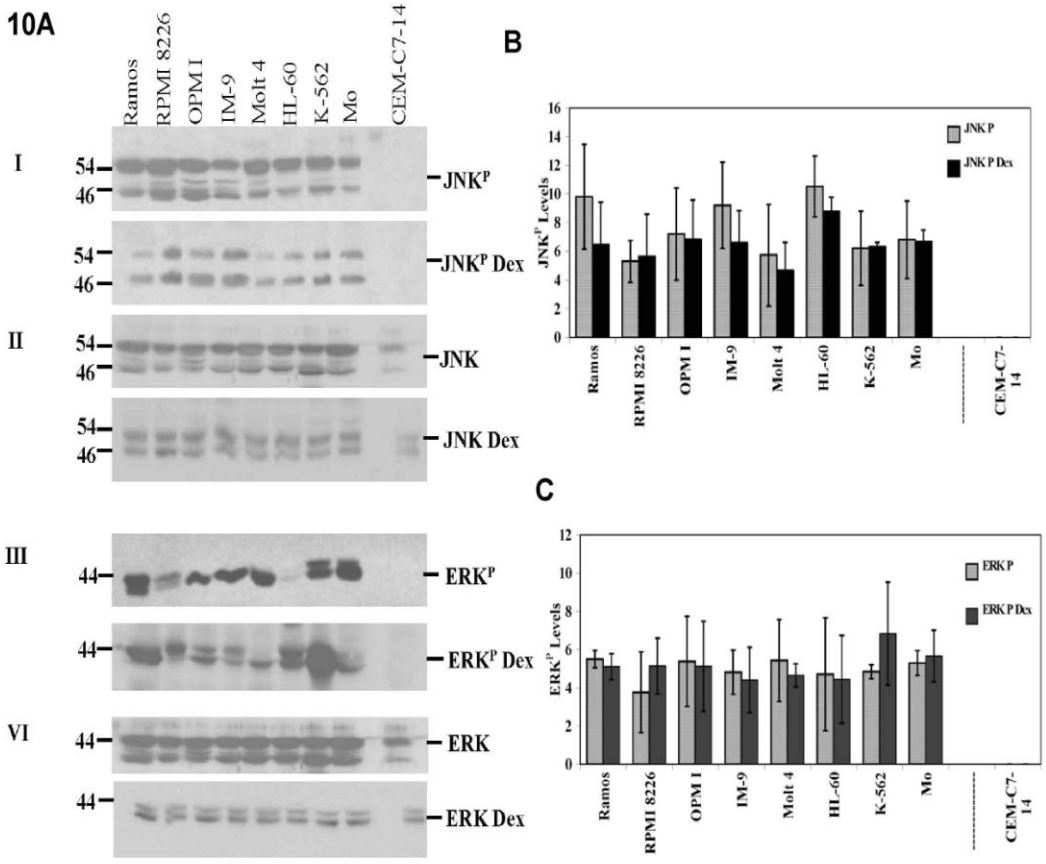



Figure 10: All the Dex-resistant cell lines have high levels of $JNK^P$ and $ERK^P$ relative to Dex-sensitive CEM-C7-14 cells.

Cells were plated at $3x10^5$ viable cells/ml. After exposure to 1 μM Dex or vehicle for 24 h, cell lysates were prepared and analyzed by immunoblot for total and phosphorylated ERK and JNK. Each filter was subsequently blotted for β-actin (not shown). **A.** Characteristic blots from two experiments, Experiment 1, filters I and II, for phosphorylated ($JNK^P$) and total

JNK, each ± Dex. Experiment 2, filters III and IV, for $ERK^P$ and total ERK ± Dex. **B.** Average $JNK^P$ and $ERK^P$ levels, ± Dex, each cell line. Means from 3 independent experiments. Images were analyzed densitometrically and normalized to β-actin. Ordinate: Ratio of densitometry units, MAPK/actin. Error bars =1 standard deviation of average n=3 independent experiments; p value based on two-tailed students t-test using Excel.

***Manipulation of several signaling pathways can restore sensitivity to Dex in resistant lymphoid malignancies.***

Cell growth and viability were evaluated by several methods, including phase contrast microscopy, counts of viable cells and flow cytometry for DNA content.
ERK activity was pharmacologically blocked by inhibiting its upstream MEK with U0126, and JNK activity was inhibited by either SP600125 or i.p. (inhibitory peptide). Inhibition of ERK and JNK slowed cell growth with minimal effects on viability. In five of the eight tested cell lines addition of Dex then caused apoptosis, with a marked reduction of total viable cells and obvious cellular death seen by phase contrast microscopy (Figure 11A, E). These cell lines were the Burkitt's lymphoma (Ramos), 3 myeloma lines (RPMI 8226, IM-9, OPM-I), and the T-cell leukemia Molt-4 (Fig. 11*A*). The bar graphs show total cell counts after 96 hours and distinguish residual viable cells (open bar) from countable but non-viable (shaded bar) Trypan blue-positive cells. The combination of both inhibitors (Fig. 2A, SpU0) had a greater sensitizing effect in the B-lineage cell lines, where viable cell counts were reduced after Dex (SpU0Dex) by 66% - 87% than in the T cell derived Molt-4 cells, where the block of ERK and JNK followed by Dex resulted in a 45% decrease. To test for non-specific drug effects, we also employed a peptide specifically inhibitory to JNK in Ramos and RPMI 8226 cells. The combination of JNK inhibitory peptide and ERK inhibitor U0126 also rendered the cells sensitive to Dex-dependent apoptosis (data not shown). Treatment of the hairy cell leukemia line Mo cells with inhibitors of ERK and JNK alone reduced cell growth and

viable cell numbers; however, no additional Dex effect was observed (Figure 9a). Finally, the myelogenous leukemias HL-60 and K-562 showed no response to any of the treatments (Figure 9b and 9c respectively).

Rapamycin, an inhibitor of mTOR, has been reported to stimulate $G_1$ arrest in cycling B-chronic lymphoblastic leukemia cells and to interfere with many molecules important for cell cycle regulation (Decker et al., 2003). We also confirm and extend prior studies linking rapamycin treatment to Dex induced apoptosis, in two resistant myeloma lines (Strömberg et al., 2004) and one T-cell leukemia line (Miller et al., 2007). Following pretreatment with rapamycin for 24 hours, Dex was added to the cell cultures and the effect was followed by counting viable cells after a total treatment time of 96 hours. The same five cell lines that had shown Dex sensitization after blocking ERK and JNK were also sensitized by rapamycin (Figure 11B). The extend of cell death was greatest in Ramos cells (87% decrease in viable cells), followed by myelomas RPMI 8226, and OPM-I, IM-9 cells, and finally, the T-cell line Molt-4 (38% decrease).

Fsk activates adenylate cyclase and thus cAMP production, which in turn causes activation of PKA (Medh et al., 1998; Tanaka et al., 2006). Loss of PKA activity has been demonstrated to cause a significant decrease in GC sensitivity (Gruol et al., 1989). PKA and GCs act synergistically to overcome resistance in Dex-resistant CEM cells and this correlates with *c-myc* and Hedgehog pathway suppression (Medh et al., 1998; Tanaka et al., 2006). We now extend these results by showing a synergistic effect of PKA with GCs to induce apoptosis in several lymphoid malignancies (Fig. 11C, Supplementary Figure 2). Ramos and IM-9 cells showed the greatest sensitivity to combined treatment with Fsk plus Dex, with decreases of viable cells by 97 and 93%, respectively. The treatment resulted in a reduction of drug matched viable cells by 83, 69 and 60% in RPMI 8226, OPM-I and Molt-4, respectively. Significant growth inhibitory effect by Fsk alone was only seen in RPMI 8226 when compared to Dex treatment. Thus of the three pathways, the PKA path provided the greatest sensitization to Dex and the least effect on

cell growth. The myeloid cell lines exhibited no response to Dex when Fsk was present (Figure 9b,c).

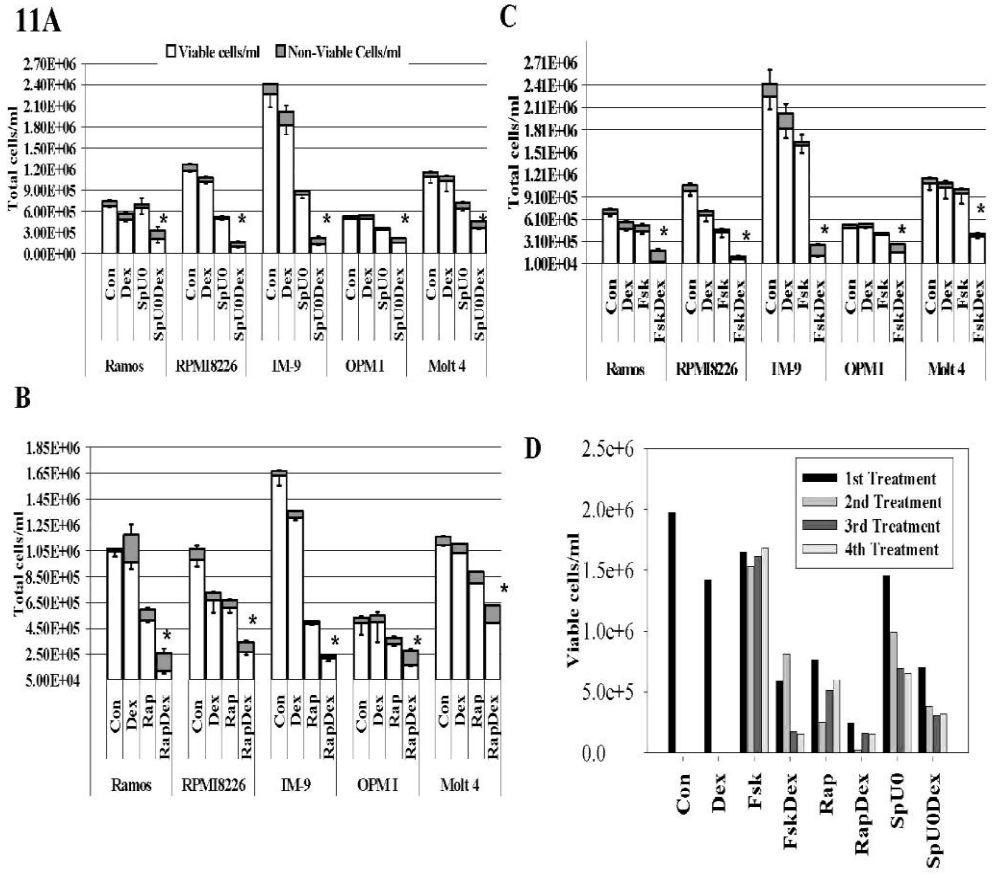



Figure 11: Manipulation of three signaling pathways can sensitize lymphoid cells to Dex.

Equal numbers of growing cells were plated in triplicate in 12-well tissue culture plates. In each experiment, the pathway-altering agents at empirically determined concentrations that caused little or no reduction in viability were added first, and after a suitable interval, Dex was added to 1 μM. Control wells (Con) received <0.1% by volume ethanol vehicle. Total, viable and non-viable

(Trypan-blue stained) cells were counted (Vi-cell Counter, Beckman) 96 h after addition of Dex. Results are shown as averages from three such experiments. Error bars correspond to 1 standard deviation: upward, for non-viable cells; downward, for viable cells. Where no error bars are shown, the standard deviation fell within the margins of the bar. Error bars= 1 standard deviation of average experiments, n=3 each performed in triplicates; p value based on two-tailed students t-test for matched drug treatments using Excel. A. Inhibition of ERK and JNK. ERK inhibitor via upstream MEK: U0126 (U0); JNK inhibitor: SP600125 (Sp). Combined treatment, SpU0. Comparing SpU0 treated cells with SpU0 + Dex gave p values for the reduction in viable cells (*) ranging from 0.02 to 0.007. B. Rapamycin (Rap). Rap vs. Rap + Dex comparisons gave p values 0.02-0.001. C. Forskolin (Fsk). Fsk vs. Fsk + Dex comparisons gave p 0.02-0.00006. D. Repeated treatments with pathway-altering combinations**.** IM-9 cells were treated with each drug or combination, counted 96 h later, allowed to recover, and retreated four times in succession. Each bar shows the results from one cycle. Con and Dex only show one bar because after cycle one, the cells overgrew the wells. Ramos and Molt-4 cells were tested using the same protocol and showed similar results.

***Repeated cycles of treatment with sensitizing drugs followed by Dex leads to increased cell kill.***

Since the restored sensitivity of these cell lines did not result in a complete loss of viable cells during a single round of treatment, we evaluated the sensitivity of the residual cell populations. The possibility of a completely resistant subpopulation was excluded by re-treating the cell population of Ramos, IM-9 and Molt-4 after 2-3 days of recovery time, when cell viability of the population had returned to ≥ 85%. All three cell lines responded with a similar or greater degree of cell kill when given up to 4 additional cycles of combination drugs. This response is shown for IM-9 cells in Fig. 12D, as

typical of the three cell lines. No significant population of resistant cells appears to have emerged after four rounds of treatment.

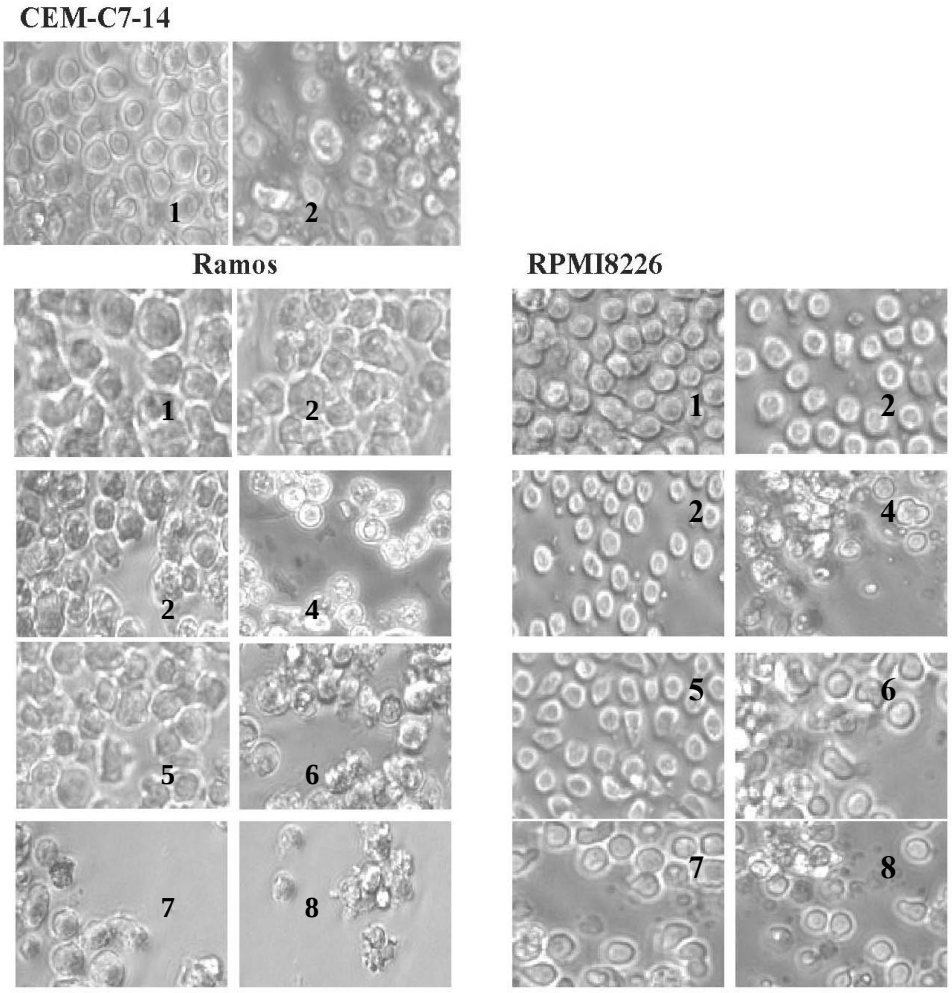



Figure 12: Overt apoptosis in cells reverted to Dex sensitivity.

Examples are shown of three cell lines: As a positive control, CEM-C7-14 cells were given vehicle only (1) or Dex (2) for 96 h. Below, results for Ramos and RPMI 8226 cells. Vehicle (1), Dex only (2), Fsk (3), FskDex (4), Rap (5),

RapDex (6), SpU0 (7), SpU0Dex (8). Phase contrast photomicrographs of typical fields.

***Flow cytometry and caspase 3 activity (FACS) indicate that Dex causes apoptosis in inhibitor treated cells.***

The appearance of apoptosis in microscopic visualization of cells reverted to Dex sensitivity was confirmed by documenting two characteristic features of apoptosis: lysis of DNA karyorrhexis within intact plasma membranes and activation of caspases. Flow cytometry of PI-stained cells showed that none of the single treatments increased the population of cells with sub-diploid amounts of DNA. Each combination of drug(s) plus Dex, however, resulted in a clear increase of sub-diploid cells, a classic indicator of apoptosis. Results with RPMI 8226 and Ramos cells are shown, with the Dex-treated, Dex-sensitive clone CEM-C7-14 as positive control (Fig 13A). An increase over controls by each combination was confirmed in all 5 lines convertible to Dex-sensitive and was not observed in the 3 lines that did not convert (not shown).

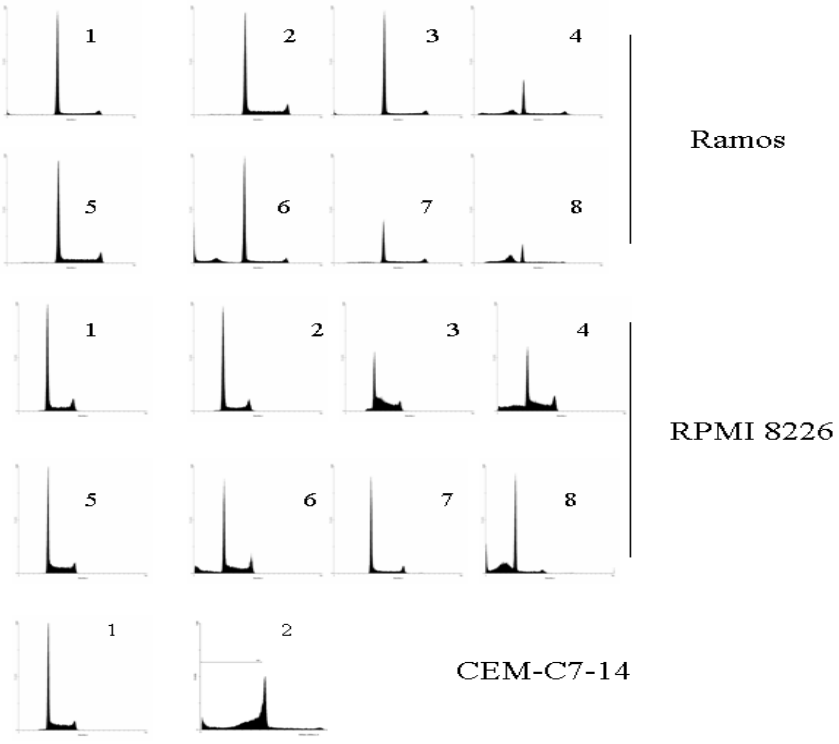


Figure 13A: Biochemical markers indicate Dex-dependant cell death is apoptotic after sensitizing treatments.

Acquisition of sud-diploid DNA content. FACS analysis of PI stained cells. RPMI 8226 and Ramos cells treated for 96 h. 1- vehicle control, 2- Dex, 3- Fsk, 4- FskDex, 5- Rap, 6-Rap+Dex, 7- Sp600125+U0126, 8- Sp600125+U0126+Dex.

FACS identified cells containing activated caspase 3 after exposure to purified polyclonal antibodies specific for the cleaved protease raised against amino acid sequence 163-175 of murine caspase 3. A time course analysis was performed in Ramos cells, following each of the three sensitizing treatments: block ERK and JNK activity, inhibition of mTOR or activation of PKA, alone and with the addition of Dex (Fig. 13B). Alone, Dex, Fsk, rapamycin, and the SP600125 plus UO126 combination each caused a modest increase in caspase 3 positive cells, control also increased over time. Dex in conjunction with each of the other treatments resulted after ≥ 20 hours in a two to three fold increase in caspase positive cells relative to drug-matched controls. This substantiates a late apoptotic event, consistent with the lag before overt apoptosis seen microscopically or by cell counting. Treatment for 36h was therefore chosen to screen IM-9 and Molt-4, representing the other two classes of cells that could be sensitized to Dex. Caspase 3 positive cells in IM-9 ranged from 3-5% after single-variable treatments and rose to 11-20% when Dex was added to the respective sensitizing treatments. Molt-4 showed 2-5% caspase 3 positive cells in single variable circumstances, and this rose to 7-10% after Dex was added to the other drugs (data not shown). The inherently Dex-sensitive cell line CEM-C7-14 was used as a positive control. Dex-sensitive CEM-C7-14 cells were 10% caspase 3 positive after 36 hours with Dex. This single time-point may not show the maximum effect in each cell line, but the data clearly indicate a "snapshot" sensitizing effect of each of the three pathway inhibitors to Dex-dependent activation of caspase 3.

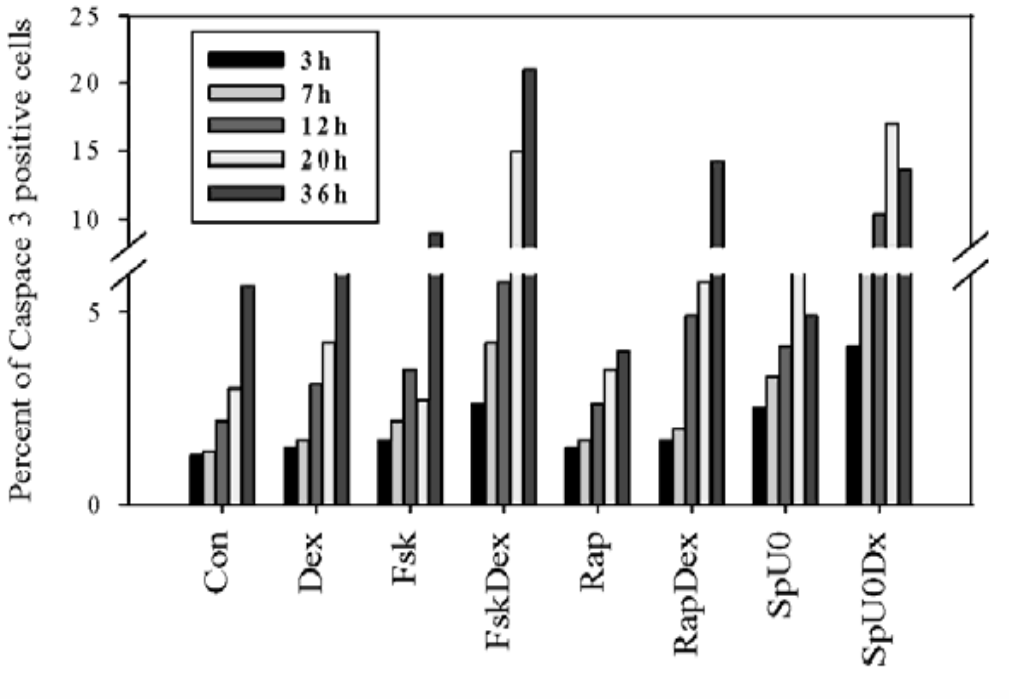



Figure 13B Biochemical markers indicate Dex-dependant cell death is apoptotic after sensitizing treatments.

Time course, activation of caspase 3. Percent cells positive for activated caspase 3 at times indicated after each treatment. Note break scale of ordinate. Ramos cells.

***Inhibition of caspase activity by Z-VAD blocks the death of cells sensitized to Dex***

To confirm the causative role of caspases in these apoptotic events, we inhibited caspase activity in cell cultures by addition of Z-VAD, and then carried out sensitizing treatments followed by Dex challenge. Dex-sensitive CEM-C7-14 cells, given Dex, which are known to die by apoptosis, were also treated with Z-VAD to confirm reversal of Dex sensitivity. In these and in all three tested cell lines- Ramos, IM-9 and Molt-4, there was a statistically significant reduction in Dex-dependent death when caspases were blocked by Z-VAD (Figure 13C).

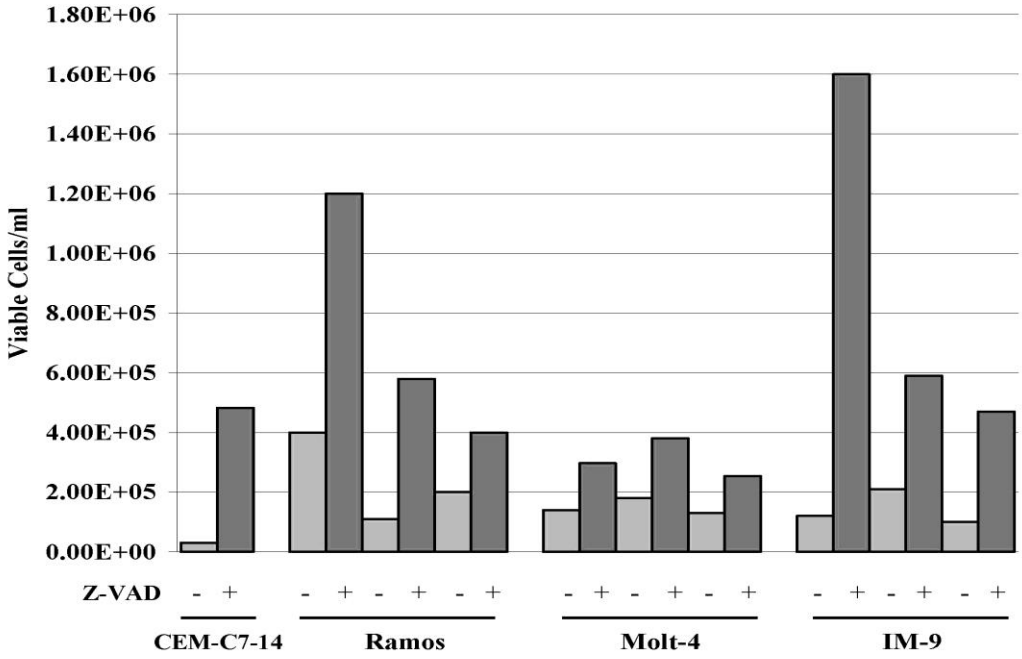



Figure 13C: Biochemical markers indicate Dex-dependant cell death is apoptotic after sensitizing treatments.

Inhibition of Dex-dependant apoptosis by Z-VAD. CEM-C7-14 cells (Dex-sensitive controls) were exposed to Dex ± Z-VAD to block caspase activity. Dex-resistant, Ramos, Molt-4 and IM-9 were treated with Fsk+ Dex, Rap+ Dex and SpU0 + Dex, each ± Z-VAD and viable cells counted 96 h later. Averages from three experiments, error bars= 1 standard deviation of the average n=3 each performed in triplicates; p value based on two-tailed students t-test using Excel. In all conditions, comparing Z-VAD with non Z-VAD treatments gave p value ranging from 0.05 to 0.0003.

***Dex-sensitizing treatments alter the balance between anti- and pro-apoptotic MAPK's.***

In CEM clones, inhibition of ERK and JNK enhances, while inhibition of p38 activity opposes, GC-induced apoptosis (Miller et al., 2007). We hypothesized that this balance of MAPK activities is more general and evaluated the relative levels of total and activated MAPKs in all five sensitizable cell lines. Each of the Dex-sensitizing treatments alters the balance between the three major MAPK signal transduction pathways as hypothesized. Total protein levels of the three MAPKs did not change, regardless of the treatment. This allowed comparison of proportions of the phosphorylated p38, ERK and JNK within each cell line (Fig. 14-18). In all, the proportions of $JNK^P$ and $ERK^P$ diminished while $p38^P$ levels increased after the Dex-sensitizing treatments. Figure 14 shows representative immunoblots of Ramos cell proteins after all eight treatments, analyzed for total and phosphorylated MAPK levels. The cells were extracted after a total treatment time of 24 hours, a time just prior to the onset of apoptosis. As immunoblots show, total JNK, ERK, and p38 varied little, but phosphorylated MAPK quantities varied considerably, depending on the treatments. The large pie charts below the blots show proportions of phosphorylated p-38 ($p38^P$) and ($JNK^P$ + $ERK^P$), averaged from three experiments. Figures 15-18 show the proportions of phosphorylated MAPKs for all other cell lines that became Dex-sensitive with combined treatments. In each case, the sensitizing treatments increased pro-apoptotic $p38^P$ but decreased the anti-apoptotic MAPKs, $JNK^P$ and $ERK^P$.

Figure 14

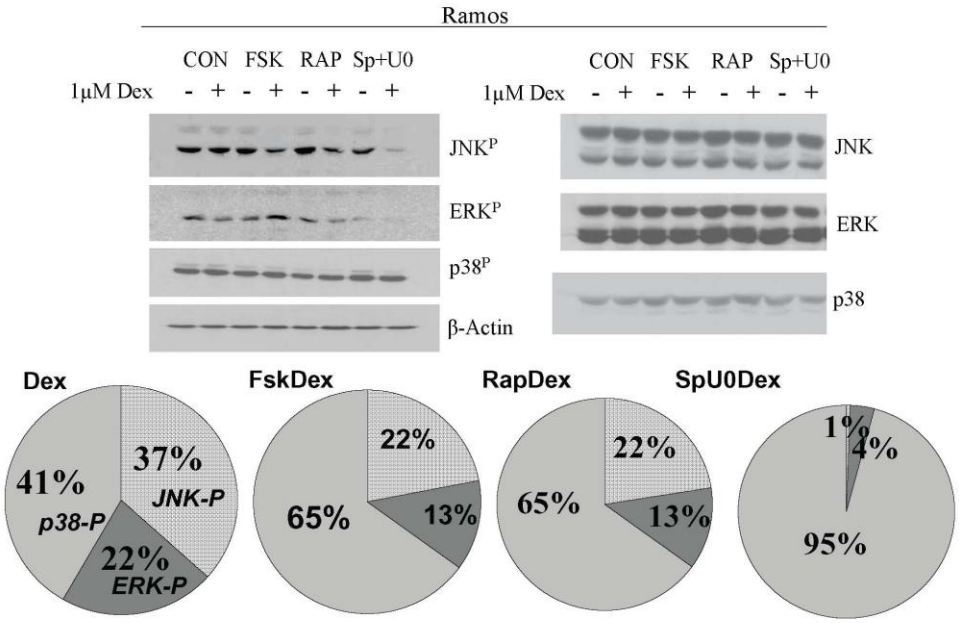



Figure 14: Altered balance of $JNK^P$, $ERK^P$, and $p38^P$ correlates with a shift to a Dex sensitive phenotype.

Ramos Cells: Immunoblots from one experiment showing in alternate lanes levels of indicated components in control and variously treated extracts. Extracts prepared after 24 h drug exposure, before apoptosis begins. JNK, ERK, p38 indicate total protein of each. $JNK^P$, $ERK^P$, and $p38^P$ indicate phosphorylated (active) forms. Actin: loading control. Large pie charts below show average proportions of the phosphorylated forms of three such experiments (for detailed description see Methods). Labels in leftward chart identify the pattern code; labels at top indicate treatment.

Figure 15

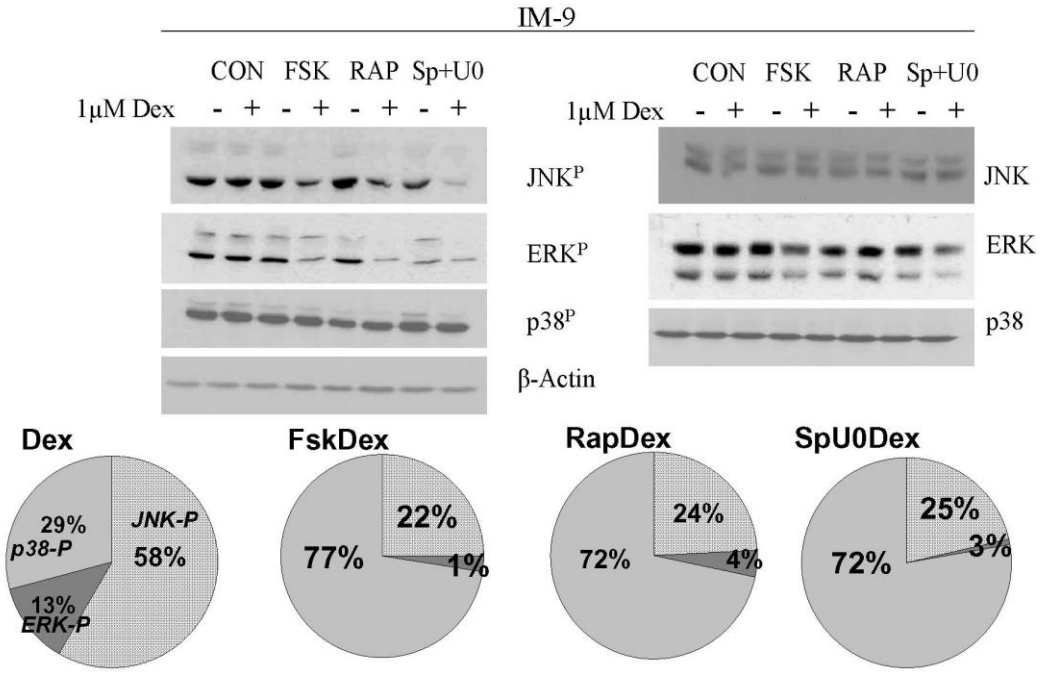



Figure 15: Altered balance of $JNK^P$, $ERK^P$, and $p38^P$ correlates with a shift to a Dex sensitive phenotype.

IM-9 Cells: Immunoblots from one experiment showing in alternate lanes levels of indicated components in control and variously treated extracts. Extracts prepared after 24 h drug exposure, before apoptosis begins. JNK, ERK, p38 indicate total protein of each. $JNK^P$, $ERK^P$, and $p38^P$ indicate phosphorylated (active) forms. Actin: loading control. Large pie charts below show average proportions of the phosphorylated forms of three such experiments (for detailed description see Methods). Labels in leftward chart identify the pattern code; labels at top indicate treatment.

Figure 16

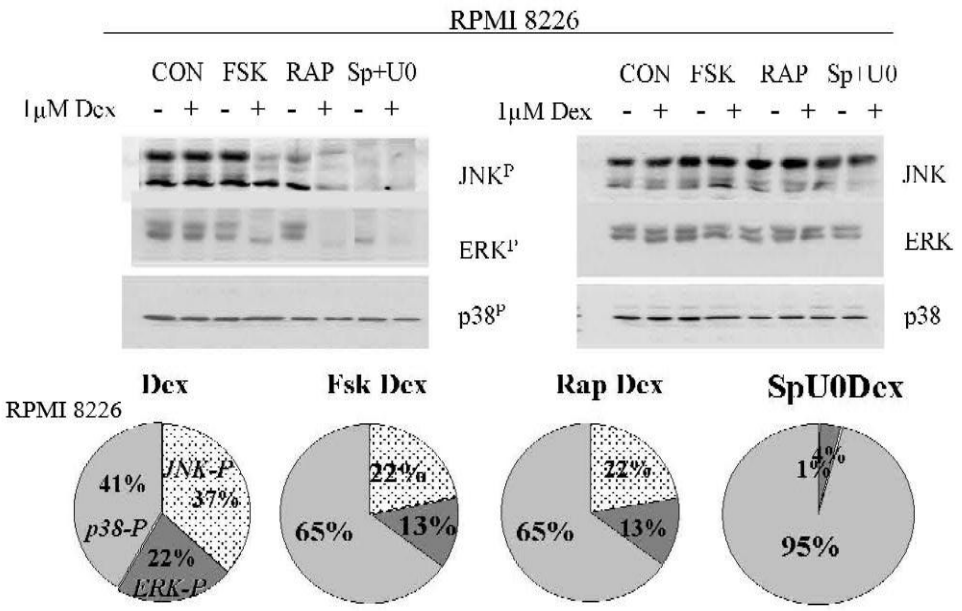



Figure 16: Altered balance of $JNK^P$, $ERK^P$, and $p38^P$ correlates with a shift to a Dex sensitive phenotype.

RPMI 8226 Cells: Immunoblots from one experiment showing in alternate lanes levels of indicated components in control and variously treated extracts. Extracts prepared after 24 h drug exposure, before apoptosis begins. JNK, ERK, p38 indicate total protein of each. $JNK^P$, $ERK^P$, and $p38^P$ indicate phosphorylated (active) forms. Actin: loading control. Large pie charts below show average proportions of the phosphorylated forms of three such experiments (for detailed description see Methods). Labels in leftward chart identify the pattern code; labels at top indicate treatment.

Figure 17

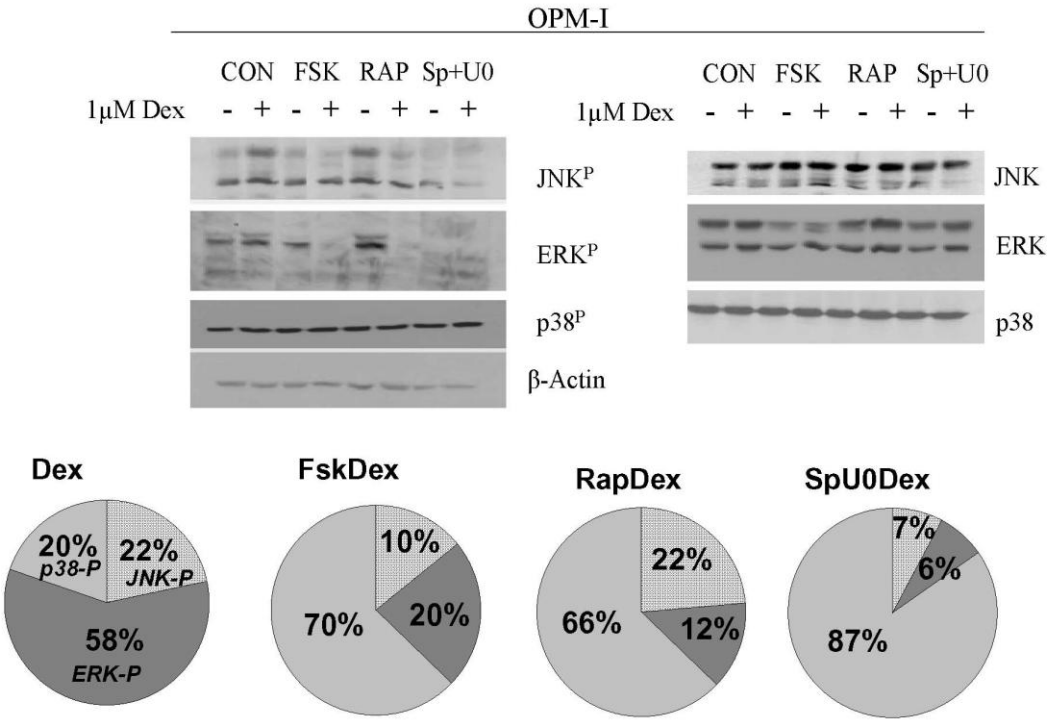



Figure 17: Altered balance of $JNK^P$, $ERK^P$, and $p38^P$ correlates with a shift to a Dex sensitive phenotype.

OPM-I Cells: Immunoblots from one experiment showing in alternate lanes levels of indicated components in control and variously treated extracts. Extracts prepared after 24 h drug exposure, before apoptosis begins. JNK, ERK, p38 indicate total protein of each. $JNK^P$, $ERK^P$, and $p38^P$ indicate phosphorylated (active) forms. Actin: loading control. Large pie charts below show average proportions of the phosphorylated forms of three such experiments (for detailed description see Methods). Labels in leftward chart identify the pattern code; labels at top indicate treatment.

Figure 18

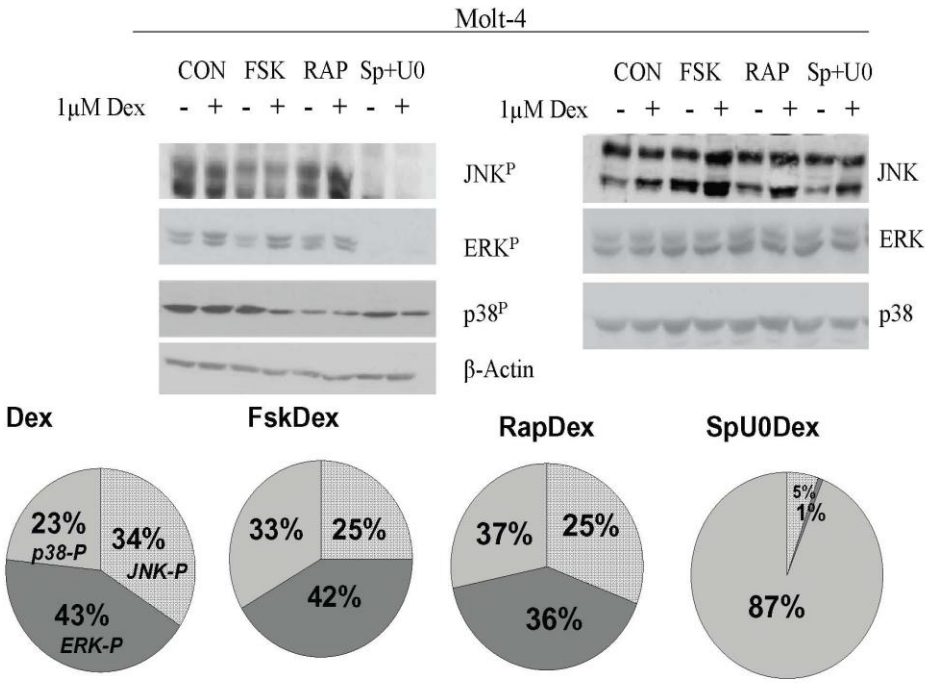



Figure 18: Altered balance of $JNK^P$, $ERK^P$, and $p38^P$ correlates with a shift to a Dex sensitive phenotype.

Molt-4 Cells: Immunoblots from one experiment showing in alternate lanes levels of indicated components in control and variously treated extracts. Extracts prepared after 24 h drug exposure, before apoptosis begins. JNK, ERK, p38 indicate total protein of each. $JNK^P$, $ERK^P$, and $p38^P$ indicate phosphorylated (active) forms. Actin: loading control. Large pie charts below show average proportions of the phosphorylated forms of three such experiments (for detailed description see Methods). Labels in leftward chart identify the pattern code; labels at top indicate treatment.

The myeloid lineage cell lines HL-60 (Figure 19) and K-562 (Figure 20) that did not undergo apoptosis as a result of the treatment regimen, were unaffected as to the distribution of $ERK^P$, $JNK^P$ and $p38^P$. The Fsk+Dex and the Rap+Dex treatments did not diminish the high $(ERK+JNK)^P$ levels and did not increase those of $p38^P$ . This further strengthens our hypothesis that myeloid derived cells are not dependant on the proposed interactions. In Mo, inhibiting active ERK and JNK by SP+U0 decreased their levels but no additional Dex induced decrease was observed (Figure 21).

**Auto-induction of GR site-specific and activating phosphorylation of GR correlated with conversion to Dex sensitivity.**

In several lymphoid systems ligand-driven transcriptional autoinduction of GR is important for GC-induced cell death (Ramdas et al., 1999; Tonko et al., 2001). We tested the generality of this effect by analyzing the cell lines herein converted to be Dex-responsive. Inhibition of ERK and JNK followed by addition of Dex increased GR protein levels in all five cell lines by at least 2.5-fold. Rapamycin plus Dex increased GR protein levels by 2.2-2.7-fold in Ramos, IM-9, RPMI 8226, and OPM-I cells. All these increases were statistically significant compared to Dex or drugs only (Figures 22-25).

Figure 19

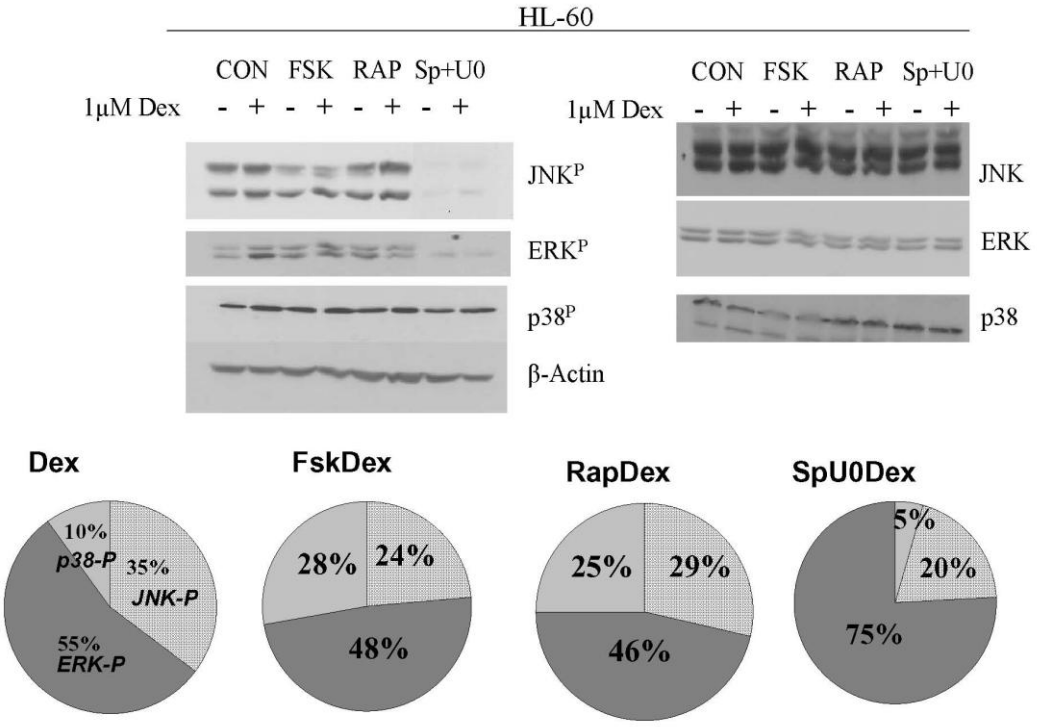



Figure 19: Altered balance of $JNK^P$, $ERK^P$, and $p38^P$ correlates with a shift to a Dex sensitive phenotype.

HL-60 Cells: Immunoblots from one experiment showing in alternate lanes levels of indicated components in control and variously treated extracts. Extracts prepared after 24 h drug exposure, before apoptosis begins. JNK, ERK, p38 indicate total protein of each. $JNK^P$, $ERK^P$, and $p38^P$ indicate phosphorylated (active) forms. Actin: loading control. Large pie charts below show average proportions of the phosphorylated forms of three such experiments

(for detailed description see Methods). Labels in leftward chart identify the pattern code; labels at top indicate treatment.

Figure 20

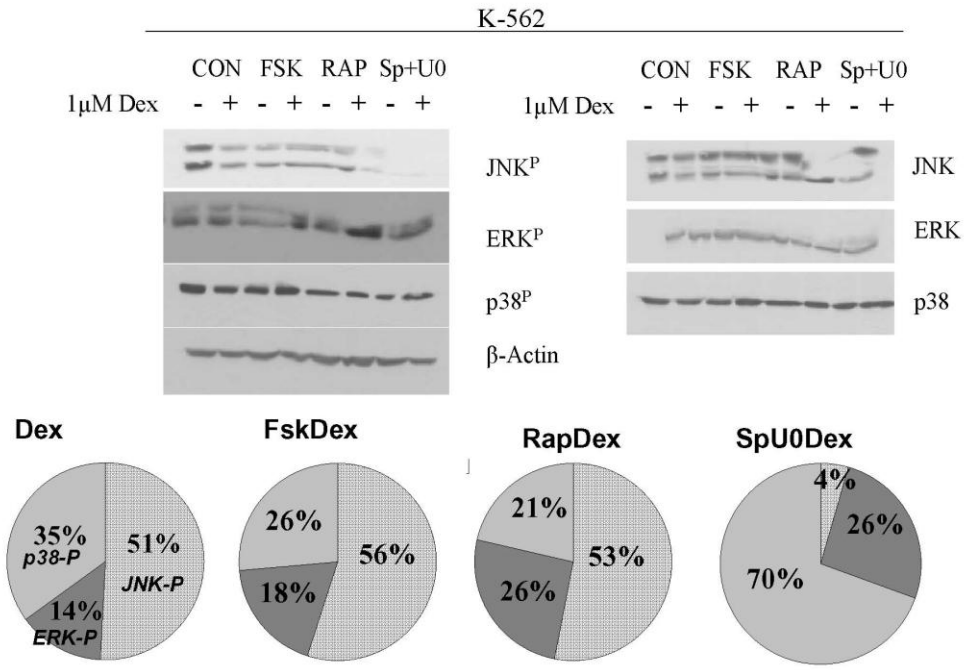



Figure 20: Altered balance of $JNK^P$, $ERK^P$, and $p38^P$ correlates with a shift to a Dex sensitive phenotype.

K-562 Cells: Immunoblots from one experiment showing in alternate lanes levels of indicated components in control and variously treated extracts. Extracts prepared after 24 h drug exposure, before apoptosis begins. JNK, ERK, p38 indicate total protein of each. $JNK^P$, $ERK^P$, and $p38^P$ indicate phosphorylated (active) forms. Actin: loading control. Large pie charts below show average proportions of the phosphorylated forms of three such experiments (for detailed description see Methods). Labels in leftward chart identify the pattern code; labels at top indicate treatment.

Figure 21

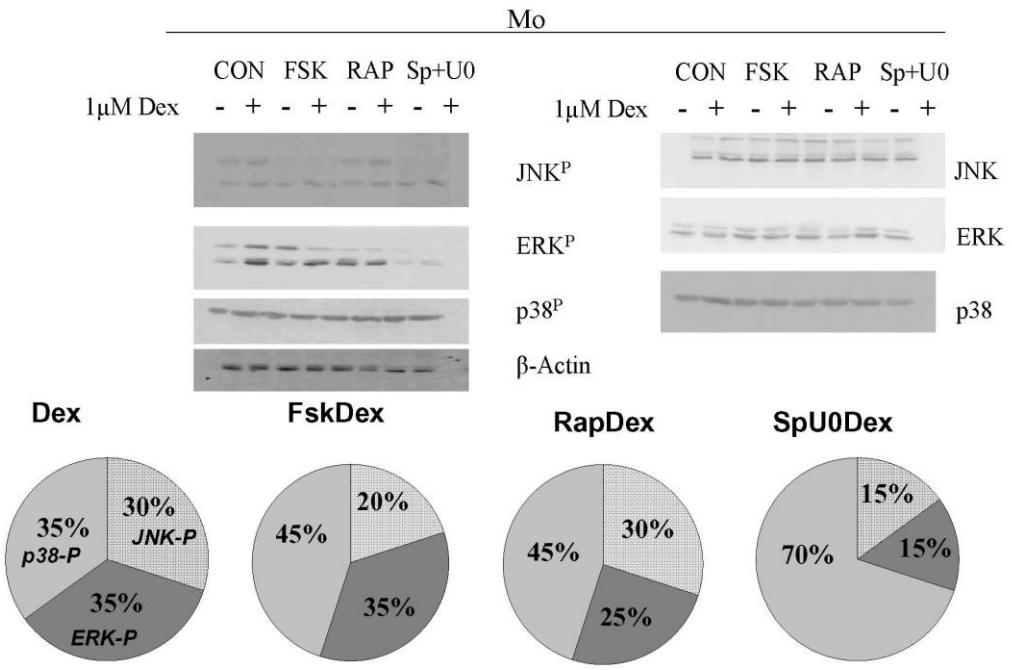



Figure 21: Altered balance of $JNK^P$, $ERK^P$, and $p38^P$ correlates with a shift to a Dex sensitive phenotype.

Mo Cells: Immunoblots from one experiment showing in alternate lanes levels of indicated components in control and variously treated extracts. Extracts prepared after 24 h drug exposure, before apoptosis begins. JNK, ERK, p38 indicate total protein of each. $JNK^P$, $ERK^P$, and $p38^P$ indicate phosphorylated (active) forms. Actin: loading control. Large pie charts below show average proportions of the phosphorylated forms of three such experiments (for detailed description see Methods). Labels in leftward chart identify the pattern code; labels at top indicate treatment.

Figure 22

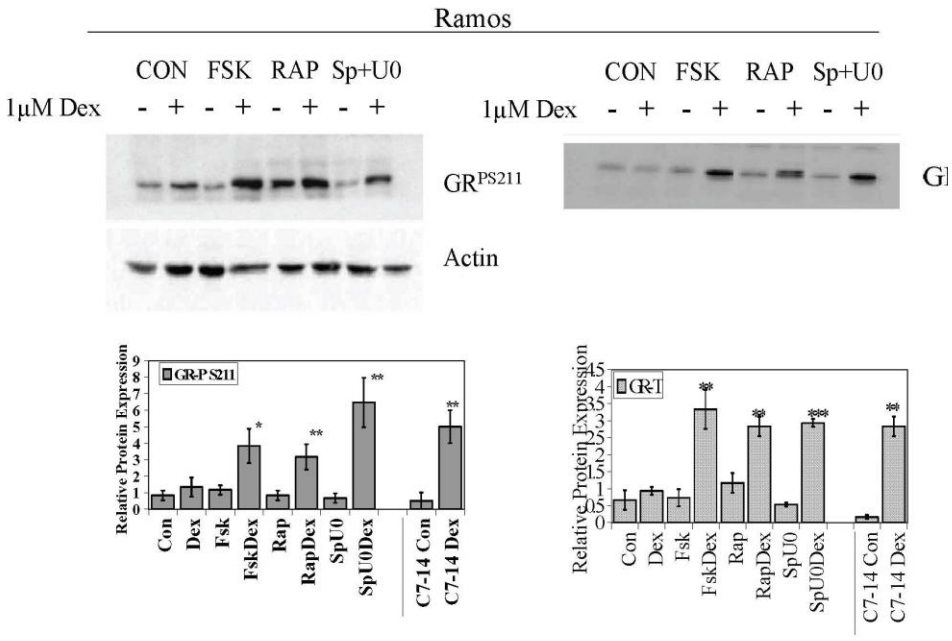



Figure 22: Cells converted to GC sensitivity show Dex-driven increase in total GR, $GR^P$ S211

Ramos Cells were treated with the MAPK path inhibitors SP600125 (Sp) plus U0126 (U0), the mTOR inhibitor Rapamycin (Rap), or the PKA pathway stimulator Forskolin (Fsk) for 6 h, followed by Dex for an additional 18 h. Cell extracts were immunoblotted for total GR, $GR^P$ S211 ($GR^P$). Immunoblots typical of those from all experiments are shown. Densitometric scans of blots from three independent experiments were normalized to actin and plotted in the bar graphs. Cell lines are indicated. Error bars= 1 standard deviation of the independent experiments, p value based on two tailed students t-test using Excel. * Indicates $p< 0.05$ and ** indicates $p< 0.009$ obtained from drug matched controls.

Figure 23

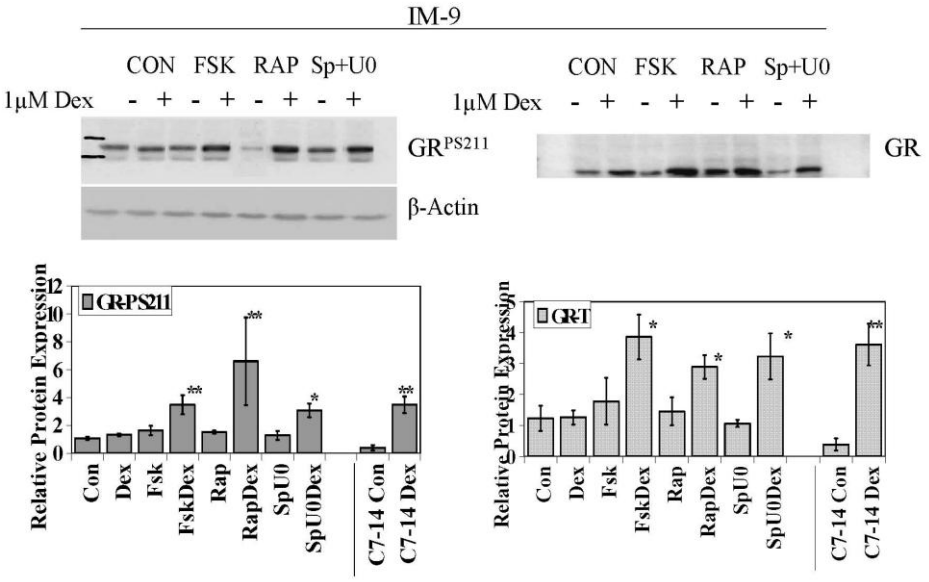



Figure 23: Cells converted to GC sensitivity show Dex-driven increase in total GR, $GR^P$ S211

IM-9 Cells were treated with the MAPK path inhibitors SP600125 (Sp) plus U0126 (U0), the mTOR inhibitor Rapamycin (Rap), or the PKA pathway stimulator Forskolin (Fsk) for 6 h, followed by Dex for an additional 18 h. Cell extracts were immunoblotted for total GR, $GR^P$ S211 ($GR^P$). Immunoblots typical of those from all experiments are shown. Densitometric scans of blots from three independent experiments were normalized to actin and plotted in the bar graphs. Cell lines are indicated. Error bars= 1 standard deviation of the independent experiments, p value based on two tailed students t-test using Excel. * Indicates $p< 0.05$ and ** indicates $p< 0.009$ obtained from drug matched controls.

Figure 24

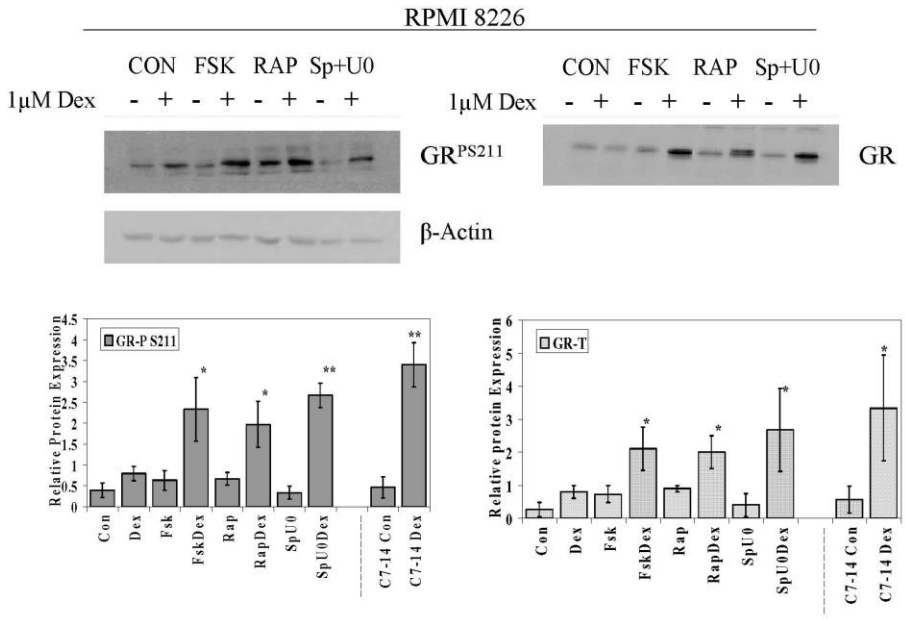



Figure 24: Cells converted to GC sensitivity show Dex-driven increase in total GR, $GR^P$ S211

RPMI 8226 Cells were treated with the MAPK path inhibitors SP600125 (Sp) plus U0126 (U0), the mTOR inhibitor Rapamycin (Rap), or the PKA pathway stimulator Forskolin (Fsk) for 6 h, followed by Dex for an additional 18 h. Cell extracts were immunoblotted for total GR, $GR^P$ S211 ($GR^P$). Immunoblots typical of those from all experiments are shown. Densitometric scans of blots from three independent experiments were normalized to actin and plotted in the bar graphs. Cell lines are indicated. Error bars= 1 standard deviation of the independent experiments, p value based on two tailed students t-test using Excel. * Indicates $p< 0.05$ and ** indicates $p< 0.009$ obtained from drug matched controls.

Figure 25

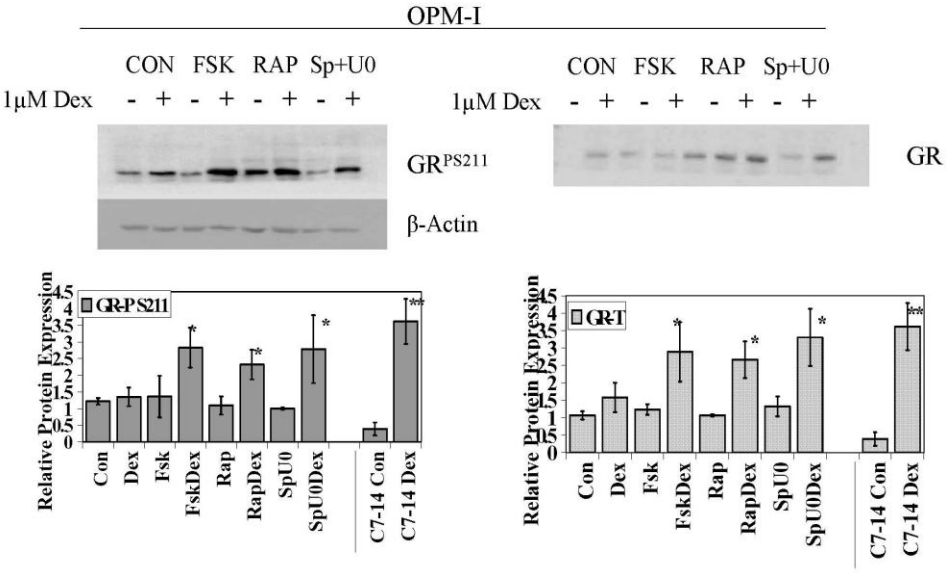



Figure 25: Cells converted to GC sensitivity show Dex-driven increase in total GR, $GR^P$ S211

OPM I Cells were treated with the MAPK path inhibitors SP600125 (Sp) plus U0126 (U0), the mTOR inhibitor Rapamycin (Rap), or the PKA pathway stimulator Forskolin (Fsk) for 6 h, followed by Dex for an additional 18 h. Cell extracts were immunoblotted for total GR, $GR^P$ S211 ($GR^P$). Immunoblots typical of those from all experiments are shown. Densitometric scans of blots from three independent experiments were normalized to actin and plotted in the bar graphs. Cell lines are indicated. Error bars= 1 standard deviation of the independent experiments, p value based on two tailed students t-test using Excel. * Indicates $p< 0.05$ and ** indicates $p< 0.009$ obtained from drug matched controls.

In Molt-4 the average 1.7-fold increase did not reach statistical significance (Figure 26). Fsk plus Dex increased GR levels significantly with respect to Dex or Fsk

alone in Ramos, RPMI 8226 and IM-9 and cells (Figures 22-24). In OPM-I cells, the increase reached significance with respect to Fsk alone but not to Dex-only GR levels (Figure 25). In Molt-4 cells, Fsk alone increased GR levels (Fig. 26). Thus there was a clear tendency for the sensitized cells to show Dex-driven induction of GR.

To cause apoptosis, the quantity of active GR and not just GR protein is critical. The phosphoprotein GR is a target of site-specific phosphorylation by p38 MAPK and $GR^P$ is involved in the apoptotic progress in CEM cells (Gruol et al., 1989; Miller et al., 2005). Therefore we assayed for the effect of the sensitizing treatments on phosphorylation of GR S211, the critical site. Phosphorylation of S211 was identified by imunoblotting with a site-specific anti-serum to $GR^P$ S211. In every case, after treatment with sensitizing compounds, addition of Dex resulted in a 2-7 -fold increase of $GR^P$ 211 (Fig. 22-26). These increases were all statistically significant with the sole exception of Fsk plus Dex in Molt-4 cells, wherein the 2-fold averaged increase showed too much variability. Thus, in five cell lines converted to be sensitive to Dex-driven apoptosis, Dex causes increased total and site-specific phosphorylated GR (Figure 22-26).

Figure 26

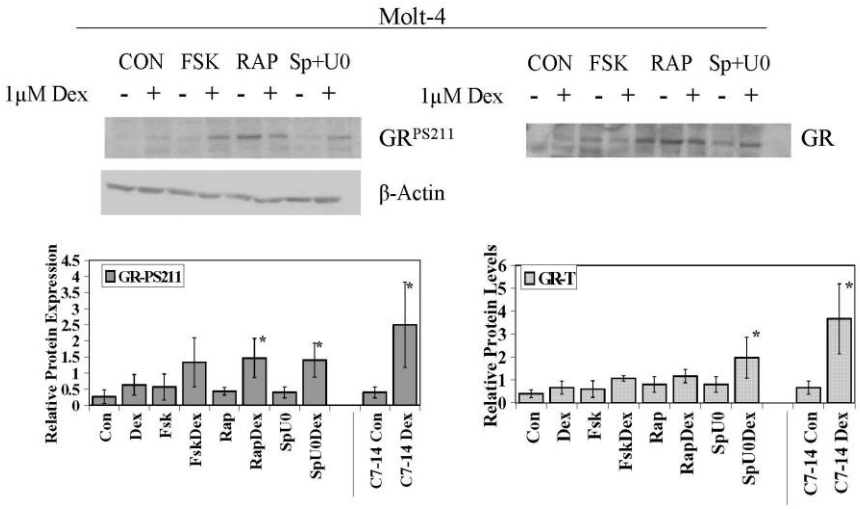



Figure 26: Cells converted to GC sensitivity show Dex-driven increase in total GR, $GR^P$ S211

Molt-4 Cells were treated with the MAPK path inhibitors SP600125 (Sp) plus U0126 (U0), the mTOR inhibitor Rapamycin (Rap), or the PKA pathway stimulator Forskolin (Fsk) for 6 h, followed by Dex for an additional 18 h. Cell extracts were immunoblotted for total GR, $GR^P$ S211 ($GR^P$). Immunoblots typical of those from all experiments are shown. Densitometric scans of blots from three independent experiments were normalized to actin and plotted in the bar graphs. Cell lines are indicated. Error bars= 1 standard deviation of the independent experiments, p value based on two tailed students t-test using Excel. * Indicates $p< 0.05$ and ** indicates $p< 0.009$ obtained from drug matched controls.

The myeloid lines and Mo were unresponsive to treatment regimen and no increase in $GR^P$ levels or GR and Bim protein was observed, consistent with the conclusion that these cell lines do not signal for apoptosis through the GR (Figure 27, 28, 29).

Figure 27

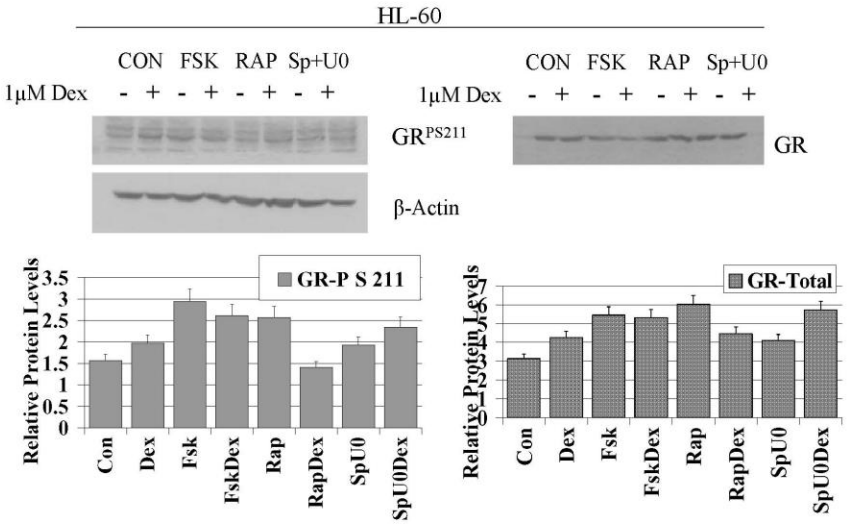



Figure 27: Myeloid Cells not converted to GC sensitivity do not show Dex-driven increase in total GR, $GR^P$ S211

Cells were treated like in figures 22-26.

Figure 28

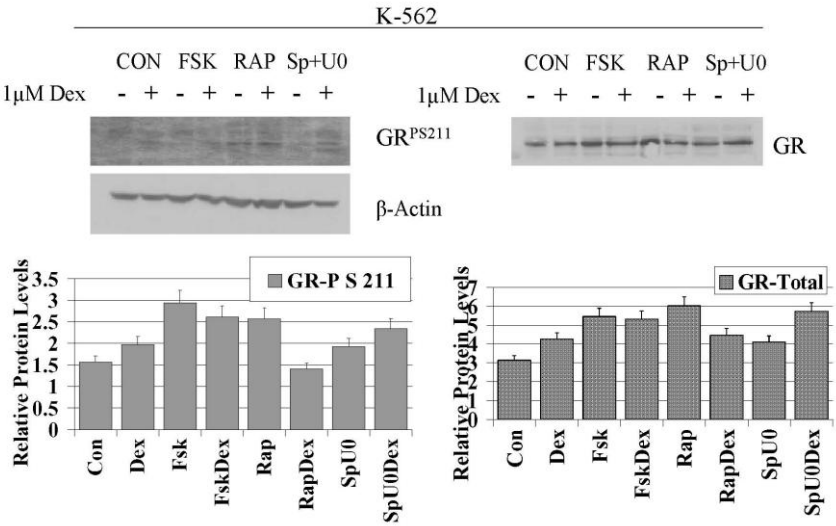



Figure 28: Myeloid Cells not converted to GC sensitivity do not show Dex-driven increase in total GR, $GR^P$ S211

Cells were treated like in figures 22-26.

Figure 29

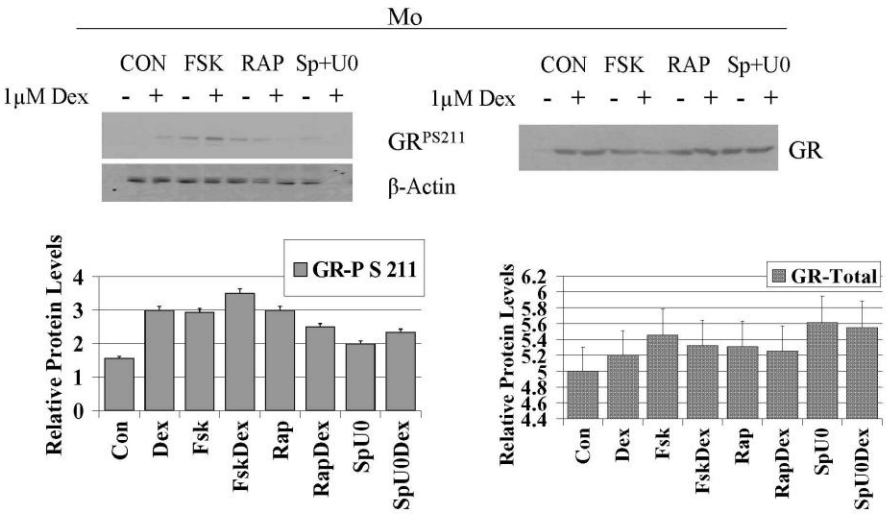



Figure 29: Hairy Cell Leumekia Cell Line not converted to GC sensitivity do not show Dex-driven increase in total GR, $GR^{P}$ S211

Cells were treated like in figures 22-26.

***In sensitized cells, a Dex-dependent increase in Bim precedes apoptosis.***

Mounting evidence implicates the pro-apoptotic Bcl-2 family protein Bim is an important precipitating factor in GC-induced apoptosis. By gene array studies of CEM C7 cells, we discovered strong Dex-dependent induction of Bim mRNA just prior to the onset of apoptosis (Medh et al., 2003). This was shown almost simultaneously in an independent study (Wang et al., 2003). We hypothesized that we would find Bim increased in the Dex-resistant cell-lines converted to Dex-sensitivity. Indeed, in four out of the five cell lines, pharmacological manipulation of each of the three signaling pathways led to Dex-dependant increases in Bim. Ramos, IM-9, OPM-I and RPMI 8226 cells all displayed statistically significant increases in Bim protein levels after Dex when compared to their respective drug-treated controls (Fig. 30). Molt-4 cells were an exception and showed significant increases of Bim levels only in the SpU0/SpU0 plus Dex treatment group (Figure 30). Fsk or Rap alone seemed to increase Bim in these cells, though the increases were not found to be statistically significant (Figure 30). This exception of the pattern suggests that induction of Bim may not be the sole mechanism for Dex-dependant apoptosis.

Figure 30

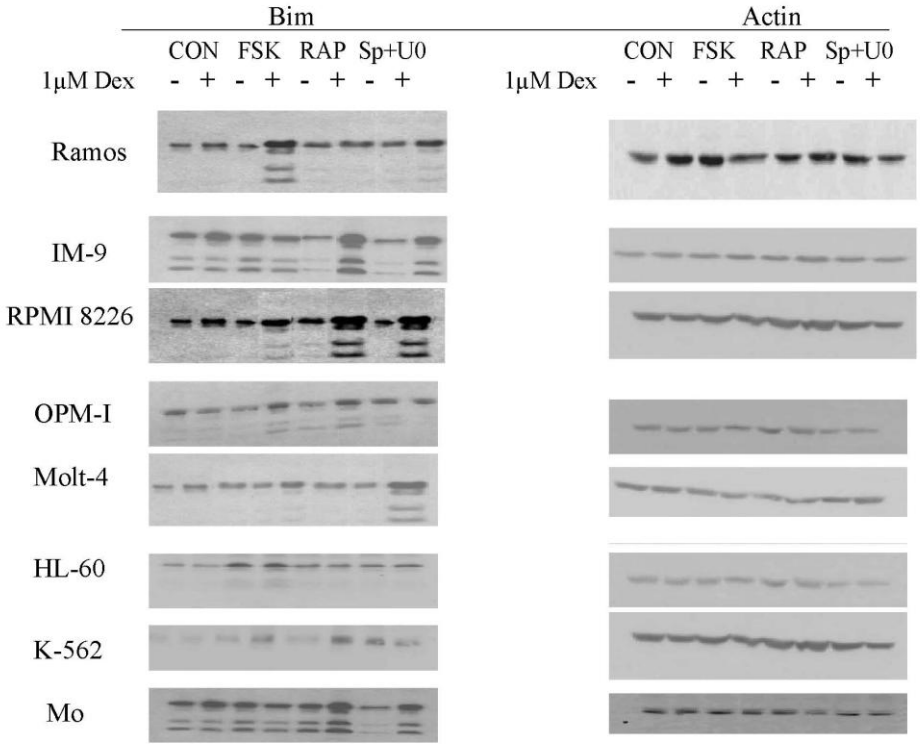

Bim
Actin
CON FSK RAP Sp+U0
1μM Dex - + - + - + - +
Ramos
IM-9
RPMI 8226
OPM-I
Molt-4
HL-60
K-562
Mo

Figure 30

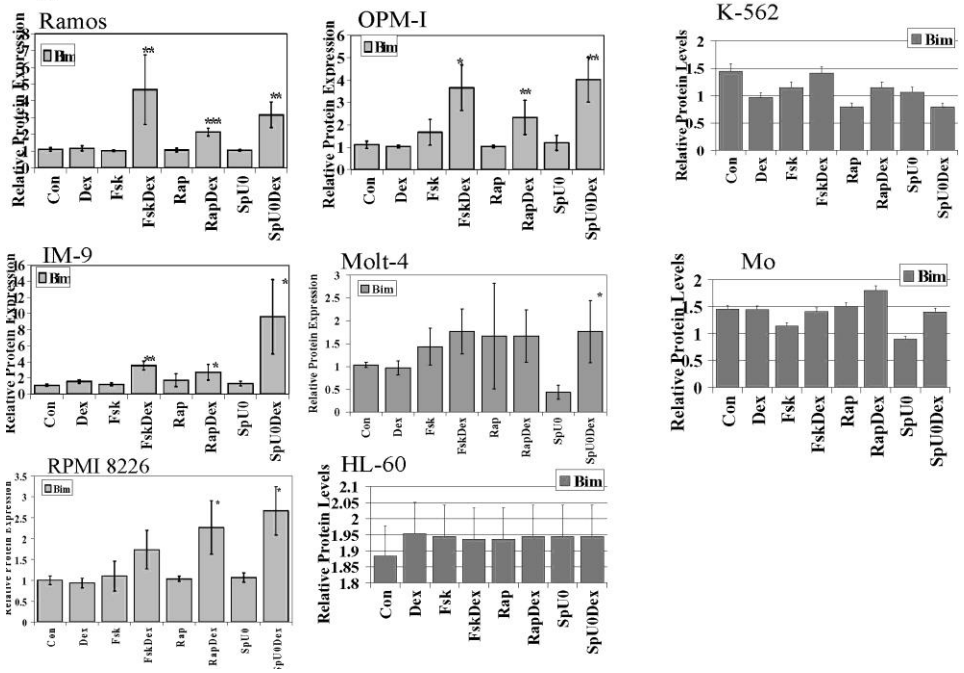



Figure 30: Cells converted to GC sensitivity show Dex-driven increase in Bim protein levels.

Cells from 5 cell lines were treated with the MAPK path inhibitors SP600125 (Sp) plus U0126 (U0), the mTOR inhibitor Rapamycin (Rap), or the PKA pathway stimulator Forskolin (Fsk) for 6 h, followed by Dex for an additional 18 h. Cell extracts were immunoblotted for total Bim isoforms EL, L and S (Bim). Densitometric scans of blots from three independent experiments were normalized to actin and plotted in the bar graphs. Cell lines are indicated. Error bars= 1 standard deviation of the independent experiments, p value based on two tailed students t-test using Excel. * Indicates $p < 0.05$ and ** indicates $p < 0.009$ obtained from drug matched controls.

## DISCUSSION

GCs such as Dex are widely used in the treatment of hematological malignancies. The presence of GR in the malignant cells is necessary but not sufficient for the steroids to evoke cell death. Too often, the cells are GR-positive but steroid resistant. Recently, studies have shown that GR function can be affected by interactions with other signal transduction pathways (Medh et al., 1998; Rogatsky et al., 1998b; Kyriakis, 2000; Bruna et al., 2003; Strömberg et al., 2004; Wada and Penninger, 2004; Ji et al., 2007). In the CEM cell system of closely related Dex-sensitive or Dex-resistant clones, we have shown that the MAPK and PKA pathways are heavily involved in this regulation of GR function. These pathway interconnections are most vividly revealed by treatments which can convert GR-positive but Dex-resistant CEM cells to cells that are killed by the steroid. Stimulating the PKA pathway does so (Medh et al., 2003; Meyers et al., 2007) and so does blocking the ERK/JNK pathways (Miller et al., 2007). Armstrong *et al*. showed that the mTOR blocker rapamycin also converts GR-positive cells to Dex-sensitive (Wei et al., 2006) and we confirmed this and found that rapamycin also causes a reduction in phosphorylated JNK (Miller et al., 2007).

These results encouraged an examination of the generality of these effects. CEM cells are of an early T-cell lineage. We selected eight GR-positive cell lines, all resistant to Dex-evoked cell death, from various lineages. Molt-4 cells, also T-lineage, were chosen to see whether the studied effects are specific to that sub-group of cells. MoT cells, from hairy cell leukemia, represent a very specialized sub-set. B-lineage cells were represented by myeloma lines and Ramos cells derived from Burkitt's lymphoma. Two myeloid-derived lines were found in HL-60 and K-562.

We tried to rebalance the three major MAPK paths by inhibiting ERK and JNK (anti-apoptotic in CEM cells). We blocked mTOR with rapamycin (which in CEM cells also lowers activated JNK) and we stimulated PKA by use of Fsk. Together with our collaborators we have recently shown that this also suppresses the hedgehog (Hh) pathway (Ji et al., 2007). Consistent with results from the resistant CEM clone C1-15, all

the resistant lines had very high ERK and JNK levels relative to sensitive CEM clone C7-14. Treatment with Dex alone had no effect on these or on phosphorylated ERK and JNK levels. Alone, none of the treatments caused significant cell death in the absence of steroid, although several dramatically slowed cell growth.

Each of these manipulations succeeded in conferring sensitivity to Dex-induced apoptotic death on five of the eight test lines. The two myeloid lines and the hairy cell MoT line were resistant to all treatments. We explored these known aspects of mechanisms for this shift to Dex sensitivity. First, each of the treatments shifted the ratio of phosphorylated/activated MAPKs in favor of pro-apoptotic $p38^P$ and away from anti-apoptotic $JNK^P$ / $ERK^P$. Second, the sensitizing treatments caused Dex to promote GR autoinduction and phosphorylation of GR at a site known to enhance the transcriptional and apoptotic actions of the receptor. We note that p38 is among the kinases that phosphorylate this site (Miller et al., 2005). More GR favors a stronger response and greater sensitivity to a given concentration of GC (Simons, 2006). Third, we found that the sensitizing treatments to alter pathways influential on the GR path caused almost all the sensitized cells to respond to Dex by increasing levels of the pro-apoptotic protein Bim. Bim has been implicated repeatedly in steroid-induced apoptosis of lymphoid cells (Medh et al., 2003; Wang et al., 2003). The exception is instructive; Fsk or Rap alone in Molt-4 cells tended to raise Bim levels, without causing apoptosis. This suggests that Bim alone may not always be both necessary and sufficient to bring about apoptosis.

## CONCLUSIONS

We conclude that several types of T- and B-lineage malignant cells can be converted from Dex-resistant to Dex-sensitive by certain pharmacological manipulations. These include suppressing ERK and JNK activity, blocking m-TOR (and suppressing $JNK^P$) with rapamycin, and stimulating PKA. The successful conversion to Dex-sensitive results in induction of GR, at a specific phosphorylation dependant activating site, and

induction of Bim. Very recently, it has been shown that activated JNK promotes Bim EL degradation (Leung et al., 2008). Our results are consistent with this finding. Treatments that reduce $JNK^P$ enhance Dex induction of Bim. Several compounds that have these actions on the cross-talk pathways are in clinical use or trials (Davies et al., 2007; Mita et al., 2008). Others may be suggested by our results. We hope that these results offer encouragement to those who might envisage improved treatments for certain lymphoid malignancies.

# Chapter 5: Conclusions and Future Directions

The initial discoveries that p38 MAPK could specifically phosphorylate the human GR, specifically at position S211, lead to many additional questions. It was additionally established that this S211 phosphorylation enhances the ability of the receptor to regulate transcription and apoptosis (Wang et al., 2002; Miller et al., 2005). One of the questions that was answered in this thesis was whether site specific phosphorylation plays a role in functional conformational changes in the intrinsically disordered AF1 domain, which houses these phosphorylation sites.

We found that phosphorylated AF1 at S211 adopts significantly higher secondary structural elements in it with helical content increased by ~50% at the expense of random coil compared with unphosphorylated AF1. Further, we found that quantum yield of fluorescence is significantly increased in the phosphorylated AF1 at S211 compared with unphosphorylated AF1 at S211, with corresponding blue shifts in the emission maxima. In addition we gathered partial proteolysis data that indicated protection of peptides in phosphorylated AF1, suggesting that site-specific phosphorylation of AF1 results in a compact structure formation. Based on this data S211 is responsible for secondary and tertiary structure formation in AF1, since AF1-S211A when phosphorylated with p38 did not induce any conformational changes determined by CD, florescence spectroscopy and partial proteolysis. Immunoblot analysis of the phosphorylation status for each experiment was performed and indicated that the other two sited were also phosphorylated with p38. Keeping in mind though that the GRS203 antibody is less specific and tends to recognize total GR protein as well.

However the exact role of the other two (thus far known) phosphorylation sites S203 and S226 remains unclear. We know that S211 is important but whether or not are the other two are supportive, inhibitory, or equally as important for structure and function remains to be determined. In an attempt to shed some light on this topic, I generated AF1-S226A, AF1-S226E, AF1-S203A and AF1-S203E recombinant protein using site directed mutagenesis and purified the protein in a bacterial expression system.

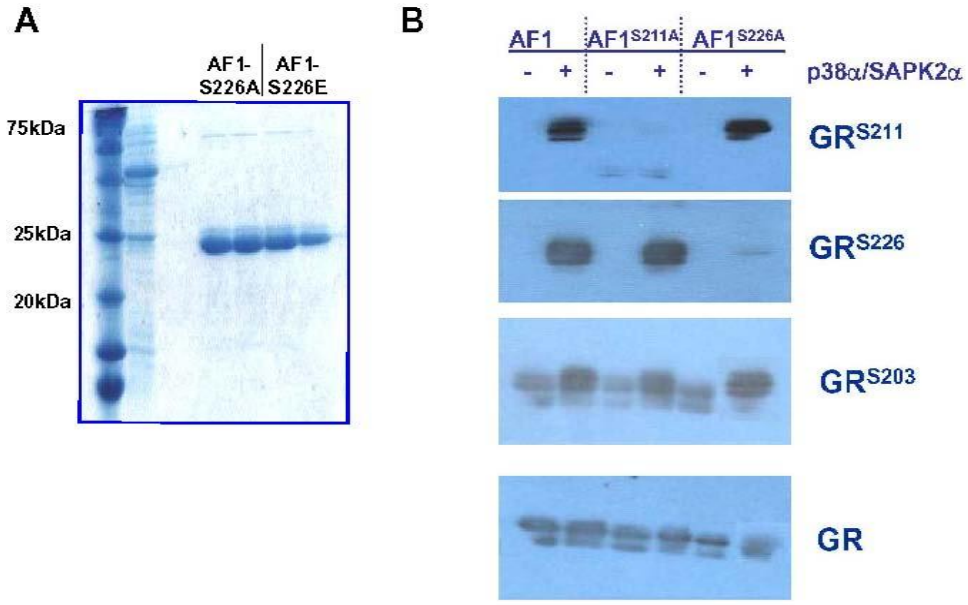



Figure 31: Generation of AF1-S226A and AF1-S226E protein and *in vitro* phosphorylation with p28 MAPK.

Purified AF1, AF1-S211A and AF1-S226A protein were incubated with assay dilution buffer alone and/or p38 active kinase. ATP cocktail was added to all samples except the buffer control. Samples were then incubated in a 30°C water bath and analyzed after two hours. Immunoblot analysis with antiserum to hGR and antisera raised against S211, S226, and S203 phosphorylation sites in the AF1 of the GR indicated phosphorylation of all sites with p38 kinase. Buffer +AF1 (unphosphorylated) yielded no positive reactions.

This data confirmed our previous findings that active p38 MAPK can phosphorylate all three relevant Serine sites in the AF1. Now the question remains as to

what the roles of the S226 and S203 phosphorylation sites are when looking at conformational changes and more ordered structure formation in GR's AF1.

Recently published data by Dr. Garabedian's laboratory showed that GR phosphorylation at S211 and S226 affects receptor transcriptional activation in a gene-specific manner. In addition they found that this regulation can be both positive and negative, and the relative level of phosphorylation at S211 *vs*. S226 is an important determinant of receptor activity (Chen et al., 2008). Further they concluded based on current and previous findings that phosphorylation of GR S226 by JNK causes receptor nuclear export when no hormone is supplied, a condition that favors low S211 and high S226 phosphorylation levels and that phosphorylation at S226 reduces GR transcriptional activation through some general mechanism such as the enhanced nuclear export (Rogatsky et al., 1998b; Itoh et al., 2002; Chen et al., 2008).

Based on these as well as my own observations I decided to examine the role of S226 site on the structure of the AF1 protein. I performed proteolytic digestions with trypsin or chymotrypsin on phosphorylated vs. un-phosphorylated protein to determine influence on tertiary structure formation. As determined in Figure 31, p38 MAPK phosphorylated the S226 site but not the S226A mutant. This data will give us some initial insight to the role this site (S226) plays on tertiary structure formation in the recombinant protein *in vitro* system.

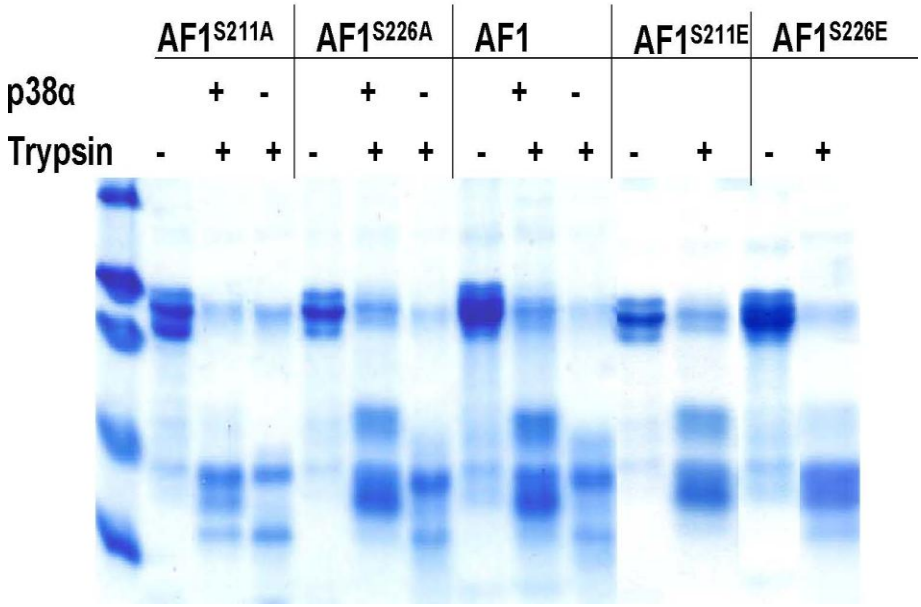



Figure 32: Partial Proteolysis of AF1 and the S211 and S226 mutant protein

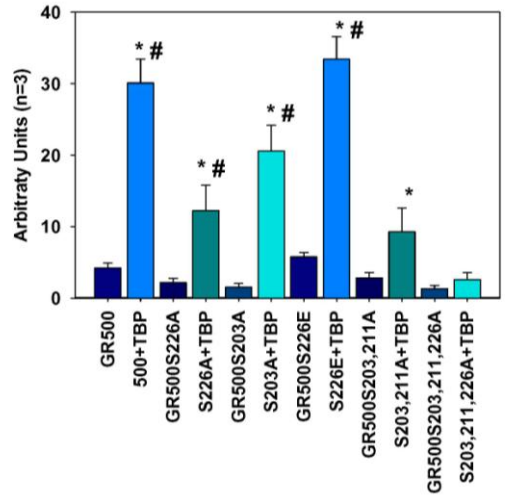
Digestion of 10µg purified AF1, AF1-S211A, S226A and AF1-S211E, S226E was carried out using sequencing grade trypsin (Sigma-Aldrich) at 4°C for 5 in 20mM Tris, 50mM NaCl, at a protein: enzyme mass ratio of 100:1. Reactions were terminated by adding SDS loading buffer and boiling for 5minutes. Digested samples were run on SDS-PAGE gel and stained by Coomassie R-250. $AF1^{P}$; $S211A^{P}$; $S226A^{P}$ were in vitro phosphorylated by p38 kinase. SDS-PAGE gel is a representative of four independent experiments with similar results.

Partial proteolysis experiments of AF1 and its mutant show that AF1-S211E, AF1-S226E, AF1-P and AF1-S226A-P are all protected from complete proteolysis by trypsin. Furthermore, AF1-S211A-P and AF1 are almost completely digested after 5 minutes trypsin incubation. AF1 when phosphorylated at S211, S226 and S203 is less digested than the S211A mutant, when phosphorylated at S226 and S203. AF1S226A, when phosphorylated at S211 is only minimally less or as protected as wild type AF1. AF1-S211E is more protected than AF1-S226E, confirming S211 as the "more important" site for folding. Overall, the data confirm that S211 is the main phosphorylation site that is responsible for conformational changes in AF1. Furthermore, the negative charge on S211E and S226E seems to aid in tertiary structure formation.

This finding reestablishes S211 as the major site for conformational change induction and is enough preliminary evidence to satisfy the Aim of this project. However, it will be interesting and valuable to see the contribution of the S203 site to make a final conclusion. Even though, one might expect there may not be more changes since previous data established the phosphorylated S203 site did not contribute to secondary and tertiary structure formation when the S211 site was mutated to alanine. Also all these experiments focused on the influence of p38 MAPK phosphorylation, and as already discussed, evidence exists that other kinases also play a role in the transcriptional activity of the GR and apoptosis. So, further studies should focus on the effect of ERK and JNK on conformational change induced in AF1.

The next question that arises after further establishing and confirming the role of the other two phosphorylation sites (S203, S226) on the structure of AF1 is their effect on function of the receptor. Immunoprecipitation results show that phosphorylation of AF1 significantly facilitates its interaction with all the three coregulators tested.

As already established in Chapter 3 of this thesis, AF1 interacts directly with TBP, CBP, and SRC-1 in the nuclei of GR-deficient CV-1 cells measures by FRET. The enhancement of GR-induced transcription by TBP, SRC-1 or CBP is achieved through the AF1 region. Furthermore, mutation S211A significantly decreases or even diminishes interactions with coregulators and GR-induced transcription. But once again, what about the S203 and S226 sites? To test the effects of S203 and S226 on protein-protein interactions and GR-induced transcription the following mutants were generated: 1) GR500S226A, and –E; 2) GR500S203A; 3) GR500S203,211A; 4) GR500S203,211,226A. These mutants were used to carry out FRET and SEAP assays to measure biological activity. CV-1 cells that do not have endogenous GR were used to transiently transfect the GR500 constructs with or without the co-regulatory protein.



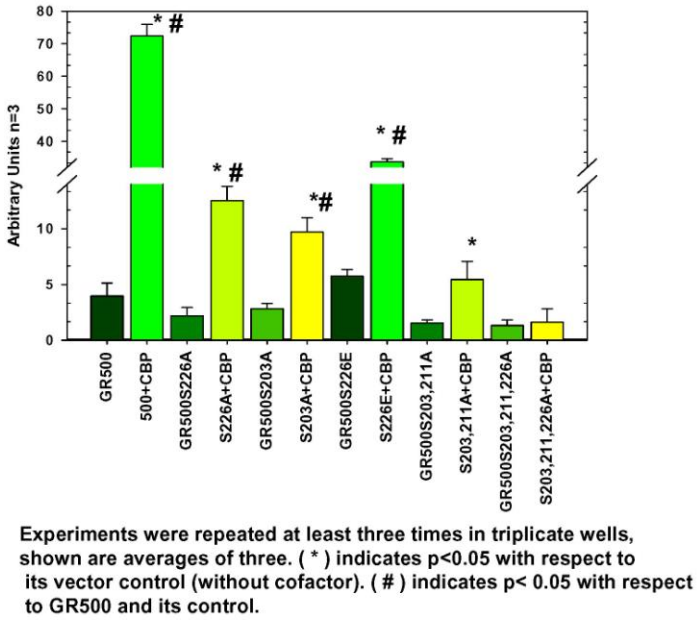

Experiments were repeated at least three times in triplicate wells, shown are averages of three. ( * ) indicates p<0.05 with respect to its vector control (without cofactor). ( # ) indicates p< 0.05 with respect to GR500 and its control.

Figure 33: Effect of S203 and S226 on GRE dependant GR transcriptional activity with and without co-transfection of TBP.

CV-1 cells constitutively expressing AF1 in a two domain GR fragment containing entire N-terminal and DNA-binding domains (GR500), or indicated mutants were cotransfected with DNA of the pGRE-SEAP plasmid alone or plus DNA for TBP. SEAP activity was measured after 24 and 48h of transfection and activity was normalized to transfection efficiency.

The experiments resulted in the following observations: 1) GR500S203A and -S226A alone decrease GR-mediated basal transcription activity just like 211A does; 2) co-transfection with TBP, however, increases the transcriptional activity significantly beyond the basal levels of GR500 (unlike S211A+TBP); 3) TBP induced increase of the S203A and S226A mutant does not reach the GR500+TBP levels; 4) GR500S203,211A also shows significant increase in transcriptional activity when co-transfected with TBP but this increase is not significant with respect to GR500 activity; 5) the triple mutant looses most of its activity regardless the addition of TBP.

The same experiments were repeated with addition of CBP and SRC-1.



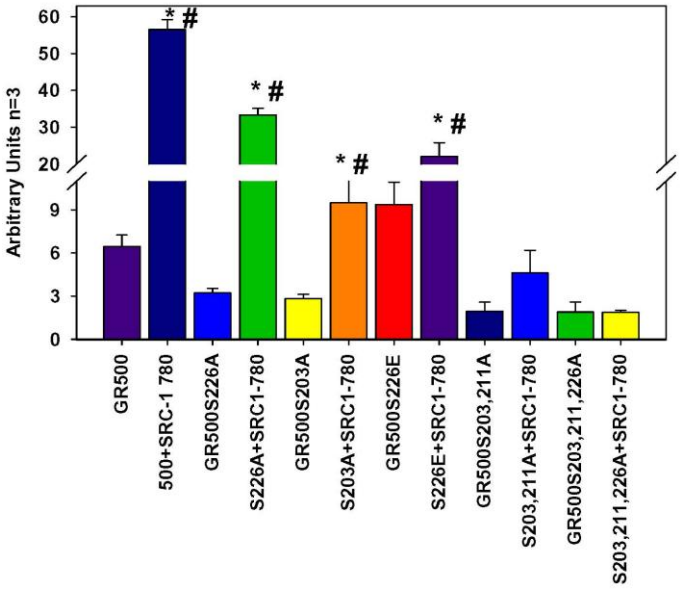

**Experiments were repeated at least three times in triplicate wells, shown are averages of three. ( * ) indicates p<0.05 with respect to its vector control (without cofactor). ( # ) indicates p< 0.05 with respect to GR500 and its control.**

Figure 34: Effect of S203 and S226 on GRE dependant GR transcriptional activity with and without co-transfection of CBP.

CV-1 cells constitutively expressing AF1 in a two domain GR fragment containing entire N-terminal and DNA-binding domains (GR500), or indicated mutants were cotransfected with DNA of the pGRE-SEAP plasmid alone or plus DNA for CBP. SEAP activity was measured after 24 and 48h of transfection and activity was normalized to transfection efficiency.

These set of data revealed that GR500S203A and -S226A alone decrease GR-mediated basal transcription activity just like 211A does. Furthermore, co-transfection

with CBP, however, increases the transcriptional activity significantly beyond the basal levels of GR500 (unlike S211A+CBP). TBP induced increase of the S203A and S226A mutant does not reach the GR500+CBP levels. GR500S203, 211A also shows significant increase in transcriptional activity when co-transfected with CBP but this increase is not significant to GR500 activity. Finally, the triple mutant looses most of its activity regardless addition of CBP.




**Experiments were repeated at least three times in triplicate wells, shown are averages of three. ( * ) indicates p<0.05 with respect to its vector control (without cofactor). ( # ) indicates p< 0.05 with respect to GR500 and its control.**

Figure 35: Effect of S203 and S226 on GRE dependant GR transcriptional activity with and without co-transfection of SRC-1.

CV-1 cells constitutively expressing AF1 in a two domain GR fragment containing entire N-terminal and DNA-binding domains (GR500), or indicated mutants were cotransfected with DNA of the pGRE-SEAP plasmid

alone or plus DNA for SRC-1. SEAP activity was measured after 24 and 48h of transfection and activity was normalized to transfection efficiency.

Similarly the experiments indicated in Figures 32 and 33, I found that GR500S203A and -S226A alone decrease GR-mediated basal transcription activity just like 211A does. Co-transfection with SRC-1, however, increases the transcriptional activity significantly beyond the basal levels of GR500 (unlike S211A+SRC-1). SRC-1 induced increase of the S203A and S226A mutant does not reach the GR500+SRC-1 levels. GR500S203, 211A does not significantly increase transcriptional activity when co-transfected with SRC-1 (unlike with TBP and CBP). The triple mutant looses most of its activity regardless of the addition of SRC-1.

I next used FRET to examine protein-protein interactions between GR500 and co-regulators (TBP, CBP and SRC-1). CV-1 cells were co-transfected with GR500-CFP and its various mutants and TBP-YFP, CBP-YFP, SRC-1-YFP co-regulators. Experiments were repeated three times and for each condition 10 random cells were chosen and indicated regions were bleached and the increase in fluorescence after photo bleach in the CFP channel was measured. Bar graphs show calculated FRET efficiency and error bars are SD of mean from the three experiments and 10 cells.

Examples of representative cells and corresponding are shown next; $p < 0.05$ (*); $p < 0.001$ (**); $p < 0.0001$ (***).

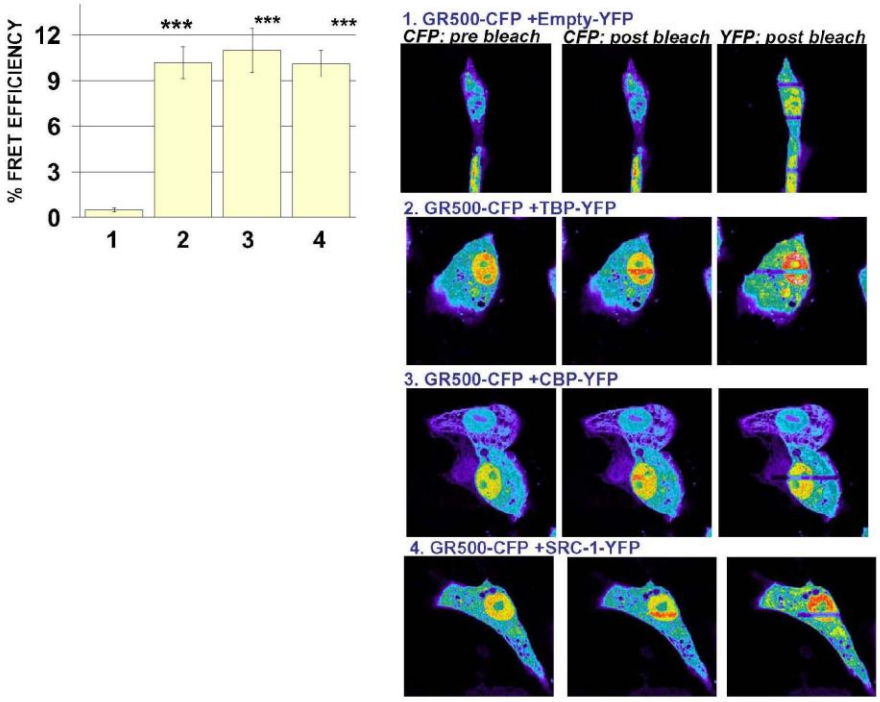



Figure 36 A: GR500 interacts with TBP, CBP and SRC-1

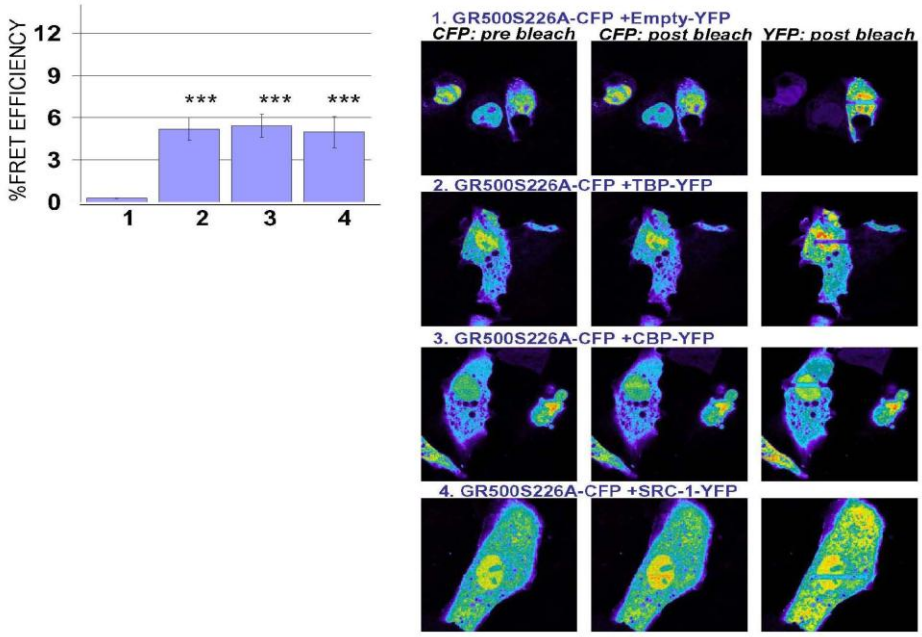



Figure 36 B: GR500S226A interacts with TBP, CBP and SRC-1 .

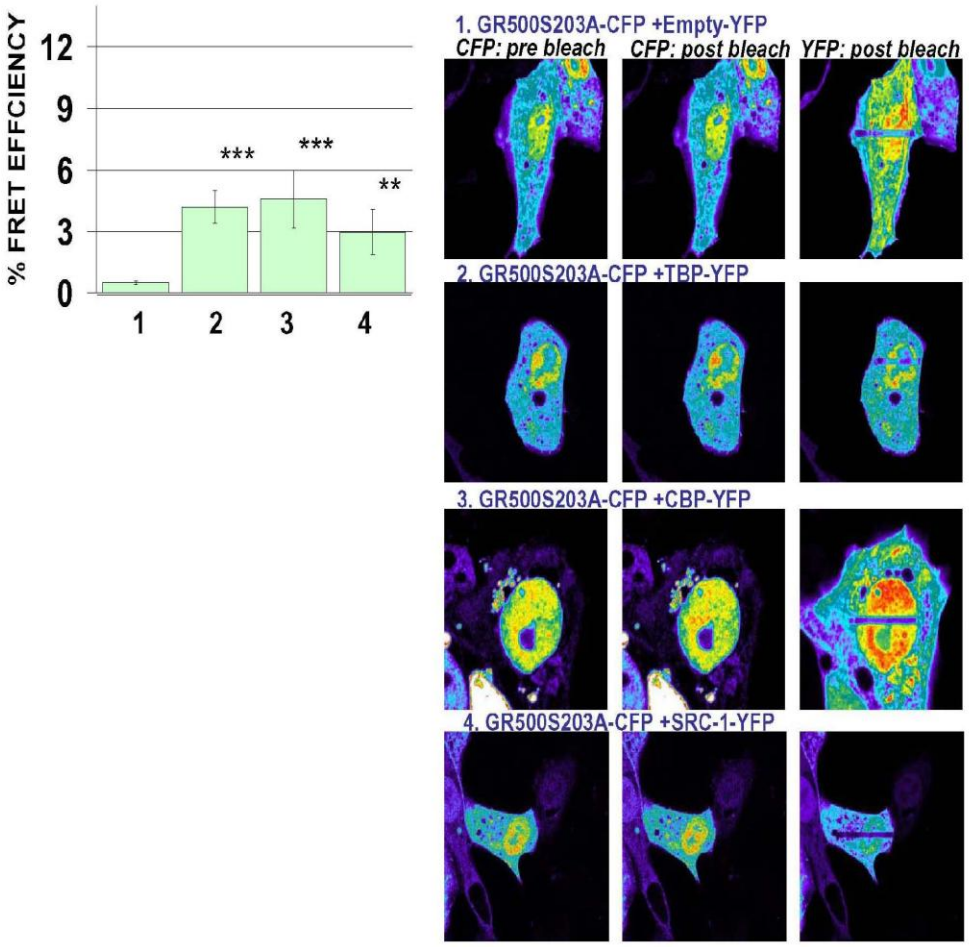



Figure 36 C: GR500S203A interacts with TBP, CBP and SRC-1

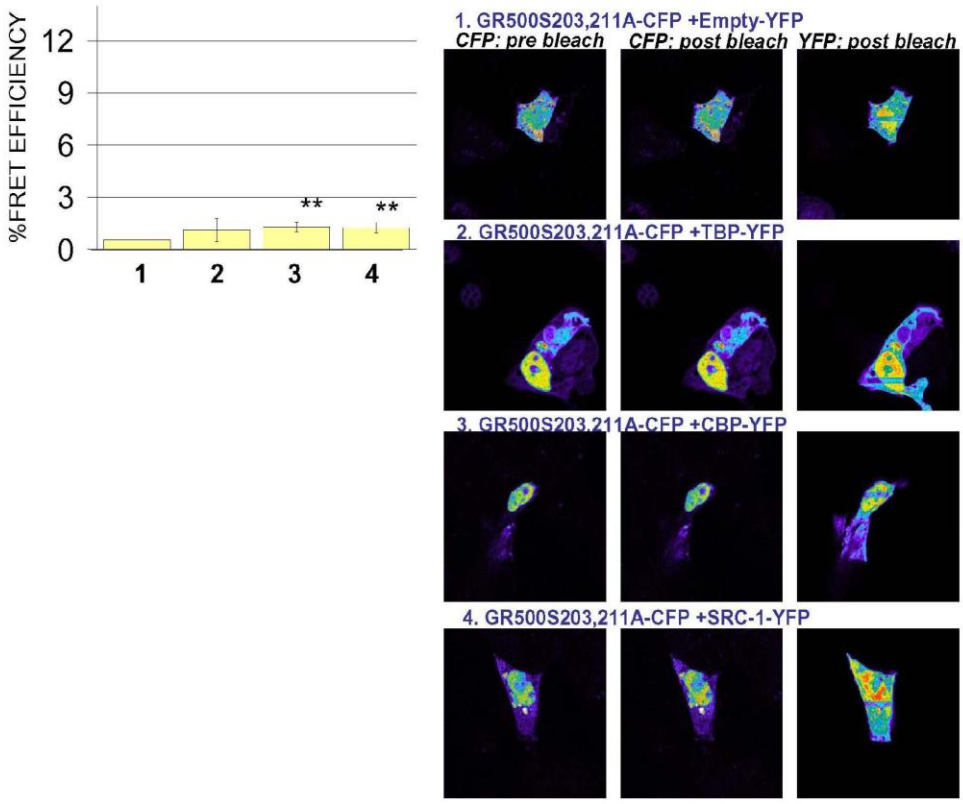



Figure 36 D GR500S203, 211A does not interact with TBP, CBP or SRC-1.

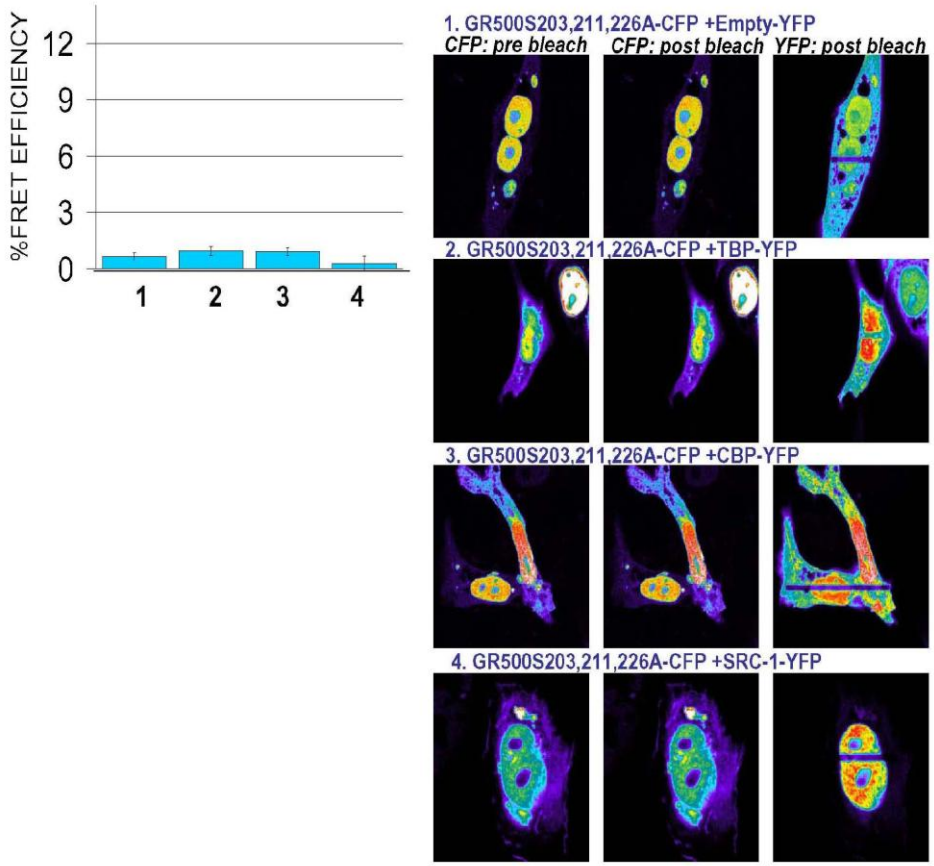



Figure 36 E GR500203, 211, 226A does not interact with TBP, CBP or SRC-1.

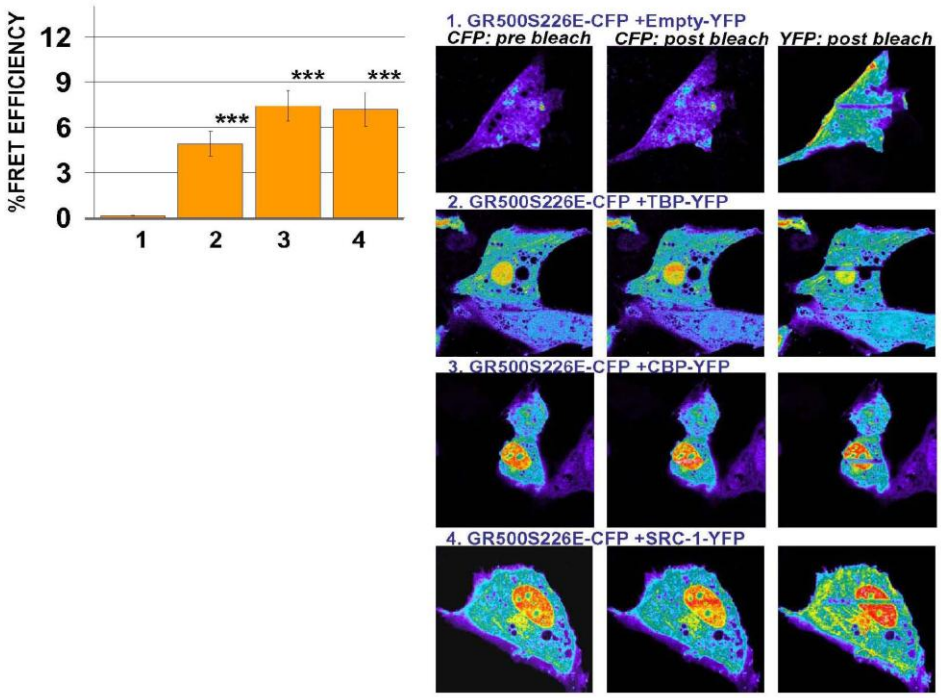



Figure 36 F: GR500S226E interacts with TBP, CBP and SRC-1.

The abscissa numbers correspond to the numbered conditions. To the right representative same-cell images in the donor (CFP) channel before and after PB; and YFP channel image is shown post bleach to demonstrate bleach efficiency. **A**, 1:GR500-CFP+ YFP-empty; 2:GR500-CFP+ TBP-YFP; 3: GR500+ CBP-YFP; 4: GR500+ SRC1-YFP. **B,** 1: GR500-S226A+ YFP-empty; 2: GR500-S226A+ TBP-YFP; 3: GR500S226A-CFP+ CBP-YFP; 4:GR500S226A-CFP+ SRC1-YFP. **C,** 1: GR500-S203A+ YFP-empty; 2: GR500S203A+ TBP-YFP ; 3: GR500-S203A+ CBP-YFP; 4: GR500-S203A+ SRC1-YFP. **D,** 1: GR500S203,211A-CFP+ YFP-empty; 2:GR500S203,211A-CFP+ TBP-YFP; 3: GR500-S203,211A+ SRC-1-YFP. **E,** 1: GR500-S203,211,226A+ YFP-empty; 2: GR500-S203,211,226A+ TBP-YFP; 3: GR500-S203,211,226A+ SRC-1-YFP.

**F,** 1: GR500-S226E+ YFP-empty; 2: GR500-S226E+ TBP-YFP; 3: GR500S226E-CFP+ CBP-YFP; 4:GR500S226E-CFP+ SRC1-YFP.

GR500S203A and -S226A co-transfected with CBP, TBP or SCR-1 show increased FRET. TBP induced FRET increase of the S203A and S226A mutant does not reach the GR500+coregulator levels. GR500S203, 211A does not shows significant FRET increase when co-transfected with TBP, CBP or SRC-1. The triple mutant looses most of its interaction capability regardless of which coregulator is introduced.

In conclusion, immunoprecipitation results show that phosphorylation [S211] of AF1 significantly facilitates its interaction with all the three coregulators tested. Furthermore, AF1 interacts directly with TBP, CBP, and SRC-1 in the nuclei of GR-deficient CV-1 cells as measured by FRET. The enhancement of GR-induced transcription by TBP, SRC-1 or CBP is achieved through the AF1 region. Finally, the S211A mutation has the greatest inhibitory effect on biological activity of AF1. We show for the first time, that ID AF1 domain of glucocorticoid receptor (GR) adopts a functionally folded conformation due to site-specific phosphorylation by p38 MAPK that we have earlier shown to be involved in the apoptotic and gene-inductive events initiated by GR.

This set of data has established that site specific phosphorylation has an effect on structure and function of the GR in an *in vitro* system. Moreover I found and described in chapter 4 that this activating phosphorylation is controlled by balanced MAPK activity in *in vitro* cell line models providing an additional mechanism for resistance. Despite all advances and progress in the field, the exact mechanism of GRs action and resistance is still unknown. Previous reports have shown that the GR is necessary but not sufficient for GC-dependant lymphoid cell death. Other known facts about GC driven apoptosis in lymphoid cells include the knowledge that GC's induce cell cycle arrest at $G_1$- or $G_0$ and that once this inhibition begins it is directly linked to apoptosis. GC's are involved in primary or secondary gene regulation. The first is rapid and involves the entry of the ligand-activated receptor into the nucleus (within minutes) and binding to open

regulatory sites on the DNA. Leading to altered transcription and protein expression of GC controlled targets, the total process is thought to take up to two hours. Secondary regulation requires new protein synthesis. Here, first the GC bound GR will undergo the same steps as in primary regulation but will also result in production of new proteins that will then regulate genes. The actions of cells exposed to GC's and destined for apoptosis strongly suggests that the slower mechanisms are involved. This can mean that the GC receptor must constantly act to modulate gene expression and that a gene network is probably involved. Recently, a wealth of data has been presented, showing important connections between steroid-driven and other signal transduction networks (Lange et al., 1999; Katzenellenbogen, 2000; Björnström and Sjöberg, 2005; Silva and Shupnik, 2007; Weigel and Moore, 2007). Our work based on CEM cell clones and extended to other cell lines, as well as that of others, has begun to reveal this network. An important action of GCs in the sensitive clones is to activate p38, a member of the mitogen-activated protein kinase (MAPK) system. Further Miller et al. found that p38 MAPK specifically phosphorylates the serine at position 211 in the human glucocorticoid receptor (Miller et al., 2005). Phosphorylation of S211 enhances the ability of the receptor to regulate transcription and apoptosis (Wang et al., 2002; Miller et al., 2005). Several pathways that modulate GC-dependent apoptotic sensitivity converge on the MAPK pathway (Miller et al., 2007). Many of these GC-regulated events depend on transcription; others are post-transcriptional protein modifications that affect enzymic activities. Several types of T- and B-lineage malignant cells can be converted from Dex-resistant to Dex-sensitive by certain pharmacological manipulations; including inhibition of ERK and JNK activity, blocking m-TOR (and suppressing $JNK^{P}$) with Rapamycin, and stimulating PKA. Successful conversion to Dex-sensitive results in induction of GR, at Ser211 phosphorylation dependant activating site, and Bim. Recently, it has been shown that activated JNK promotes Bim EL degradation (Leung et al., 2008). Treatments that reduce JNKP enhance Dex induction of Bim.

I have proven my hypothesis and shown that: ID AF1 domain of glucocorticoid receptor (GR) adopts a functionally folded conformation due to site-specific phosphorylation by p38 MAPK. p38 MAPK has been shown to be involved in the apoptotic and gene-inductive events initiated by GR. These conformational changes are important for AF1's interaction with coregulatory proteins, and subsequent GRE mediated transcriptional activity of the GR. This activating phosphorylation, specifically at S211, is controlled by balanced MAPK activity in *in vitro* cell line models providing and additional mechanism for resistance. In this scenario phosphorylated p38 levels are high relative to low ERK and JNK activity levels, further suggesting that p38 MAPK activity plays a role in structural and functional consequences of the GR.

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# Vita

Anna Stwora de Garza was born on January 23 1982 in Bytom, Poland. She is the daughter of Teresa and Mieczyslaw Stwora. She completed elementary and High School education in Berlin, Germany completing the German Abitur in 2001. She then moved to Edinburg, Texas where she received a bachelors of science majoring in biology, and graduated suma cum lauda, and completed the university honors program at the University of Texas Pan American in 2005. Since the beginning of her graduate school career she has been published as a second author in a peer reviewed journal and has two additional publications in submission where she is the first author. She presented her research at several international meetings and published numerous abstracts. She has gained experience in teaching by supervising several summer and graduate rotation students.

**Articles in Peer-Reviewed Journals:**

1. Miller AL, Garza S. A., Johnson BH, Thompson EB. Pathway interactions between MAPKs, mTOR, PKA, and the glucocorticoid receptor in lymphoid cells. Cancer Cell International 2007; 7-3.

2. Garza S. A., Miller AL, Johnson BH, Thompson EB. Converting cell lines representing hematological malignancies from glucocorticoid-resistant to glucocorticoid-sensitive: signaling pathway interactions. Article in Submission: Leukemia Research

3. Garza S. A., Kumar, R. Site-specific Phosphorylation of an Intrinsically Disordered Activation Domain of the Glucocorticoid Receptor Leads to Structural and Functional Consequences. Article in Submission Molecular Cell.

4. Garza S. A., Ahmad N., Kumar R. Role of Intrinsically disordered Protein Regions/Domains in Transcriptional Regulation. Review Article in Preparation.

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