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Functional analysis of glucocorticoid receptor phosphorylation

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Functional analysis of glucocorticoid receptor phosphorylation

by

Marija Krstic

DISSERTATION

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DOCTOR OF PHILOSOPHY

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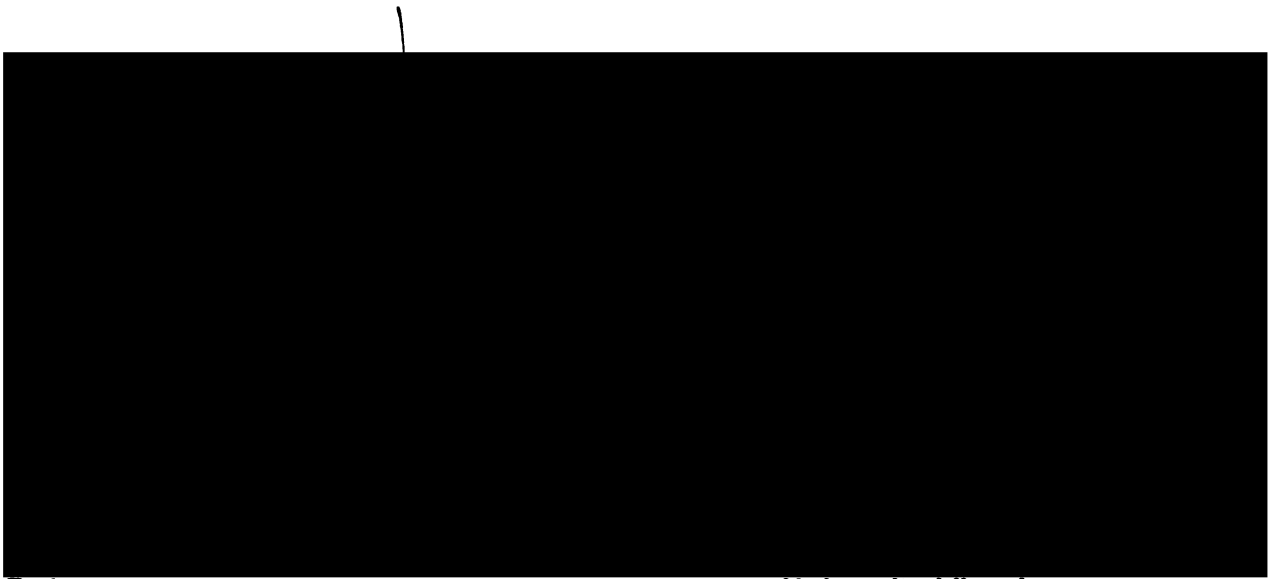
San Francisco



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Dedicated to my parents Koviljka and Dragomir

ACKNOWLEDGEMENTS

Fist, I want to thank many people for making graduate school easier for me. I want to thank my parents, who were always there for me with their support and their unconditional love; without them it would have been much more difficult to go through graduate school. I want to thank my friends, Branka, Lidija, Zeljko, Ljilja, Prota, Kristina, Sneza, and Jelena for providing emotional and financial support whenever they could, for which I am very grateful. I owe special thanks to Branka who cooked for me back in those days when I did not know how to bring lunch to Yamamoto lab group meetings. My friends Ann, Ravinder and Sandra helped me to understand American culture and enjoy its diversity and beauty. I want to thank Michael Garabedian who I worked with for a long time in the lab and who taught me how to do experiments over and over with the patience that he only has. I also owe my thanks to members of the Yamamoto lab for dealing very well with my daily mood swings and for discussing science with me even when they had better things to do. I thank members of Herskowitz and Guthrie lab, specially Mattias and Bruce for explaining yeast genetics to me and providing plates, toothpicks, and tubes, sometimes even without knowing it. People in Physiology Department Richard Weiner and Marry Dahlman were my student advisors and were always sympathetic to a big winner like me, Maurine and Pam helped me through administrative traps of UCSF, and Brad and Mike from Biochemistry Department were big help with purchasing all those tomorrow needed chemicals.

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Finally, I want no thank Keith for his brilliance, support and for suggesting ser-thr switch experiment.

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FUNCTIONAL ANALYSIS OF GLUCOCORTICOID RECEPTOR PHOSPHORYLATION

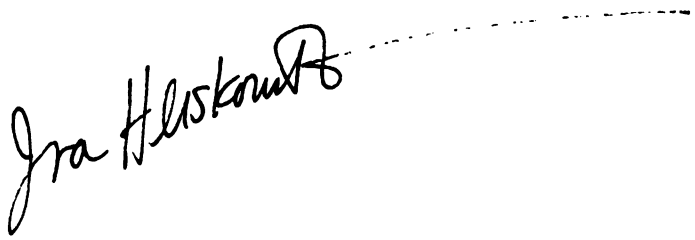
MARIJA KRSTIC

ABSTRACT

We have studied the role of phosphorylation in glucocorticoid receptor function using various experimental approaches involving both mammalian and yeast cells; in particular we studied how receptor function is modulated by phosphorylation, and the kinases involved in receptor phosphorylation.

In the first part of our study we examined receptor functions affected by phosphorylation. The rat glucocorticoid receptor is competent for signal transduction and transcriptional regulation when expressed in *Saccharomyces cerevisiae*. We found that the receptor in yeast, as in mammalian cells, was phosphorylated in the absence and presence of hormone, and that additional receptor phosphorylation accompanied agonist binding. The receptor was phosphorylated on Ser and Thr residues, and two-dimensional tryptic and V8 phosphopeptide maps of receptor from yeast and mammalian cells were qualitatively similar; we showed directly that T171, S224, S232 and S246 were phosphorylated in yeast *in vivo*, as they are in animal cells. T171 and S246 were phosphorylated constitutively, whereas phosphorylation of S224 and S232 was increased in the presence of hormone agonists. In both yeast and animal cells, transcriptional enhancement was reduced by mutation of S224 and increased by mutation of S246. In contrast, transcriptional repression was unaffected by either mutation. We conclude that the glucocorticoid receptor phosphorylation affects selectively its transcriptional regulatory functions.

In the second part, we investigated the kinases responsible for receptor phosphorylation. We found that cyclin-dependent kinase (CDK) and mitogen-activated protein kinase (MAPK) phosphorylate the rat glucocorticoid receptor *in vitro* at distinct sites that together comprise the phosphorylated receptor residues observed *in vivo;* MAP kinase phosphorylates receptor residues threonine 171 and serine 246, whereas cyclin-dependent kinase modifies serines 224 and 232. Mutations in these kinases have opposite effects on receptor transcriptional activity *in vivo.* Receptor dependent transcriptional enhancement is compromised in yeast strains deficient in the catalytic (p34 CDC28) or in certain regulatory subunits (cyclins) of the cyclin dependent kinase and is potentiated in a strain of yeast devoid of the mammalian MAP kinase homologues FUS3 and KSS1. These findings indicate that the glucocorticoid receptor is a target for multiple kinases *in vivo,* which regulate either positively or negatively receptor transcriptional enhancement. We conclude that the receptor receives and integrates information from multiple regulatory inputs, including steroids, and at least two signaling pathways that modulate distinct protein kinases.



CHAPTER I: Introduction

The goal of this study is to provide insight into the role of phosphorylation in glucocorticoid receptor function; in particular, what are the potential kinases involved in receptor phosphorylation and how is receptor function modulated by phosphorylation.

Protein phosphorylation

Polypeptide chain modifications play an important role in determining the activity of a particular protein. One of the most frequent posttranslational modifications of proteins is phosphorylation of serines and threonines and somewhat less often on tyrosines. There are thousands of proteins expressed in a typical mammalian cell, and many contain phosphorylated amino acids (Hubbard and Cohen, 1993). This reversible and relatively fast addition of phosphate groups onto proteins is an important mechanism by which physiological functions are coordinated in the cell. For example, the information carried out by extracellular peptide hormones can be transduced by membrane receptors and further amplified and transferred through a cascade of events to a final target protein (Treisman, 1994); activation of this protein then leads to a physiological response in the cell such as a change in the expression of genes involved in controlling metabolic rate, growth, differentiation etc.

Cellular enzymes that control protein phosphorylation are called kinases and phosphatases (Hunter, 1991). Kinases transfer a phosphate group from ATP onto target proteins and phosphatases remove the phosphate group from target proteins. About 200 protein kinases and 100 protein phosphatases have already been identified and recent genomic sequencing has revealed that perhaps 2-3% of

all eukaryotic genes may code for protein kinases (Hubbard and Cohen, 1993). For a certain number of kinases physiological substrates are well defined, as well as the amino acid sequences that are their target. Many protein kinases and phosphatases have multiple target proteins, and phosphatases typically show broader substrate specificity than kinases. Generally, these enzymes are specific for either serine and threonine or for tyrosine residues.

Kinases are involved in regulation of numerous cellular processes. For example, several signal transduction pathways involving a cascade of protein kinases converge on serine/threonine protein kinases referred to as mitogen activated protein kinases, MAPK, also known as extracellular regulated kinase-ERK (Figure 1.1., Marshall, 1994; Cooper, 1994). This kinase can be activated by phosphorylation on both tyrosine and threonine, by the so-called dual specificity kinase, MAPKK or MEK, which itself is phosphorylated and regulated by serine/threonine protein kinases. The activity of MAPKK appears to be regulated by several upstream pathways. For example, raf p74, the product of the c-raf proto-oncogene can phosphorylate and activate MAPKK (Kyriakis et al., 1992) as can Mos and MEKK. This signaling pathway starts with membrane receptors with seven transmembrane helices, or receptor tyrosine kinases. The activity of MAP kinases can also be regulated negatively by protein kinase A and protein phosphatase CL 100. This pathway in yeast is represented by three members of the MAP kinase family that control the mating pheromone response (SPK1 in S. pombe, FUS3/KSS1 in S. cerevisiae), cell wall biosynthesis (MPK1) and sensing of hyperosmotic environments (HOG). The mating pheromone response is the best studied and most resembles tyrosine kinase linked pathways in higher eukaryotes (Herskowitz, 1995). The fact that MAP kinase is involved in regulation of multiple processes in yeast initiated a search for homologous MAP kinases in mammalian cells and led to the discovery of mammalian stress activated and osmoregulated

Figure 1.1: Mitogen activated protein kinase (MAPK) and its regulation (from Cooper, 1994). MAP kinase can be stimulated by stress, mitogens and osmotic signals. It appears that in both yeast and mammalian cells MAP kinase pathways are involved in similar processes.

Figure 1.1

Budding yeast
Mammalian cells
Stimulus
Receptor
MAPKKK
MAPKK
MAP kinase
Response
Pheromone (haploid cells)
Heptahelical receptor
Gγ
STE20
STE11
STE7
FUS3
KSS1
Cell-cycle arrest
Mating-type gene expression
Low nitrogen
Pseudo-hyphal growth
Hypertonicity
Two-component system
PBS2
HOG1
Glycerol synthesis
Cell enlargement
Protein kinase C1
BCK1
MKK1
MKK2
MPK1
Cell-wall remodelling
Mitogenic stimuli
G-protein- and tyrosine kinase-linked receptors
Protein kinase C
Ras
Raf
MEKK
MEK1
MEK2
ERK1
ERK2
Immediate early gene expression
Proliferation or differentiation
Stresses
p38
SAPKs/JNKs

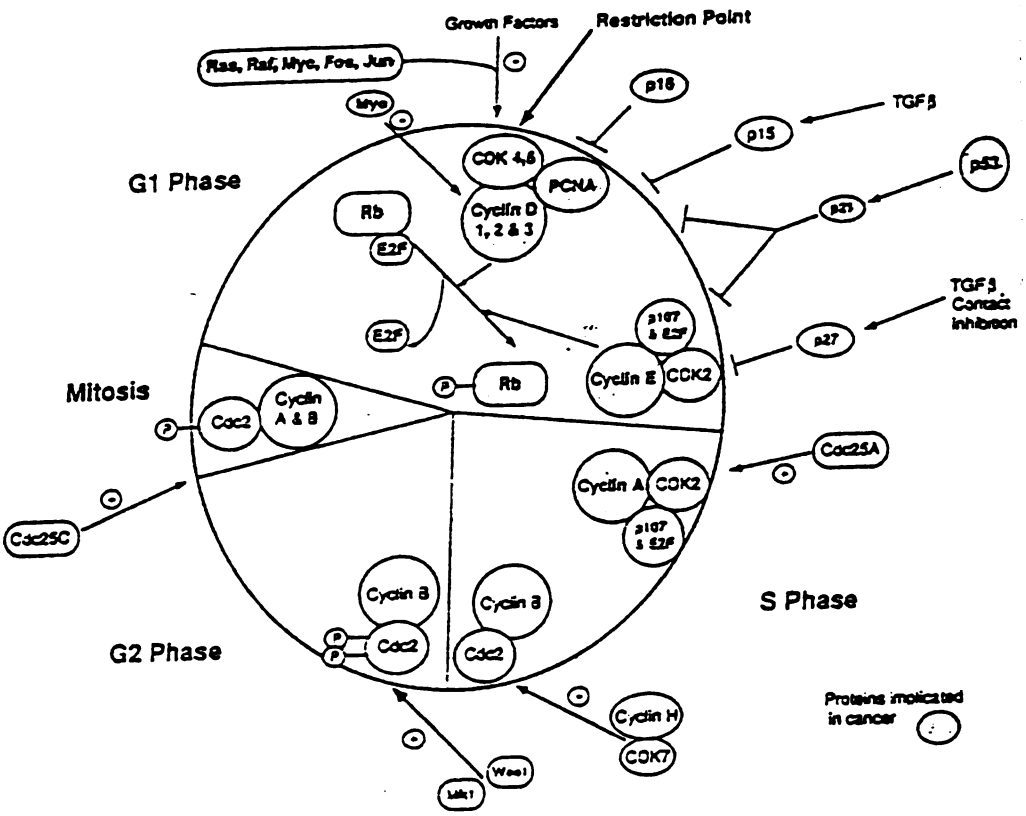
MAP kinases. MAP kinase potential substrates include cytosolic phospholipase A2, kinase p90 rsk, and number of transcription factors such as c-myc, c-jun, elk-1 in mammalian cells and proteins STE 12 and FAR1 in *S. cerevisiae.* As for all other kinases, whether these targets are true in vivo substrates remains to be determined.

Another example of a kinase with multiple family members is p34/CDC2/CDC28 cyclin dependent kinase (CDK), first discovered as a regulator of the cell cycle (Fig. 1.2) (Murray, 1994; Hunter and Pines, 1994). Eukaryotic cells contain several homologs of this kinase that seem to control different checkpoints of the cell cycle. This kinase is composed of a catalytic subunit, p34 and a regulatory subunit, cyclin. Cyclins were originally discovered by virtue of oscillations in their protein levels in sea urchin cleavage stage embryos. There are at least two groups of cyclins: G1 cyclins, which are required for activation of the kinase at START (G1 to S transition, when cells commit itself to another round of DNA replication) and G2 cyclins, which act at the G2-M boundary (when cells prepare for mitosis).

CDK enzymatic activity is stimulated by CDK association with cyclins and by dephosphorylation with the phosphatase CDC25, and can be inhibited by phosphorylation of CDC2 by wee1/mik1 related protein kinases in yeast. In addition, several macromolecular inhibitors of cdk-cyclin complexes have recently been isolated in mammalian cells. These small molecular weight proteins (p16, p15, p21, p27) inhibit the kinase function and target different CDK-cyclin complexes. The activity of many of these inhibitors can in turn be regulated by extracellular signals. For example, p15 mRNA and protein are induced more than 30-fold by TGF-β treatment of HACAT keratinocytes. Importantly, CDK-cyclin enzymes regulate processes other than the cell cycle. For example, the Pho85/Pho80 CDK-cyclin complex is involved in regulation of phosphate metabolism in *Saccharomyces cerevisiae* (Kaffman et al., 1994); in mammalian cells CDK5 is

Figure 1.2: Cyclin dependent kinase (CDK) and its regulation (from Hunter and Pines, 1994). Transitions between different cell cycle states are regulated at checkpoints by multiple cyclin-CDK complexes. These complexes are controlled by regulatory networks of growth factors, tumor suppressors and oncogenes.

Figure 1.2


Growth Factors
Restriction Point
Ras, Raf, Myc, Fos, Jun
Myc
p16
TGFβ
p15
p53
p21
G1 Phase
COK 4,6
PCNA
Cyclin D 1, 2 & 3
Rb
E2F
TGFβ Contact inhibition
p27
p107 & E2F
Cyclin E
COK2
Mitosis
Rb
Cdc2
Cyclin A & B
Cdc25A
Cdc25C
Cyclin A
COK2
p107 & E2F
Cyclin B
Cdc2
S Phase
G2 Phase
Cyclin H
COK7
Wee1
Mik1
Proteins implicated in cancer

highly expressed in postmitotic neurons, and it is likely involved in controlling functions other than cell cycle (Tsai et al., 1994).

There are many potential target proteins for CDK (Nigg, 1993). One molecule regulated by phosphorylation by these kinases is the tumor suppressor protein Rb. The Rb is underphosphorylated throughout G1 phase, phosphorylated before S phase and remains phosphorylated until late mitosis. Hypophosphorylated RB arrests cells in G1 phase and its phosphorylation relieves this inhibition. In addition, these kinases reportedly phosphorylate transcription factors SWI5, c-myb, c-fos/jun and Oct1 (Moll et al., 1991; Lucher and Eisenman, 1992; Abate, et al., 1991; Roberts et al., 1991).

Phosphorylation of transcriptional regulatory factors

Transcription can be divided into three phases, initiation, elongation and termination (Yanofsky, 1992). These three phases of transcription are complex and can be controlled by several types of regulatory proteins. In the key regulated event in the initiation of mRNA synthesis in eukaryotes, so called general transcription factors assemble and function at the promoter in ways that typically involve stimulation or repression by transcription regulatory factors.

Many transcriptional regulatory factors are phosphoproteins. There are at least three predominant effects of phosphorylation on activity of regulatory factors. First, transcription factors can be sequestered in the cytoplasm and their nucleocytoplasmic shuttling can be modulated by phosphorylation. For example, phosphorylation regulates nuclear localization of the SWI5 transcription factor by phosphorylation (Moll et al, 1991). Its activity is required for cell cycle regulation of mating type switching in *Saccharomyces cerevisiae*. SWI5 is held in the cytoplasm by phosphorylation by CDC28 kinase. In the G1 phase of the cell cycle, SWI5

localizes to the nucleus in its active, unphosphorylated form. Another example is regulation of NFkB nuclear localization. NFkB is a transcription factor that is complexed in the cytoplasm with IkB, an inhibitor protein (Ghosh and Baltimore, 1990). Phosphorylation of IkB appears to be necessary for dissociation from NFkB, which translocates to the nucleus and activates transcription of target genes. A recent report from Finco et al. (1994) suggests that there may be additional events, such as phosphorylation of NFkB, that are also involved in controlling association and dissociation of IkB/NFkB complexes.

A second activity of transcription factors that can be regulated positively or negatively by phosphorylation is DNA binding. For example, the serum response factor, SRF binds and activates the c-fos promoter through the SRE sequence (Treisman, 1994). Mutational analysis suggests that phosphorylation of several serines plays important role in the kinetic effects on DNA binding *in vitro* (Janknecht et al. 1992; Marais et al., 1992). Phosphorylation of the *Rhizobium meliloti* FixJ protein (involved in the transcriptional activation of nitrogen fixation genes) induces its binding to a regulatory region at the FixK promoter (Galinier et al., 1994). In contrast, phosphorylation of Oct-1 protein correlates with inhibition of Oct-1 DNA binding activity *in vivo* and *in vitro*. Oct-1 is a transcription factor involved in cell cycle regulation of histone H2B gene transcription, and is hyperphosphorylated in mitosis. A mitosis-specific phosphorylation site is in the homeodomain of Oct-1 and is phosphorylated *in vitro* by PKA (Segil et al., 1991).

Several mechanisms may account for these effects of phosphorylation on DNA binding. In cases where phosphorylation negatively influences the binding, it is possible that phosphates that are negatively charged interfere with the binding by electrostatic repulsion or that phosphorylation alters the conformation of a protein in a way that would change its DNA binding properties. In principle, DNA binding can also be affected if dimerization of the factor is modulated by phosphorylation.

In fact, there are several examples of both positive and negative influence of phosphorylation on oligomerization. Heterodimerization of human thyroid receptor β1 (hTRβ1) and retinoid receptor (RXRβ) appears to be enhanced by phosphorylation of thyroid receptor on serine residues (Bhat et al., 1994). The IL-4STAT transcription factor is phosphorylated on tyrosine residues, and this phosphorylation regulates its activity by enhancing dimerization and mediating protein-protein contact with the IL-4 receptor (Hou et al, 1994). In fact, an SH2 domain both free and complexed with a phosphotyrosine peptide has been recently crystallized. The high affinity SH2-peptide complex resembles a two-hole socket (the SH2 domain), engaging a two-pronged plug (the phosphopeptide). Comparison of the complexed and uncomplexed form of Src SH2 reveals that highly localized and relatively small conformational changes occur upon peptide binding (Waksman et al, 1993). However, similar information for phosphoserine or phosphothreonine is not available yet.

Third, phosphorylation may affect enhancement or repression functions of transcriptional regulators. The CRE- binding protein, CREB is part of a signaling pathway in which the increase in cAMP leads to the activation of CRE-containing genes. Increased cAMP activates PKA which in turn phosphorylates CREB on Ser 133 (Gonzales and Montminy, 1989); this phosphorylation event stimulates the interaction of CREB, which is already CRE-bound, with CBP (CREB-binding protein), which activates transcription (Nordheim, 1994). Another example of signal transduction from the membrane to the specific target genes via phosphorylation is regulation of the c-fos promoter. Growth factor stimulation causes phosphorylation of transcription factor Elk-1, a component of a ternary complex that is involved in regulation of this promoter (Marais et al, 1993). Phosphorylation by MAP kinase of several ser/thr-pro motifs localized in transcriptional activation domain increases ability of ELK-1 to activate

transcription. In other cases, transcriptional regulatory activity can be inhibited by phosphorylation. For example, the phosphorylation by PKA of ADR1, a yeast transcription factor that regulates ADH2 expression, blocks its transcriptional activation function (Cherry et al., 1989).

The effect of phosphorylation on transcriptional repression is less clear. Phosphorylation has been suggested to negatively regulate ETS-related transcription factor yan required for Drosophila eye development (Brunner et al., 1994). In this model, Ras1/MAP kinase phosphorylates and down regulates either DNA binding or transcriptional repression activity of this protein. Similarly, disruption of the PKA consensus sequence markedly diminishes DNA binding, transcriptional repression and transformation by v-rel oncogene. However, mutation of the serine 275 at the mentioned PKA consensus site had no effect on v-rel function, suggesting that phosphorylation of this site is not the key determinant of transcriptional repression (Walker et al., 1992).

Two basic mechanisms have been proposed to account for the effects of phosphorylation on enhancement. First, when phosphorylation affects transcription factor enhancement positively, it may increase its affinity for the component of a basal transcriptional machinery. This can happen by increasing the charge of an activation domain or by changing its conformation. Second, when phosphorylation exibits negative effects on factor activity, phosphorylation might mediate association of the regulator with an inhibitory protein.

In summary, most transcription factors are phosphoproteins and it has been shown in some cases that their activities can be altered by phosphorylation. However, in few cases have molecular mechanisms of phosphorylation-mediated modulatory effects been described.

Steroid hormones and receptors

Intercellular communication is mediated by various regulatory molecules that transduce chemical information. These molecules are involved in the coordination of complex metabolic processes, such as adaptation to the changing environment and preparing the organism for reproduction. A major class of these regulatory molecules are hormones, which can be divided into at least two subclasses: protein hormones and steroid hormones. In principle, these hormones elicit their effects on target cells by different mechanisms: protein hormones bind to membrane receptors that in turn activate a cascade of second messengers, while steroid hormones bind to intracellular receptors, which directly regulate expression of specific genes.

Steroid hormones are derivatives of cholesterol and include glucocorticoids, progestins, estrogens, mineralocorticoids, and androgens. These hormones are secreted by gonads and the adrenal gland into circulation and are distributed to target tissues where they elicit specific cell responses. In the circulatory system, steroid hormones are present both free and bound to plasma proteins; circulating glucocorticoid hormone levels oscillate over 24 hr intervals (circadian rhythm). Circulating glucocorticoids are mostly bound to corticosteroid-binding globulin (CBG or transcortin), which likely keeps the hormone in circulation and serves as a buffer for availability of free hormone (Baxter and Tyrell, 1987).

A subclass of sex steroids that includes androgens, progestins and estrogens affects gene expression in reproductive tissues. In contrast, glucocorticoids regulate intermediary metabolism and an array of other processes in a wide variety of cells, and mineralocorticoids regulate electrolyte homeostasis in particular cell types.

The main focus of this study is on glucocorticoid receptor function, but many of the principles of their action are in common with the other steroid receptors.

Glucocorticoid hormones are important physiological regulators and are essential for survival. They were named for their glucose regulatory properties and influence carbohydrate, lipid, protein and DNA metabolism, they elicit anti-inflamatory and anti-allergenic effects and are permissive to the action of catecholamines and glucagon. Glucocorticoid target tissues can be divided into two classes: catabolic and anabolic. In catabolic tissues (the lymphatic system, skin, muscles, connective and adipose tissues), glucocorticoids cause the repression of synthesis of particular proteins and their faster degradation, catabolism of lipids and nucleic acids and lowered levels of glucose and amino acid usage (Milward, 1980; Fain, 1979). Anabolic effects of glucocorticoid hormones are the most obvious in the liver and kidney where they increase synthesis of enzymes that are involved in deamination of amino acids and in gluconeogenesis. Glucocorticoids also increase the transport of amino acids and glucose into these tissues (Rousseau, 1972).

Figure 1.3 shows a simplified view of the steroid hormone receptor signal transduction pathway. In this scheme, once the glucocorticoid hormone reaches the cytoplasm, the hormone binds to the glucocorticoid receptor protein. Hormone-receptor complex translocates to the nucleus, binds DNA and regulates transcription of target genes by increasing or decreasing the rate of transcriptional initiation. In detail, the transduction mechanism is more complex as the receptor interacts with several other proteins (Fig. 1.4). For example, heat shock protein 90 (Hsp90) is involved in ligand binding (Bohen and Yamamoto, 1994) and there is genetic evidence suggesting that steroids can be actively transported through the membrane (Kralli, A., Bohen, S., Yamamoto, K.,R., in press). The focus of this study is on interaction of serine/threonine kinases with the glucocorticoid receptor. Specifically, I will describe receptor phosphorylation before or after ligand binding and the signals downstream and upstream from these kinases.

Glucocorticoid receptor was originally purified from the rat liver by using its ability to bind DNA (Wrange et al., 1979). This 90 kD protein can be divided into three functional domains, as also seen in other steroid receptors (Fig. 1.5) (Wrange et al., 1984). The N-terminal region of the receptor is relatively susceptible to proteolytic degradation and contains most of immunogenic epitopes. This region is involved both in transcriptional activation (Godowski et al., 1988) and repression (Pierce and Yamamoto, 1993) although these regulatory regions are not well mapped. Adjacent to it is the DNA binding region, which contains two zinc fingers and mediates receptor dimerization and displays the highest degree of homology within the steroid receptor family. The C-terminal signaling domain binds hormone and also binds heat shock protein 90 (Hsp 90); receptor mutants lacking this region are constitutive activators of transcription (Godowski et al, 1987).

Phosphorylation of steroid receptors

The glucocorticoid receptor is a member of the steroid-thyroid hormone receptor family, and most members of this protein family are phosphoproteins. The best studied receptor from the aspect of phosphorylation is the progesterone receptor, and its phosphorylation occurs on multiple serine residues both in the absence and presence of hormone. Progesterone receptors in chick oviduct, rabbit uterus and human breast cancer cells exibit significant amounts of phosphorylation in the absence of hormone and undergo an increase in phosphorylation upon hormonal stimulation (Logeat et al., 1985; Dougherty et al., 1982; Sheridan et al., 1984; Takimoto et al., 1992). In the chicken progesterone receptor, phosphorylation occurs at four sites, three of which are within the N-terminal region and a fourth site within the hinge region between the DNA binding and the hormone binding domains (Polleti and Weigel, 1993). Both the N-terminal and the hinge region of

Figure 1.3: Signal transduction by the glucocorticoid receptor: simplified view. Glucocorticoid receptor is localized in the cytoplasm in its inactive state. Upon hormone binding, ligand-receptor complex undergoes activation and translocation to the nucleus, where it binds DNA and regulates expression of target genes by increasing or decreasing the rate of transcriptional initiation.

Figure 1.3

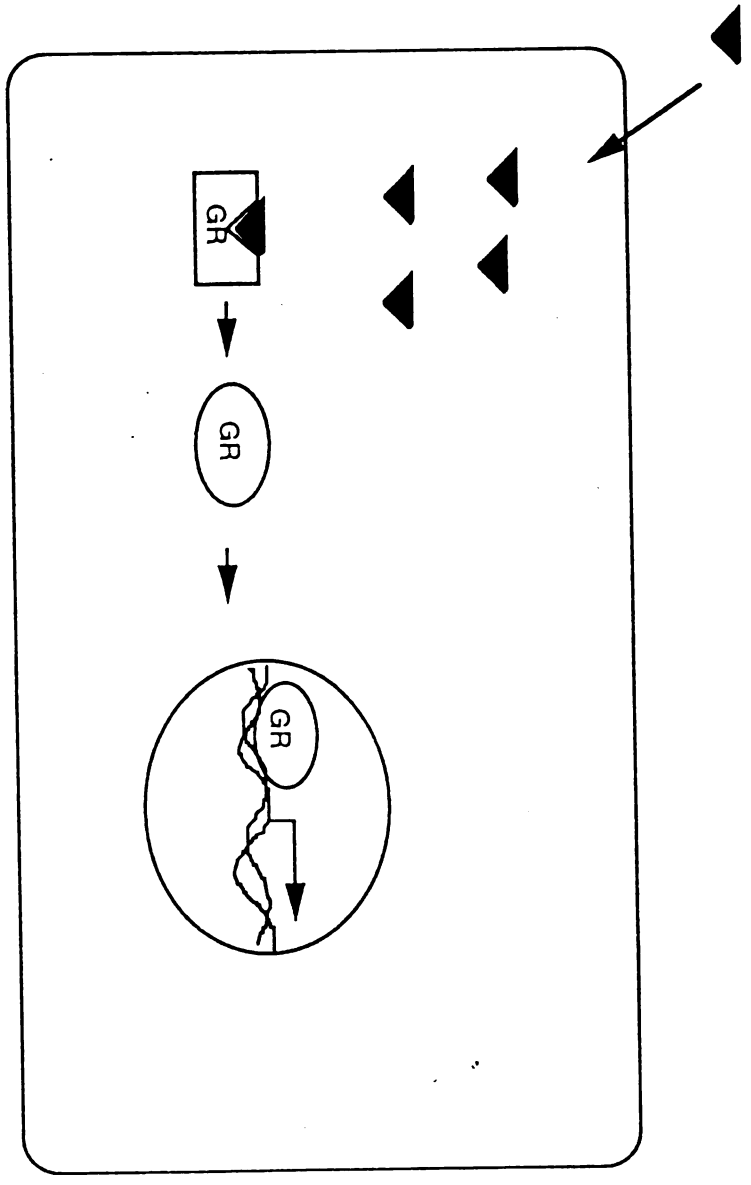


Figure 1.4: Signal transduction by the glucocorticoid receptor: recent view. Glucocorticoid receptor interacts with the network of accessory proteins, such as heat shock protein 90 (HSP 90) or serine/threonine kinases. The latter interaction and its implications is the focus of this study.

Figure 1.4

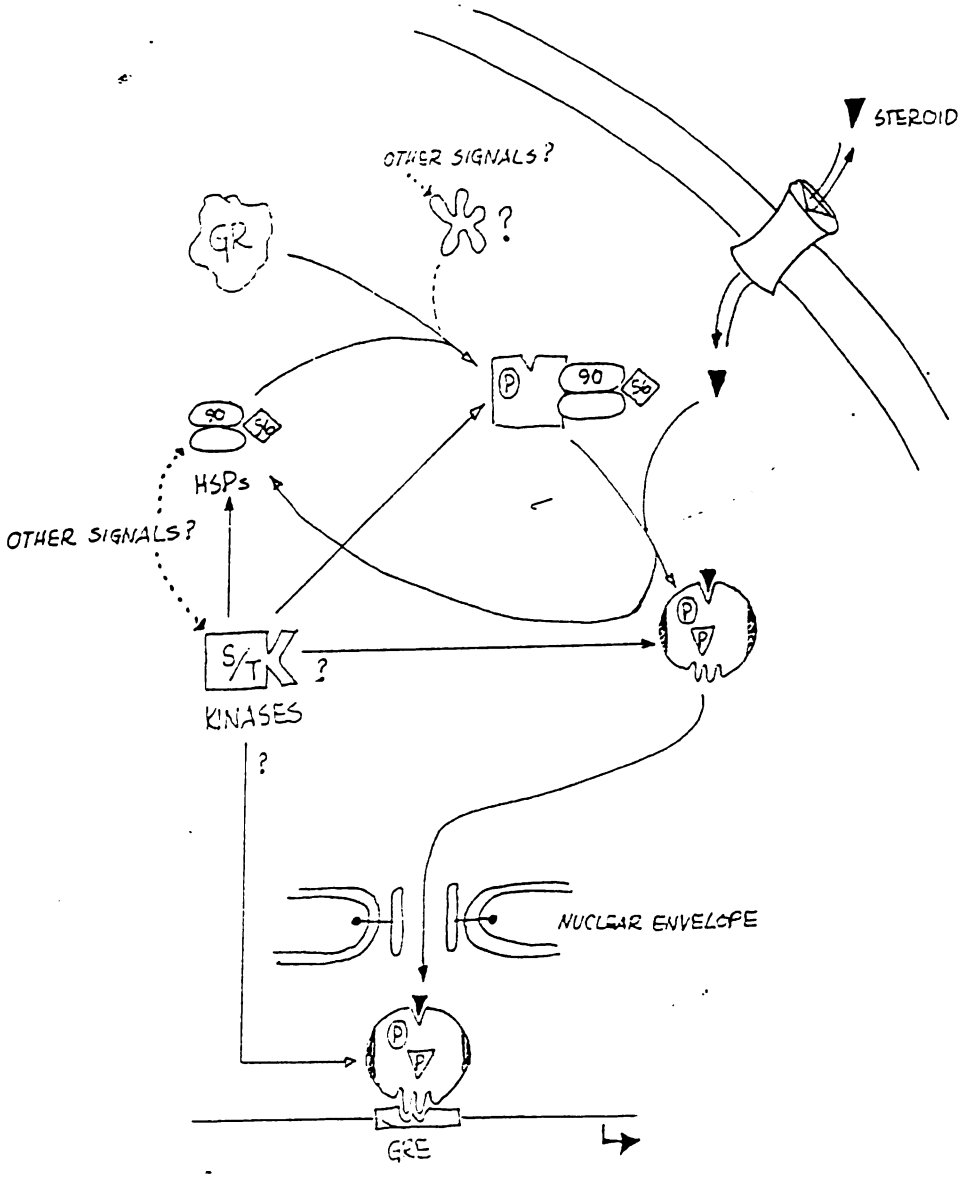
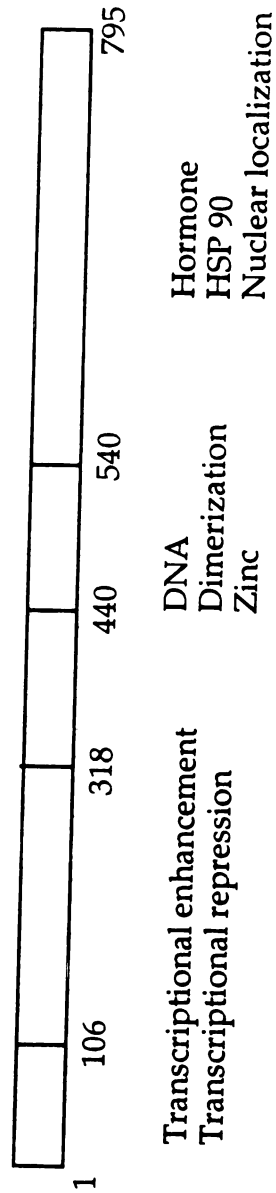

STEROID
OTHER SIGNALS?
GR
?
P
90
56
90
56
HSPs
OTHER SIGNALS?
S/T K
?
KINASES
?
P
P
NUCLEAR ENVELOPE
P
P
GRE

Figure 1.5 Domains of glucocorticoid receptor. Receptor is divided into three functional domains: the C-terminal part of protein, which binds ligand and HSP 90; the middle part which binds DNA, zinc and is involved in dimerization, and the N-terminal part, which includes sequences involved in transcriptional enhancement and repression.

Figure 1.5


1
106
318
440
540
795
Transcriptional enhancement
Transcriptional repression
DNA
Dimerization
Zinc
Hormone
HSP 90
Nuclear localization

the progesterone receptor have been suggested to be important for transcriptional regulation, suggesting that this function of the progesterone receptor may be regulated by phosphorylation.

These four phosphorylation sites contain Ser-Pro motifs, which is a minimal consensus sequence for the p34/CDC28/CDC2 kinase and MAP kinase. Two of the sites in the chicken PR show substantial basal phosphorylation, whereas the two flanking the DNA binding domain are phosphorylated extensively only in the presence of hormone (Poletti and Weigel, 1993).

The kinases and phosphatases involved in PR phosphorylation have not been identified. However, several signaling pathways appear to affect PR activity. For example, activators of protein kinase A (PKA) can activate PR expressed in monkey kidney CV1 cells even in the apparent absence of progesterone (Denner et al., 1990). PR from T47D human breast cancer cells can not be activated in a ligand independent fashion by this same treatment, but instead show a synergistic activation of 3-4 fold with progesterone (Beck, 1992). Interestingly, cAMP has been reported to convert certain progesterone antagonists into agonists (Sartorius et al., 1993). Similarly, chicken and human PR, but not GR, can be activated in a ligand-independent manner by a membrane receptor agonist, the neurotransmitter dopamine (Power et al., 1991). However, neither of above mentioned studies show that progesterone receptor is the direct target for the action of these agents. First, it is not clear whether the change in progesterone receptor activity after these treatments is the result of receptor modification or modification of some other protein involved in receptor function. Second, these agents likely activate cascades of kinases, and therefore the kinase that phosphorylates the receptor or the critical target may not be PKA but another kinase in this cascade.

Taken together, these results suggest that progesterone receptor function can be modulated through several signaling pathways. However, whether these effects

are mediated through phosphorylation of progesterone receptor and what the specific kinases involved are is not clear.

The mouse uterine estrogen receptor (ER) is phosphorylated on serine residues (Washburn et al., 1991). Similarly, ER from MCF-7 human breast cancer and calf uterine cells is also phosphorylated on serine residues (LeGoff et al., 1993). As with the progesterone receptor, certain ER phosphorylation events appear to be hormone dependent as the level of phosphorylation of human ER increases two-four fold within 20-40 min of estradiol treatment (Denton et al., 1992; LeGoff et al., 1993).

Several putative phosphorylation sites on serine residues on the human estrogen receptor have been identified, and some of those sites are localized close to the transcriptional activation domain. Mutating those residues to alanine causes a reduction in estrogen receptor-mediated transcriptional activation (LeGoff et al., 1993; Ali et al., 1993).

The kinases and phosphatases involved in estrogen receptor phosphorylation are not known. It has recently been shown that treatment of breast cancer cells with agents that increase intracellular cAMP can result in synergistic activation of estrogen receptor-mediated transcription by estradiol (Cho et al., 1993). Furthermore, PKA enhances the agonist activity of certain partial agonist hormones in a promoter-context dependent manner (Fujimoto et al., 1994). However, the role of cAMP in receptor-mediated transcriptional enhancement is not clear and may implicate contributions of both receptor phosphorylation as well as phosphorylation of other factors that may influence estrogen receptor function.

Other receptors in the nuclear receptor superfamily such as the androgen, vitamin D, v-erbA, thyroid and retinoid acid receptors are also phosphoproteins, although their phosphorylation has not been studied in detail (reviewed in Orti et al., 1992). For many of these receptors, neither modified amino acids nor the

phosphorylated regions are known.

In conclusion, there are several common features of steroid receptor phosphorylation. First, phosphorylation of many steroid receptors occurs on serines and threonines and increases with the addition of hormone. Second, phosphorylated residues are usually localized close to transcriptional regulatory regions or to the DNA binding domains. Third, although the kinases and phosphatases involved in steroid receptor phosphorylation are not known, many of the identified sites on progesterone, estrogen and glucocorticoid receptors are within ser/thr-pro motifs, known to be potential targets for the cyclin-dependent kinases and mitogen activated kinases.

Phosphorylation of glucocorticoid receptors

The first direct evidence that the glucocorticoid receptor is a phosphoprotein was provided by Housley and Pratt (1983). Mouse fibroblasts were labeled *in vivo* with ^{32}P orthophosphate, and radioactivity was found to be associated with two proteins migrating at 100 kD and 92 kD bound by dexamethasone mesylate (Housley and Pratt, 1983). Further analysis by numerous other investigators in different systems showed that the 100 kD band is indeed the glucocorticoid receptor and that it is phosphorylated *in vivo* (Singh et al., 1985; Grandics et al., 1984). It was subsequently reported that the glucocorticoid receptor contained multiple sites of phosphorylation that are predominantly phosphoserine, some phosphothreonine, and no phosphotyrosine (Housley and Pratt, 1983; Hoeck and Groner, 1990; Bodwell et al., 1991).

The physiological significance of this covalent modification is not clear, and evidence for functional changes resulting from phosphorylation is still limited. Early studies showed that hormone binding to glucocorticoid receptor rises and

falls with changes in cellular ATP levels (Bell and Munck, 1973, Munck and Holbrook, 1984). These observations led to speculation that phosphorylation may be important for receptor function and that receptors in normal cells cycle between the nucleus and the cytoplasm in an ATP-dependent manner (Mendel et al., 1986; reviewed in Orti et al., 1992). In addition, in cells treated with okadaic acid (OA), an inhibitor of protein phosphatases PP1 and PP2A, the glucocorticoid receptor is inefficiently retained in the nucleus and remained trapped in the cytoplasm (De Franco et al., 1991). These results suggest that re-entry of GR into the nucleus requires a dephosphorylation event that is catalyzed by these phosphatases and that hyperphosphorylation of certain peptides contributes to inhibition of the GR recycling. However, the concentration of OA needed to elicit an effect on GR phosphorylation is much higher than the concentration required for maximal effects on GR-mediated transcriptional activation (Moyer et al., 1993), implying that the effect is indirect.

On the other hand, GR has been reported to exist as a phosphoprotein in the absence and presence of hormone with hormone binding leading to an increase in the number of phosphates per molecule. For example, Orti et al., (1989) reported that agonists increase the average number of phosphates on the steroid binding protein from approximately 3 to 5 and that antagonists have no effect. In addition, chemical cleavage of receptor with hydroxylamine and cyanogen bromide from hormone treated and control cells localized the majority of receptor phosphorylation sites to the N-terminal region (Hoeck and Groner, 1990). The DNA binding domain was weakly phosphorylated and no phosphorylation was found in the signaling domain. Phosphorylation of the N-terminal region of the receptor was increased 2-3 fold by hormone treatment. Subsequently, Bodwell and colleagues (1991) have reported seven sites of phosphorylation on receptor isolated from hormone treated mouse cells. As the phosphorylation sites reside close to the

transcriptional regulatory domain, transcriptional regulatory function of the glucocorticoid receptor was proposed as another receptor function potentially modulated by phosphorylation.

The kinases and phosphatases involved in receptor phosphorylation are not known. However, it has been reported that stimulation or inhibition of certain signaling pathways can influence receptor function. For example, protein kinase A can potentiate glucocorticoid receptor function in developing retina (Zhang et al., !993) and in F9 embryonal carcinoma cells (Rangarajan et al., 1992) was found to promote receptor function. A link between the steroid hormone pathway and other signaling pathways has also been hypothesized on the basis of the modulation of GR dependent transcription by the activation or expression of certain oncogenes (Jaggi et al., 1986). For example, DeFranco et al. (1989) described that in v-mos transformed cells, GR was not efficiently retained in the nucleus. As mentioned above, similar results were obtained if cells were treated with the PP1 and PP2A inhibitor, okadaic acid, implicating these two phosphatases as potential enzymes involved in receptor dephosphorylation *in vivo*. However, it is unknown whether mechanisms underlying these observations involve direct effects on GR phosphorylation.

The receptor interacts with several regulatory proteins that modulate its action in a hormone dependent, cell specific and promoter specific fashion. For example, the receptor can bind glucocorticoid response elements (GREs) of at least two types: simple and composite. At simple GREs receptor alone is sufficient to confer hormonal regulation and it effects enhancement but not repression of transcription (Godowski et al., 1988). At composite GREs hormonal regulation requres a collaboration between the receptor and other transcriptional regulators. For example, at the proliferin gene composite element, the receptor represses transcription in the presence of a heterodimeric AP-1 factor cJun-cFos (Diamond et

al, 1990). Thus, the tissue and cell specificity of hormone effects derive at least in part from the interaction of the receptor with several other types of regulators. In addition, GR as well as other steroid receptors are covalently modified by phosphorylation, suggesting that another level of regulation of receptor function may exist. Studying the effects of phosphorylation on GR function could give insight into the paradox of how one ubiquitous transcription factor can effect cell-specific gene regulation.

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CHAPTER II: Functional analysis of glucocorticoid receptor phosphorylation

Part A (submitted to JBC)

FUNCTIONAL ANALYSIS OF GLUCOCORTICOID RECEPTOR PHOSPHORYLATION

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Running Title: GR phosphorylation and transcriptional regulation

SUMMARY

The rat glucocorticoid receptor is competent for signal transduction and transcriptional regulation when expressed in *Saccharomyces cerevisiae*. We found that the receptor in yeast, as in mammalian cells, was phosphorylated in the absence and presence of hormone, and that additional receptor phosphorylation accompanied agonist binding. The receptor was phosphorylated on Ser and Thr residues, and two-dimensional tryptic and V8 phosphopeptide maps of receptor from yeast and mammalian cells were qualitatively similar; we showed directly that T171, S224, S232 and S246 were phosphorylated in yeast in vivo, as they are in animal cells. T171 and S246 were phosphorylated constitutively, whereas phosphorylation of S224 and S232 was increased in the presence of hormone agonists. In both yeast and animal cells, transcriptional enhancement was reduced by mutation of S224 and increased by mutation of S246. In contrast, transcriptional repression was unaffected by either mutation. We conclude that the glucocorticoid receptor integrates information from multiple signaling pathways, and that phosphorylation of particular receptor sites affects selectively its transcriptional regulatory functions.

Keywords: phosphorylation/S. cerevisiae/steroid receptor

The glucocorticoid receptor is an intracellular protein that transduces the signaling information of cognate hormonal steroids. Thus, upon agonist binding, the hormone-receptor complex enters the nucleus, associates with specific DNA sequences termed glucocorticoid response elements (GREs), and modulates transcriptional initiation from nearby promoters (1-3). A single glucocorticoid receptor protein species appears to be expressed in virtually all mammalian cells, and to control in each cell type a distinct program of gene regulation (4). The receptor can interact selectively with other cellular proteins, and such interactions could in principle give rise to context-dependent combinatorial specificity. However, the precise mechanisms by which the receptor, or any transcriptional regulator produces distinct consequences in different cell or physiologic settings remain to be determined.

In addition to its noncovalent interactions with hormonal ligands and with various cellular proteins, the glucocorticoid receptor is covalently modified by phosphorylation (5, 6). Multiple serine and threonine residues are phosphorylated both in the absence and presence of hormone, and agonist binding appears to increase receptor phosphorylation (7, 8); similar findings have been reported for the progesterone and estrogen receptors (9-17). Seven phosphorylated residues have been mapped within the N-terminal half of the glucocorticoid receptor in hormone-treated mouse cells (18); this phosphorylated segment overlaps a ~200 aa portion of the receptor implicated both in positive and negative transcription regulation (19, 20).

Despite the prevalence of phosphorylation among eukaryotic signaling factors and transcriptional regulators, no simple rules have emerged for predicting the mechanisms by which this class of modifications alters protein function. In the relatively few cases in which the cellular processes affected by phosphorylation have been broadly determined (for review,

see 21, 22), it is apparent that the functional targets are numerous. For example: phosphorylation of the yeast Swi5 protein triggers its nuclear localization (23); phosphorylation of mammalian SRF alters its DNA binding kinetics (24, 25); phosphorylation of mammalian CREB stimulates its association with a protein cofactor, CBP (26, 27).

With respect to steroid receptors, it has been suggested that phosphorylation might modulate nucleocytoplasmic shuttling or that it might affect transcriptional regulatory functions (8, 13, 16, 28, 29, 30). However, in a survey of transcriptional enhancement by glucocorticoid receptors mutated at all putative phosphorylation sites, Mason and Housley (31) detected no significant defects. Clearly, such negative results might be obtained in some assay contexts and not in others. That is, effects might have been detected using a different combination or arrangement of promoter and GRE, or at a different concentration of receptor or hormone, or in a different cell type; such context effects have been invoked to explain the results of phosphorylation site mutations in the estrogen or progesterone receptors (13, 16, 30).

In view of such potential complexity, it seemed worthwhile to re-examine the significance and function of glucocorticoid receptor phosphorylation, and to seek an approach that might be extended eventually to an assessment of the enzymes that mediate this modification. Previous studies had demonstrated that the rat glucocorticoid receptor is competent both for signal transduction and GRE-dependent transcriptional enhancement when expressed in baker's yeast, *Saccharomyces cerevisiae* (32, 33). An inference of these findings is that any phosphorylation events that are essential for receptor action must occur both in yeast and in mammalian cells. Thus, although yeast appears to represent a significant departure from the cellular environments typically experienced by the receptor, the simpler organism presents substantial experimental advantages, including facile construction of stable strains expressing relatively high levels of wild-type and mutant receptors, and access to strains bearing mutations in genes encoding particular kinases or phosphatases. Moreover, it is a simple matter to test

systematically in animal cells any finding obtained in yeast.

In the present study, we examined the phosphorylation of the rat glucocorticoid receptor expressed in yeast and mammalian cells, determined the effects of hormone binding on receptor phosphorylation, and assessed the consequences of phosphorylation on receptor function.

MATERIALS AND METHODS

Yeast strains, cell lines and growth conditions

The triple protease deficient yeast strain, BJ2168 (a, pep 4 - 3, prc 1-417, prb 5 -1122, ura 3 - 52, trp 1, leu 2) and W303 (α, ade 2-1, trp 1-1, ura 3-1, leu 2-3,112, his 3-11,15, can 1-100) (34, 35) served as the parent for derivative strains containing various 2μ-origin-based expression and reporter vectors as described below; these plasmids are maintained at about 10-40 copies/cell in this background. Yeast cultures were propagated at 30°C in minimal yeast medium with amino acids and 2% glucose (33). Transformations were performed by the lithium acetate procedure (36).

The mammalian cell line, GRH2 (37), is a rat hepatoma HTC cell derivative transfected stably with rat glucocorticoid receptor cDNA; these cells express the receptor at elevated levels. The simian COS7 cell line, derived from CV1 cells, and the mouse F9 embryonal carcinoma cell line were used as recipients of mammalian expression and reporter vectors (described below) in transient transfection experiments. Cells were grown at 37°C in Dulbecco's modified Eagle's medium H-16, supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). For transient transfection, DNA was introduced into 50% confluent cultures by the calcium phosphate method as previously described (37). After 16 hr, F9 cells were subjected to a 2 min 15% glycerol shock, washed twice in phosphate buffered saline (PBS), and then incubated for an additional 24 hr in whole medium containing or lacking 0.1 μM dexamethasone (Sigma); COS 7 cells were treated the same way, except the glycerol shock was omitted.

Plasmids

The yeast expression plasmid pG-N795 (32, 39) contains the rat glucocorticoid receptor cDNA driven by the yeast glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter; residing on a 2 μ vector (10-40 copies per cell) bearing the TRP1 selectable marker. For lower levels of receptor expression, the same effector gene was introduced into a CEN/ARS vector (1-2 copies per cell; pRS314-N795).

Reporter plasmids pΔs26x and pΔs1Gs3, contain three tandem 26 base pair oligonucleotides from the tyrosine aminotransferase GRE or a single imperfectly-palindromic GRE, respectively, inserted upstream of a minimal yeast cytochrome c1 (CYC1) promoter, retaining only a TATA box region and the transcription initiation site, fused to the *E. coli* β-galactosidase (Lac Z) coding sequences. The 2 μ vector carries the URA3 selectable marker, a bacterial origin of replication and the bacterial ampicillin resistance gene (32, 40).

The mammalian expression vector, 6RGR (20), is an SP65-based plasmid carrying the rat glucocorticoid receptor cDNA driven by the Rous sarcoma virus (RSV) promoter; the SV40 polyadenylation signal is fused downstream of the expressed sequences. Mammalian expression vectors for cJun and cFos were similarly constructed on this plasmid backbone (38), as was 6RZ (20), a β-galactosidase expression plasmid (6RZ) used as an internal control to monitor transfection efficiency.

Reporter plasmid ΔGTCO (20) contains two synthetic GREs (41) derived from the mouse mammary tumor virus long terminal repeat (MMTV LTR) upstream of the thymidine kinase (TK) promoter driving the chloramphenicol acetyltransferase (CAT) gene.

Reporter plasmid plfG3 CAT (42) carries three composite GREs from the proliferin gene

inserted upstream of a minimal ADH promoter driving the CAT gene.

Metabolic labeling

The yeast strain BJ2168 containing the receptor expression vector pG-N795 and reporter vector pΔs26x were grown to O.D.$_{600}$ 0.4–0.7 in 50 ml minimal selective yeast medium with amino acids and 2% glucose. The cells were centrifuged, washed once in the same medium lacking phosphate, and incubated for 30 min at 30°C in 50 ml phosphate-free medium. The cells were centrifuged, resuspended in 5 ml of phosphate-free medium and divided into two equal portions. To each was added [^{32}P] orthophosphate (25 mCi/ml, carrier free, NEN) to a final concentration of 1 mCi/ml; one portion was brought to 10 μM deoxycorticosterone (Sigma). After 2 hr at 30°C, cells were harvested by centrifugation, washed in 5 ml of cold PBS and resuspended in 350 μl of high salt lysis buffer (400 mM NaCl, 45 mM HEPES, pH 7.5, 1 mM EDTA, 2 mM DTT, 10% glycerol, 0.5 % NP40, 25 mM sodium fluoride, 20 mM β-glycerophosphate, 5 mM sodium pyrophosphate and a protease inhibitor cocktail containing 1μg/ml each of aprotinin, leupeptin, and pepstatin A, and 1 mM PMSF). An equal volume of acid washed glass beads was added and cells were vortexed for 15 min using a horizontal bead beater (Eppendorf). Cell lysates were cleared by centrifugation at 12 000g for 10 min at 4°C. The supernatant was placed in a fresh tube for immunoprecipitation of the receptor.

The mammalian cell line GRH2 was grown in 10 cm dishes to near confluency in DMEH-16 medium (1g/L glucose) supplemented with 10% fetal calf serum; one plate of cells was used per experiment. Cells were washed twice with 5 ml of phosphate free medium and preincubated in medium lacking phosphate for 30 min at 37°C; 5 ml of fresh phosphate free medium (without serum) was added along with 0.5 mCi/ml of [^{32}P] orthophosphate and 0.1 μM dexamethasone where indicated. After 2 hr at 37°C, cells were washed once with cold PBS and lysed on the plate by the addition of 0.5 ml of high salt lysis buffer. Mammalian cell

lysates were prepared for immunoprecipitation as described below.

Immunoprecipitation and immunoblotting

To immunoprecipitate the glucocorticoid receptor, an equal volume of 2x RIPA buffer (1x RIPA = 150 mM NaCl, 10 mM HEPES pH 7.5, 0.1% SDS, 1% deoxycholic acid-disodium salt, 1% Triton X-100) was added to cleared cell lysates, together with 5 µl of ascities fluid containing the rat glucocorticoid receptor specific monoclonal antibody BUGR2 (43). After 1-2 hr at 4°C, 100 µl of 50 mg/ml protein A Sepharose (Sigma) equilibrated in 1X RIPA was added and incubated for an additional 2 hr with gentle agitation at 4°C. Protein A sepharose was collected by centrifugation, washed 4 times with ice cold 1x RIPA buffer, and once with PBS. Bound protein was released in 2x SDS sample buffer (0.125 M Tris pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol and 0.004% bromphenol blue), displayed on 7.5% SDS polyacrylamide gels, and transferred to Immobilon-P membranes (Millipore). Immunoblots were probed the BUGR2 antibody, and developed by using an alkaline phosphatase detection system.

Phosphopeptide mapping and phosphoamino acid analysis

Polyacrylamide gels containing the labeled receptor were washed in 500 ml of water, three times, 10 min, and dried between cellophane sheets. Following autoradiography, the glucocorticoid receptor band was excised and the gel rehydrated and eluted in 50 mM ammonium acetate, 1 mM DTT. For digestion with trypsin, the rehydrated gel slice was placed in a microcentrifuge tube containing 1 ml 50 mM ammonium acetate, 1 mM DTT and 50 µg/ml TPCK treated trypsin (Sigma), freshly prepared. After 6 hr at 37°C, an additional 50 µg of fresh trypsin (1 mg/ml in aforementioned buffer), was added and incubation was continued for 10 hr at 37°C; more extended incubations did not significantly change the

pattern of phosphotryptic peptides. For digestion with V8 protease, the rehydrated gel slice was placed into a microfuge tube at room temperature in 50 mM ammonium acetate, 1 mM DTT, and 50 μg/ml of V8 protease (Endoproteinase Glu-C, Boehringer Mannheim), adjusted to pH 4. Samples were centrifuged for 5 min at 12000 x g and the supernatant containing the digested peptides was evaporated to dryness in a Speedvac (Savant, Farmingdale, NY). Peptides were resuspended in 500 μl of water, dried and washed once more. Finally, peptides were dissolved in 10 μl of 15 % acetic acid, 5% formic acid. For trypsin phosphopeptides, >2000 cpm was used for 2-D analysis; for V8 phosphopeptides, >1000 cpm was used. Peptides were electrophoresed in 15 % acetic acid, 5% formic acid on cellulose plates (microcrystalline cellulose adsorbent without fluorescent indicator; Kodak) at 1000 volts for 50 min. Plates were then dried and subjected to ascending chromatography in the second dimension for 3 hr with 37.5% butanol, 25 % pyridine, 7.5% acetic acid, air dried, and exposed to film (44).

For phosphoamino acid analysis, [^{32}P] labeled receptor was treated as described above for peptide mapping, except that dried peptides were dissolved in 400 μl of 6M HCl (Pierce) and receptor hydrolyzed by heating to 110^{o}C for 60 min. Samples were then dried and dissolved in 10 μl of pyridine:acetic acid:H_2O (10:100:1890). The hydrolysates were spotted on a plate, along with phosphoamino acid standards (1μl of mixture of phosphoserine, phosphothreonine and phosphotyrosine (Sigma), 1 mg/ml each, and resolved on cellulose thin-layer plates (Kodak) in the first dimension by electrophoresis at 1500 V for 20 min, and in the second dimension by electrophoresis at 1300 V for 16 min. After drying, plates were sprayed with 0.2 % w/v ninhydrin in acetone, developed at 70 ^{o}C and autoradiographed.

Site directed mutagenesis

The mutagenesis system was supplied by Amersham (version 2.1, RPN. 1523). The Nco I-Sph

I fragment of the rat glucocorticoid receptor cDNA was inserted into the M13 mp18 vector, and single stranded DNA was prepared and used as template. The mutagenic oligonucleotide was annealed and extended by Klenow polymerase in the presence of T4 DNA ligase. Thionucleotide incorporation into the mutant strand during extension allowed selective removal of the nonmutant strand by nicking with restriction enzymes that cannot cleave phosphorothioate DNA, followed by exonuclease III treatment. Double stranded DNA was prepared from the mutagenized single strands, ligated into appropriate vectors to reconstruct full or partial receptors, and transformed into bacteria. Mutations were identified by sequencing. Derivatives bearing multiple mutations were constructed by recombining point mutants. To construct the double mutants T171S/S224T, T171S/S232T and T171S/S246T, the Nco I-Eco NI fragment from T171S and the Eco NI-Sph I fragment of S224T, or S232T, or S246T were inserted into pGN795 cleaved with Nco I and Sph I. The resulting constructs were confirmed by restriction digestion and sequencing.

Synthetic peptides

Phosphopeptides were synthesized on HMP resin using standard HOBT active ester/FMOC chemistry on an ABI 431-A peptide synthesizer (Applied Biosystems). Residues to be phosphorylated were coupled as alcohol-unprotected FMOC derivatives, and were phosphorylated after synthesis with di-t-butyl-N,N-diisopropylphosphoramidite / tetrazole followed by oxidation of trivalent phosphorus with t - butylhydroperoxide. Peptides were cleaved and deprotected with Reagent K, purified by reversed-phase HPLC and characterized by electrospray-ionization mass spectrometry (Hewlett-Packard model 5989A). 50µg of each peptide was dissolved in 10 µl of electrophoresis buffer, loaded on TLC plates and electrophoresis and chromatography performed as described above. Plates were dried and synthetic peptides were visualized with ninhydrin. These peptides were used as markers to identify phosphopeptides labeled in vivo by comigration.

CAT assay

Mammalian cell monolayer cultures were rinsed with 2 ml of cold PBS and harvested in 1.5 ml PBS by 10 min centrifugation at 2000 x g. Pellets were resuspended in 100µl of 250 mM Tris-HCl (pH 7.8), and lysed by three cycles of freezing (–70 ^{o}C) and thawing (37 ^{o}C); cell debris was removed by centrifugation at 12000 x g for 3 min. CAT activity was assayed as previously described (38). Briefly, a non-chromatographic assay was performed with heat treated extracts (68^{o}C for 10 min). Extracts were incubated with [^{14}C] acetyl coA for 2 hr and labeled acetylated chloramphenicol was extracted with ethyl acetate. Internal control β-galactosidase was measured as described (38). Briefly, 5µl of unheated extract was assayed for 20 min at 37^{o}C, and the reaction stopped by the addition of 1 ml of 250 mM glycine (pH 10.65). Reactants were assayed in a Hoefer DNA fluorometer. CAT values were normalized to β-galactosidase levels.

β-galactosidase assay in yeast

β-galactosidase activity was assayed as previously described (32). Briefly, yeast was collected by centrifugation from 1.5 ml cultures, and washed with LAC Z buffer (10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, and 100 mM $NaPO_4$, pH 7.0). After recentrifugation, cells were resuspended in 50 µl of LAC Z buffer and permeabilized with 50 µl of $CHCl_3$ and 20 µl of 0.1% SDS; β-galactosidase substrate (o-nitro-phenyl-β-galactoside, 0.7 ml of 2 mg/ml) was added and the reaction stopped with 0.5 ml of 1 M Na_2CO_3 after 1-10 min incubation.

RESULTS

Glucocorticoid receptor phosphorylation in yeast

As a first test of glucocorticoid receptor phosphorylation in *S. cerevisiae*, a protease deficient strain (34) stably expressing the receptor was metabolically labeled with [^{32}P]-orthophosphate in the presence and absence of 10 μM deoxycorticosterone (DOC). The receptor was immunoprecipitated with a monoclonal antibody (BUGR2; 43), fractionated by SDS polyacrylamide gel electrophoresis, and visualized by autoradiography. As in mammalian cells, we found that the receptor is phosphorylated in yeast both in the absence and in the presence of hormone, and shows a modest but reproducible reduction in electrophoretic mobility after hormone treatment (Fig. 1A). Such mobility differences commonly are indicative of additional protein phosphorylation (45).

In a second experiment, we treated unlabeled yeast cultures for 2 hr with glucocorticoid ligands that display a range of agonist potencies in yeast, isolated and fractionated the receptor as before, and visualized it by immunoblotting. The results revealed that the reduction in receptor mobility (Fig. 1B) parallels roughly the relative efficacies of the ligands to stimulate transcriptional activation by the receptor (Fig. 1C; see also ref. 33). Thus, dexamethasone (DEX), which is a weak agonist in *S. cerevisiae* under these conditions, induced little or no shift in receptor electrophoretic mobility, whereas deacylcortivazol (DAC) and deoxycorticosterone (DOC) each conferred strong transcriptional enhancement activity on the receptor, and produced more pronounced effects on receptor mobility (Fig. 1B). Treatment of receptor with calf intestine alkaline phosphatase eliminated the slower migrating receptor forms observed in the hormone treated cells, supporting the notion that the decreased receptor mobility reflects phosphorylation (data not shown). Phosphatase treatment had no effect on the mobility of the receptor from untreated cells, implying either that the constitutively

modified residues are inaccessible to the enzyme, or that they do not contribute detectably to the mobility shift.

The immunoblotting procedure visualizes the entire cellular complement of receptor molecules, whereas the metabolic labeling experiment monitors the receptors that acquire phosphate adducts during the two hour labeling period. Interestingly, it appears that most or all of the receptor is phosphorylated constitutively, and that most or all of it receives a net increase in phosphate accompanying hormone addition; the heterogeneous species observed in DOC and DAC may indicate multiple distinct phosphorylated isoforms. Taken together, these experiments imply that the rat glucocorticoid receptor is phosphorylated when it is produced in yeast, and that the extent of hormone dependent phosphorylation correlates with agonist potency.

Glucocorticoid receptor phosphorylation sites are similar in yeast and mammalian cells

To determine if similar sites are phosphorylated when the receptor is expressed in yeast and mammalian cells, we turned to phosphopeptide mapping. Hormone-treated yeast and rat hepatoma cells, each expressing the receptor, were metabolically labeled with [^{32}P]-orthophosphate, and the receptor was isolated by immunoprecipitation and digested with trypsin. The resulting phosphopeptides were separated on thin-layer plates in two dimensions by electrophoresis and chromatography, respectively (44). As seen in Fig. 2 labeling of the receptor in either species yielded qualitatively similar patterns of tryptic phosphopeptides. For ease of discussion, we denote eleven major tryptic phosphopeptides as T.1 – T.11 and seven minor phosphopeptides as T.a – T.g (Fig. 2D). Importantly, a similar phosphopeptide pattern was observed upon mixing the digested receptor from yeast and mammalian cells prior to two-dimensional separation (Fig. 2C; shown schematically in Fig. 2D). These findings suggest that the same amino acid residues on the receptor are phosphorylated in hormone-treated yeast

and mammalian cells.

Despite the evident qualitative similarities in Fig. 2A and B, the relative intensities of certain phosphopeptides differed between the two species. For example, peptides T.2, T.4 and T.5 were more intense in mammalian cells, whereas peptides T.9, T.10 and T.11 were more strongly labeled in yeast. Although these quantitative distinctions might in principle be species specific, variations of this magnitude have been observed in comparisons of different mammalian cell lines, and between stationary and actively growing cultures of one cell line (data not shown). Hence, it is premature to ascribe the variations to species differences.

As a second comparison of receptor phosphorylation in yeast and mammalian cells, we mapped receptor phosphopeptides following cleavage by V8 protease rather than trypsin. In the experiment shown, separate labeling reactions were carried out on hormone-treated and control cultures (Fig. 3). In all cases, the resulting maps were substantially simpler, reflecting fewer primary cleavage sites for this protease, as well as fewer partial products generated by incomplete digestion. In any case, the phosphopeptide patterns of receptor labeled in yeast and mammalian cells were again strikingly similar, with five major phosphopeptides generated upon V8 cleavage (Fig. 3, compare panels A and B with panels C and D). The extent of phosphorylation of these peptides seemed roughly uniform between species, except for peptide V.1, which appeared more intense in yeast than in mammalian cells; in fact, V.1 may represent a cluster of more than one peptide (Fig. 3; see also Fig. 4) whose sites are phosphorylated at low relative stoichiometries in the mouse receptor.

In general, it is apparent that both ligand dependent and ligand independent receptor phosphorylation is closely conserved between species. In both yeast and mammalian cells, peptide V.4 was phosphorylated much more intensely in hormone-treated than in control cultures (Fig. 3B and D, arrow), whereas the other phosphopeptides appeared to be unaffected

by hormone treatment. In combination, our analyses with two proteases imply that the receptor peptides (and likely the individual receptor amino acid residues) phosphorylated in mammalian cells are similarly modified in yeast. Moreover, the additional phosphorylation that accompanies hormone binding seems to be conserved between species.

Identification of receptor phosphorylation sites

Seven residues are phosphorylated, with different apparent efficiencies, on the glucocorticoid receptor in hormone-treated mouse cells (18). We mutated four predominant sites at the corresponding rat receptor residues from serine or threonine to alanine (T171A, S224A, S232A and S246A), and characterized the phosphorylation state of these receptor derivatives in metabolically labeled yeast. V8 phosphopeptide analysis of these point mutants in hormone-treated and control cultures is shown in Fig. 4. The T171A mutant lacked peptide V.1, both in the absence and presence of hormone (Fig. 4, panels 1 and 2); we assume that the residual labeling at this position results from one or more minor peptides comigrating at that position. Similarly, the S246A receptor lacked peptides V.5a and V.5b without regard to hormone treatment (Fig. 4, panels 7 and 8). Note that peptide V.3, which was weakly phosphorylated in these labelings, was detected in longer exposures of all derivatives except S224A, and was also observed in other experiments.

The phosphopeptide pattern of the S224A mutant differed from that of the wild type receptor at two positions: in untreated cells, peptide V.3 was absent, and after hormone treatment, peptides V.3 and V.4 were absent (Fig. 4, panels 3 and 4). The S232A receptor derivative also lacked the V.4 peptide, suggesting that S232 phosphorylation is also stimulated by hormone (Fig. 4, panels 5 and 6); the difference in peptide V1 labeling in panels 5 and 6 was not observed in other experiments. The simplest interpretation of these findings is that the V.4 peptide carries hormone-dependent phosphorylation on both S224 and S232 (see below). In

fact, phosphotryptic peptide mapping of the mutant receptor derivatives in both yeast and mammalian cells supports the notion that T171 and S246 are phosphorylated constitutively, whereas phosphorylation of S224 and S232 increases upon addition of hormone (MJG, unpublished; MDK, in preparation).

Thus, each of the four receptor derivatives bearing an Ala substitution in a putative phosphorylation site lacked one or two phosphopeptides relative to the wild type receptor. Alteration of two peptides after mutation of one putative target residue might result from partial proteolysis, or from interdependence of one phosphorylation event upon another. To resolve these and other possibilities, we synthesized the predicted V8 phosphopeptides of the receptor (Fig. 5A), purified them, established their identities by mass spectroscopy, and compared their positions on 2D peptide maps with those of the receptor phosphopeptide observed after metabolic labeling. The results confirmed, for example, that phosphopeptide V.5a contains a phosphoserine at position 246 (Fig. 5B, panel *i*), and that phosphopeptide V.1 includes the phosphorylated T171 residue (Fig. 5B, panel *iii*). Finally, the V.2a/2b peptides correspond to the 220-242 fragment with a single phosphorylation site at S232 or S224, whereas peptide V.4 coincides with the doubly phosphorylated version of this peptide (Fig. 5B, panel *ii*; MDK, in preparation). An interdependence of hormone stimulated phosphorylation of these two residues may account for the extent of labeling of this phosphopeptide. Together, these experiments produced an internally consistent interpretation of the specific receptor residues phosphorylated in vivo, and allowed for their identification on the phosphopeptide maps (Fig. 5C).

To examine further the receptor phosphorylation sites used in vivo, we exploited the fact that some serine/threonine kinases are rather indiscriminate in modifying either S or T residues at a given substrate site (46). We first converted threonine 171 to serine by site directed mutagenesis, to eliminate the only predicted threonine phosphorylation site on the receptor

(18). As expected, we found that this T171S receptor was phosphorylated only on serine, whereas the wild type protein contained phosphoserine together with a lesser amount of phosphothreonine (Fig. 6 A-D). Using this "all phosphoserine" receptor derivative, we then constructed double mutants containing S224T, S232T or S246T. Hormone treated and control cultures of strains carrying each receptor derivative were metabolically labeled with [^{32}P], and the isolated glucocorticoid receptors were analyzed for their phosphoaminoacid content. In the case of T171S/S246T, phosphothreonine was observed constitutively (Fig. 6I, J), whereas T171S/S224T and T171S/S232T contained increased amounts of phosphothreonine after hormone treatment (Fig. 6E-H). These findings lend additional support to our interpretations of prior mapping experiments.

Effects of phosphorylation site mutations on transcriptional regulation

We next wished to assess whether mutation of any of the phosphorylation sites on the receptor might affect its transcriptional regulatory activities. We examined two classes of GREs, "simple" and "composite" (4). A simple GRE is a DNA sequence at which receptor binding is sufficient for hormone-mediated enhancement of transcription from linked promoters; two such elements, derived from the MMTV LTR, are linked on reporter vector ΔGTCO (20, 41). Alternatively, the receptor can bind to composite GREs, but hormonal regulation at such elements requires collaboration between the receptor and a second bound regulator; for example, at the plfG3 composite GRE, the receptor represses transcription in the presence of a heterodimeric AP1 factor, cJun–cFos, also bound at that site (4, 38, 42, 47).

In transiently transfected mammalian cells, we failed to detect a significant effect of any of four individual phosphorylation site mutations, T171A, S224A, S232A and S246A, on repression by the receptor at the plfG3 composite element (Fig. 7A). In contrast, transcriptional enhancement from a simple GRE was altered by two receptor mutations (Fig.

7B): enhancement conferred by the S246A receptor was increased relative to wild type, whereas the S224A mutant displayed reduced enhancement activity; although these effects are modest, they are significant and reproducible. Parallel results were obtained in yeast for transcriptional enhancement (Fig.7C); repression was not assessed in yeast, as the plfG composite GRE does not function in yeast. Immunoblotting confirmed that mutant receptors were produced at levels similar to wild type (Fig. 7D). We conclude that mutation of certain individual receptor phosphorylation sites alters, either positively or negatively, transcriptional enhancement by the receptor, but that those same mutations are without effect on transcriptional repression. Thus, the regulatory functions of the receptor appear to be selectively modulated by the phosphorylation status of the receptor.

DISCUSSION

Virtually all eukaryotic transcription regulators studied to date are phosphoproteins (21, 22). However, with notable exceptions, the precise sites and functional consequences of the phosphate modifications have commonly not been determined. In the present study, we examined phosphorylation of the rat glucocorticoid receptor in *Saccharomyces cerevisiae*. In this heterologous setting, the receptor is competent both for signal transduction and for transcriptional regulation (32, 33), despite the fact that no yeast genes related to the intracellular receptor superfamily have yet been discovered.

Our results, which confirm, complement and extend previous findings of others (18, 48), reveal that the receptor residues phosphorylated in yeast and mammalian cells are identical for at least four (and perhaps all seven) of the reported positions (T171, S224, S232, S246). This implies that the receptor is modified by highly conserved kinases. Furthermore, a net increase in receptor phosphorylation occurs after hormone treatment in yeast, consistent with previous findings with the glucocorticoid, progesterone and estrogen receptors in mammalian cells (7-17, 27, 49). We identified two specific sites, S224 and S232, at which phosphorylation is stimulated in the presence of bound agonists; analogously, Poletti and Weigel (14) have shown that two serine residues on the progesterone receptor are phosphorylated in response to hormone.

We found that mutation of certain phosphorylation target residues to alanine altered the transcriptional enhancement activity of the glucocorticoid receptor. For example, the S246A derivative displayed a modest but consistent increase in enhancement activity from a simple GRE, both in yeast and in transfected mammalian cells, implying that phosphorylation of that position on the wild type receptor may inhibit receptor activity. In the same contexts, the S224A derivative was markedly reduced in its enhancement activity, suggesting that

phosphorylation of S224 stimulates enhancement. In contrast, neither mutation affected the magnitude of transcriptional repression by the receptor from a composite GRE in the same mammalian cells in which the effects on enhancement were observed. Thus, phosphorylation of the receptor can differentially and selectively affect its transcriptional regulatory functions.

It is notable that the phosphorylation sites on the receptor are clustered in or near a region that includes sequences involve both in transcriptional activation (19) and in repression (20). These functional domains appear to overlap, although neither has been delineated precisely. Consistent with our findings, however, an analysis of a series of internal deletions of the receptor revealed that a derivative lacking the full consensus site for phosphorylation of S246, but containing S224 and S232 kinase consensus sites activates transcription more strongly than wild type receptor; in contrast, a derivative lacking all three of the consensus sites activates transcription only weakly (50).

Our finding that receptor phosphorylation affects enhancement but not repression may facilitate dissection of this domain into particular subregions or "surfaces" dedicated to positive and negative regulation. It will be important, however, to assess the effects of the phosphorylation site mutations on other GREs and in other cell and promoter contexts, to test the simple generality that receptor phosphorylation modulates only its enhancement function. Moreover, our analysis to date has considered only the effects of phosphorylation of individual sites, without regard to the states of other positions. Bodwell et al. (18) showed that the various phosphorylation sites are modified to different relative efficiencies, and calculated that each receptor molecule contains, on average, less than 3–4 phosphorylated residues. No study has yet defined a particular combination of phosphorylated residues that appears on a given receptor molecule, or the extent of heterogeneity in the phosphorylation patterns across the receptor population. Conceivably, different combinations of phosphorylated residues may generate distinct functional consequences. Testing this possibility will be facilitated by

the availability of mutations and assays that allow the many distinct glucocorticoid receptor activities to be uncoupled and analyzed independently.

Previous studies have not converged on a simple or consistent view of the functional effects of phosphorylation on steroid receptors. Thus, it has been reported that phosphorylation is important for transcriptional enhancement by the estrogen and progesterone receptors, as phosphorylation deficient derivatives displayed reduced activity in particular promoter contexts (13, 16, 30). In contrast, Mason and Housley (31) constructed a mouse glucocorticoid receptor with alanine substitutions at all of its phosphorylation sites, and found little effect on its transcriptional enhancement activity. Whether the apparent discrepancies reflect compensating positive and negative effects of specific phosphorylation events, intrinsic differences among receptors, distinct cell or promoter contexts, or different assay methodologies remains to be determined.

In any case, our present results establish that phosphorylations of the receptor at S224 and S246 selectively alter its activity. In addition, the failure of the mutations at S224A or S246A to affect repression indicates that the early steps in receptor function, which include signal transduction activities such as hormone binding, Hsp90 release, and nuclear translocation, are retained in these mutants. Therefore, we conclude that these phosphorylation events must affect one or more late steps in receptor function, which include transcriptional regulation.

The sequence contexts of the various glucocorticoid receptor phosphorylation sites suggest that they might be substrates for several classes of protein kinases, including cyclin-dependent kinases (CDKs), mitogen activated kinase (MAPK) and glycogen synthase kinase 3 (GSK3). Interestingly, the two sites at which increased phosphorylation is observed upon hormone binding, S224 and S232, reside within perfect matches to the consensus site for CDK (Ser/Thr–Pro–X–Arg/Lys). Furthermore, recent studies (MDK, KRY and MJG, in

preparation) confirm that these residues can be phosphorylated by cyclin dependent kinases in vitro, and that mutations of particular CDK genes in yeast yield phenotypes similar to those conferred by mutation of the sites themselves. Analogously, S246 and T171 appear to be preferred targets for MAPK in vitro, and mutations of these sites or of certain yeast MAPK genes produce similar effects on receptor function.

Taken together, these results suggest that one or more enzymes from the MAPK family may be involved in constitutive phosphorylation of the receptor, and that at least one of those modifications decreases transcriptional enhancement by the receptor in yeast and mammalian cells; in contrast, one or more enzymes from the CDK family appear to introduce phosphate modifications selectively onto the hormone-receptor complex, and at least one of those phosphorylation events leads to an increase in receptor-mediated enhancement. In principle, the inhibitory and stimulatory effects of phosphorylation on receptor function allow its enhancement activity to be "tuned" by the activities of the relevant kinases. Moreover, the functional consequence of each phosphorylation site might differ in different promoter contexts or cell types. According to this view, phosphorylation increases substantially the diversity of receptor activities that can be generated even within the nucleus of a single cell.

We speculate that in addition to hormonal ligands, at least two additional signal transduction pathways converge on the receptor, one culminating in phosphorylation by a CDK, and the other producing modification by a MAPK. In this sense, the receptor could be considered an "information integrator" that receives and processes extracellular signaling information from multiple (at least three) regulatory inputs and responds with an appropriate constellation of transcriptional regulatory activities. Indeed, it is conceivable that receptor function in certain contexts might be triggered even in the absence of a hormonal signal by a particular combination of phosphorylated residues. Such an activity might account for the intriguing reports (51, 52) that progesterone receptor functions can be activated by dopamine, even in

the absence of cognate steroid ligands. If it proves possible to identify in yeast particular members of the CDK and MAPK families that effect receptor phosphorylation, the mammalian homologs of those enzymes may provide an approach to defining additional signaling pathways that affect receptor function.

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Fig. 1. Glucocorticoid receptor phosphorylation in yeast. (A) Autoradiogram of [^{32}P]-labeled receptor. Yeast expressing the rat glucocorticoid receptor were incubated with [^{32}P]-orthophosphate in the absence (lane 1) and the presence (lane 2) of 10 μM DOC. Receptor was immunoprecipitated with a monoclonal antibody, fractionated by SDS polyacrylamide gel electrophoresis, and visualized by autoradiography. (B) Immunoblot of receptor expressed in yeast. Yeast cells were incubated in phosphate free medium as described in (A), except [^{32}P] orthophosphate was omitted. Hormones were used at 10 μM: DEX, dexamethasone; DOC, deoxycorticosterone; DAC, deacylcortivazol. Receptor was immunoprecipitated, fractionated by SDS polyacrylamide electrophoresis, transferred to Immobilon-P membrane (Millipore), and visualized by immunoblotting. (C) Gene activation by the receptor under conditions of metabolic labeling. Yeast was treated as in (B), and accumulation of β-galactosidase from a GRE-linked reporter vector pΔs26x was monitored in cell extracts. Data represent average and range of two independent assays.

Figure 1A

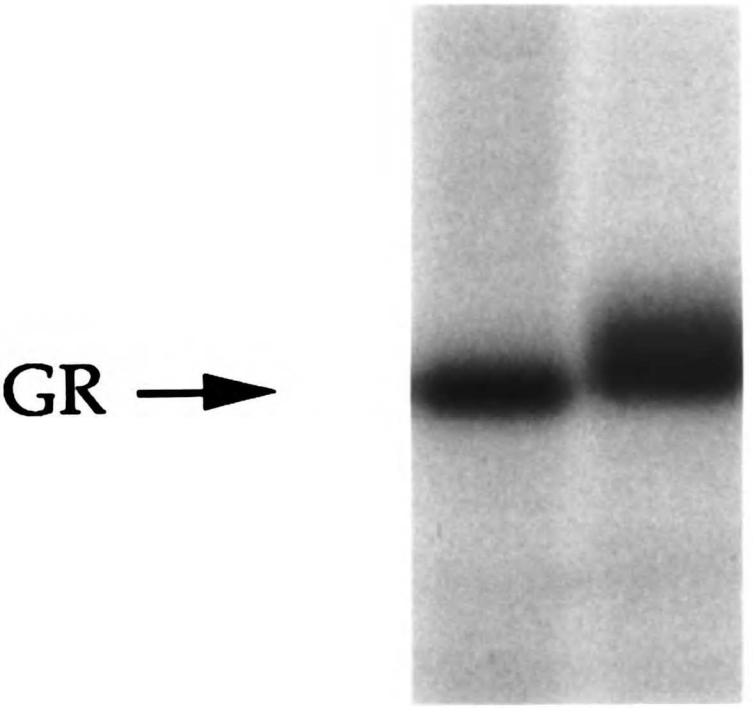


Figure 1B

Figure 1C

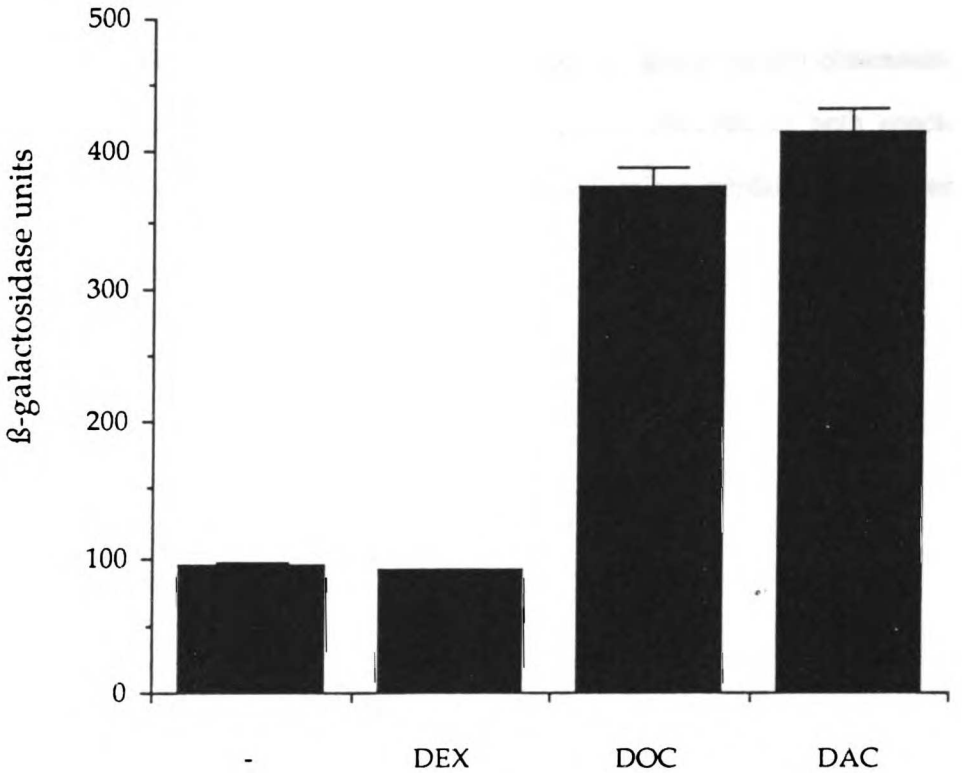


Fig. 2. Tryptic phosphopeptides of receptor expressed in yeast and mammalian cells. Yeast and mammalian cells were incubated with [^{32}P] orthophosphate and with hormone. Receptor was fractionated by gel electrophoresis, extracted from the gel and digested with trypsin. Phosphopeptides were separated in the first dimension (left to right) by electrophoresis and in the second dimension (bottom to top) by chromatography. (A) Receptor phosphopeptides from yeast. (B) Receptor phosphopeptides from mammalian cells. (C) Phosphopeptides from yeast and mammalian cells were mixed and subjected together to two-dimensional separation. (D) Schematic representation of the phosphopeptides obtained in both species; 11 major labeled peptides (1-11; cross-hatched circles) and 7 minor peptides (a-g; open circles) are displayed.

Figure 2

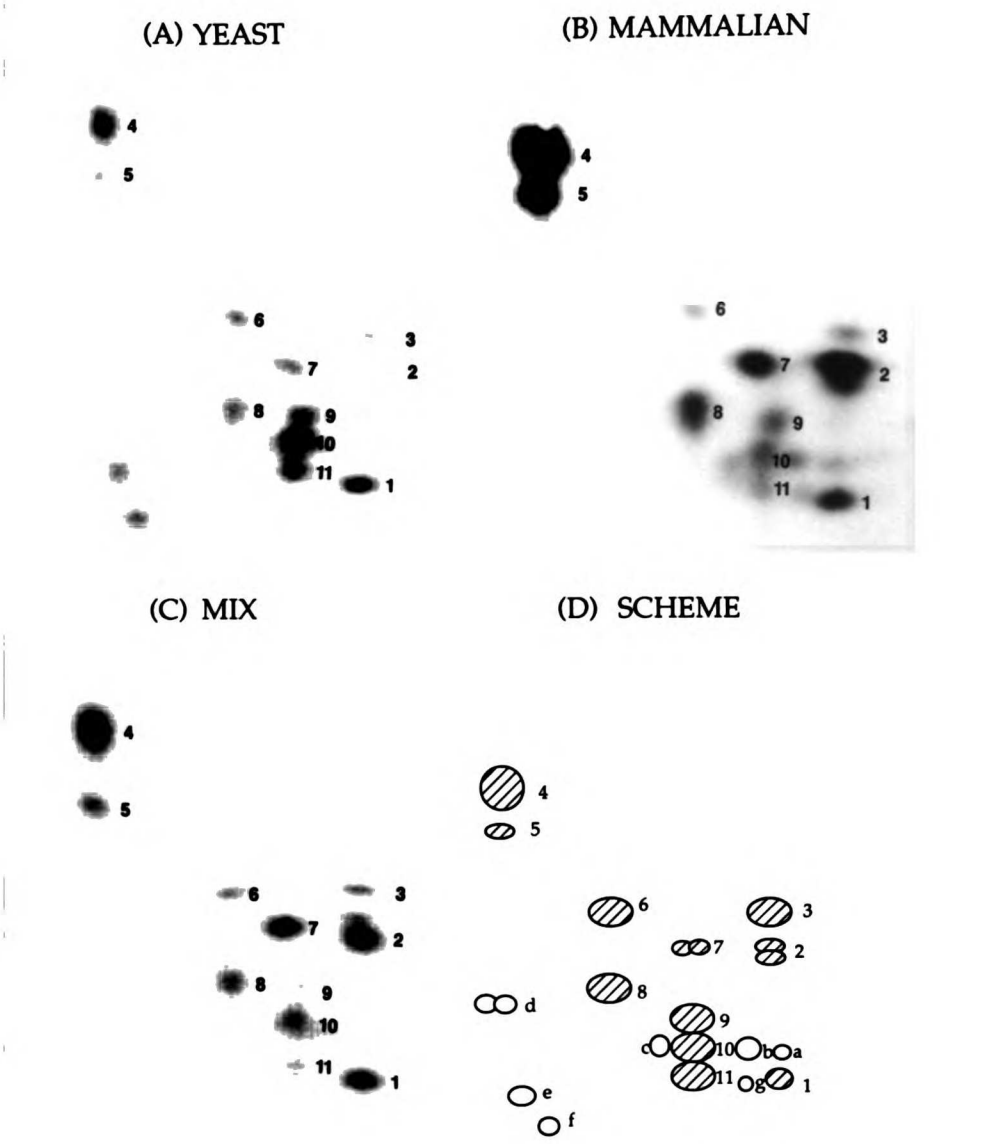


Fig. 3. V8 phosphopeptides of receptor expressed in yeast and mammalian cells in the absence and presence of hormone. Cells were treated as in Figure 2, except that receptor was cleaved with V8 protease. (A and B) Phosphopeptides from yeast. (C and D) Phosphopeptides from mammalian cells. (A and C) Cells not treated with hormone. (B and D) Cells treated with hormone. Arrow identifies peptide whose labeling is hormone dependent.

Figure 3

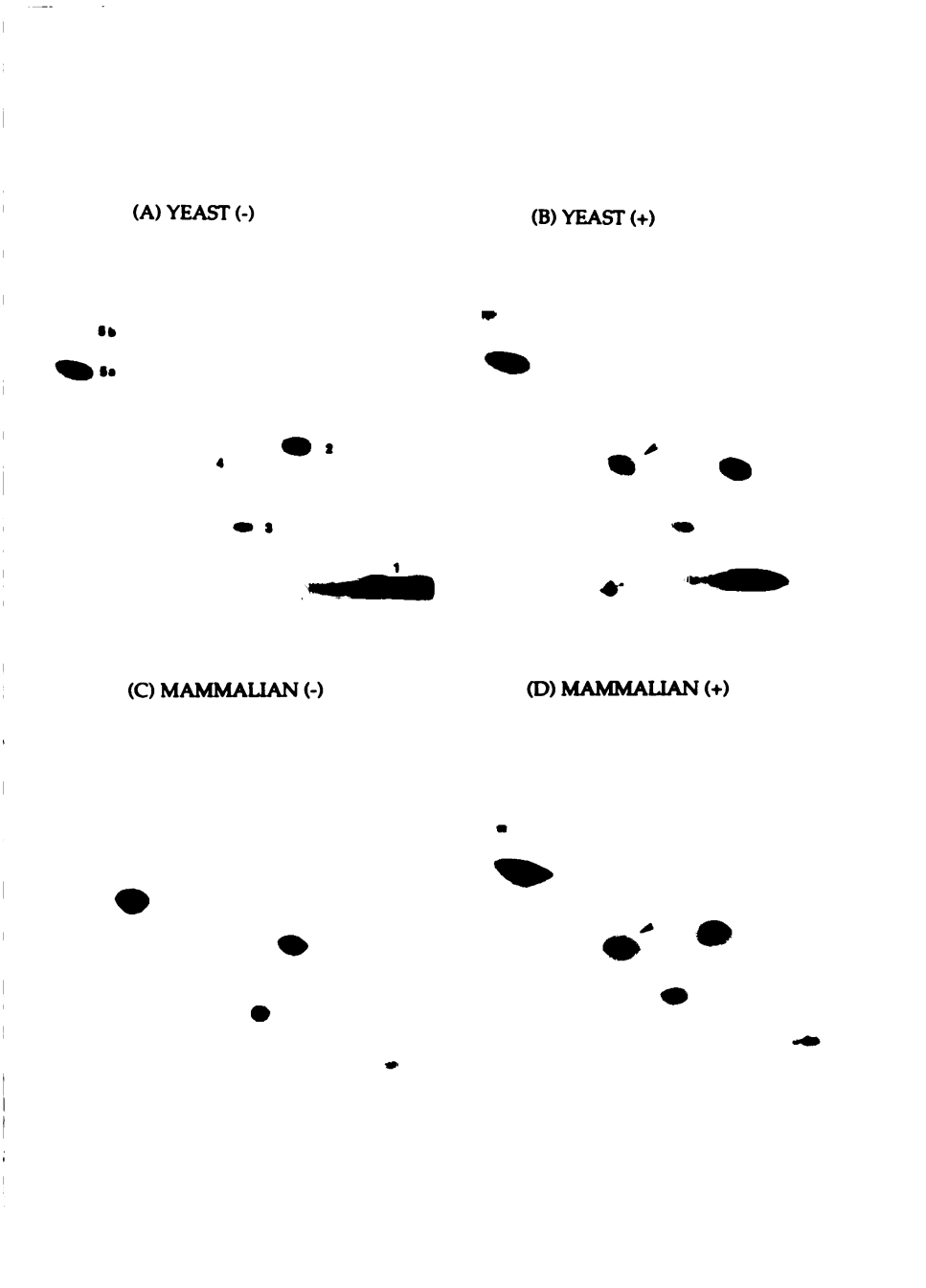


Fig. 4. Phosphopeptide mapping of the GR point mutations. Receptor mutants T171A (panels 1, 2), S224A (3, 4), S232A (panels 5, 6), and S246A (panels 7, 8) were isolated from [^{32}P]-labeled yeast and cleaved with V8 proteases as in Figure 3. Phosphopeptide maps are shown for cells incubated in the absence (panels 1, 3, 5, 7) and presence (panels 2, 4, 6, 8) of hormone. Arrows identify positions of labeled peptides present in wild type receptor, but missing from mutants.

Figure 4

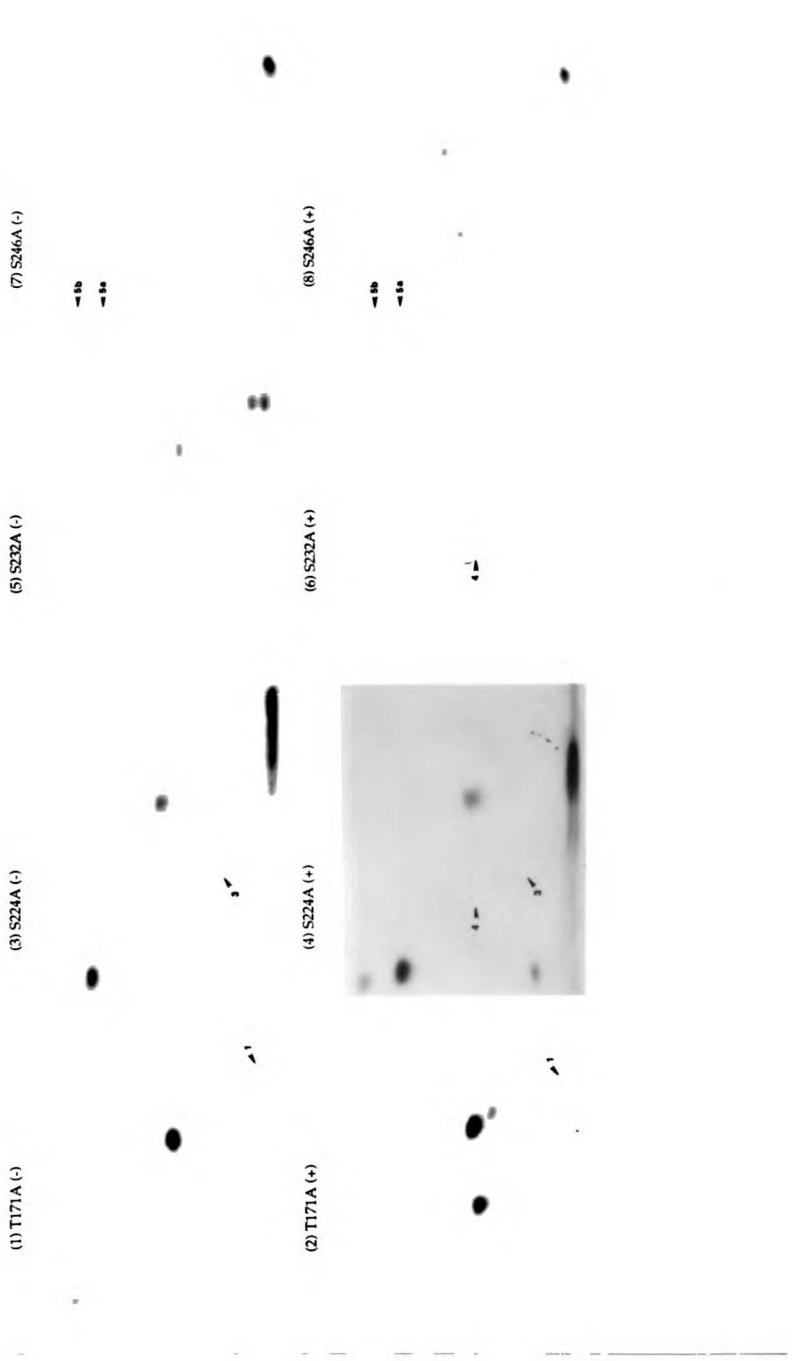


Fig. 5. Identification of V8 peptides using synthetic peptides (A) Sequences of predicted V8 phosphopeptides of the receptor; synthetic peptides corresponded to these species. (B) Individual peptides (50 μg) or subsets of peptides were fractionated by electrophoresis and chromatography, and visualized with ninhydrin. Panel *i*: Peptide V.5, carrying residue S246; panel *ii*: peptides V.2, V.3 and V.4, here fractionated together; in other experiments, they were fractionated separately to allow their individual indentification (MDK, in preparation); panel *iii*: peptide V.1; panel *iv:* receptor specific V8 phosphopeptides from in vivo labeled hormone treated cells. O denotes origin. (C) Schematic representation of V8 peptides resolved on 2D map.

Figure 5A

SYNTHETIC PEPTIDES	MAP POSITION
159 asn pro lys ser ser thr ser ala thr gly cys ala **P-thr** 171 pro thr glu 174	1
220 phe ser ala gly **P-ser** 224 pro gly lys asp thr asn glu 231	3
232 **P-ser** 232 pro trp arg ser asp leu leu ile asp glu 242	6
220 phe ser ala gly ser224 pro gly lys asp thr asn glu **P-ser** 232 pro trp arg ser asp leu leu ile asp glu 242	2
220 phe ser ala gly **P-ser**224 pro gly lys asp thr asn glu **P-ser** 232 pro trp arg ser asp leu leu ile asp glu 242	4
243 asn leu leu P-ser 246 pro leu ala gly glu 251	5

Figure 5B

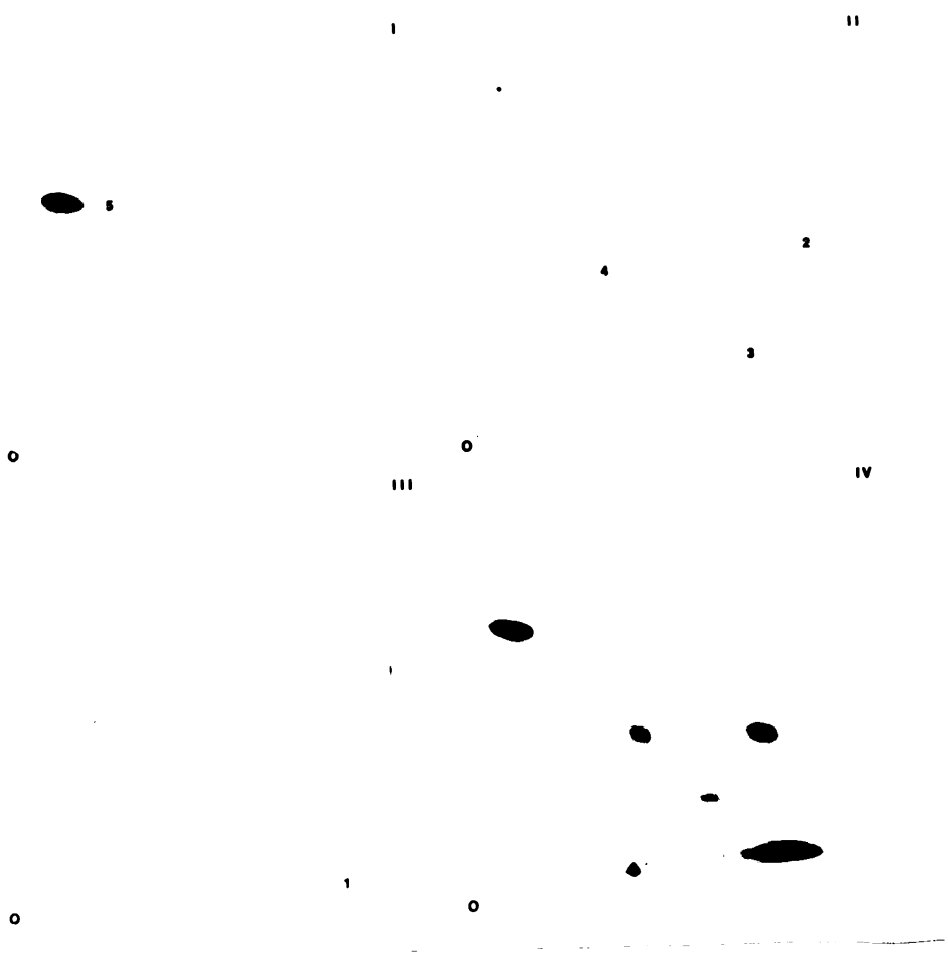


Figure 5C

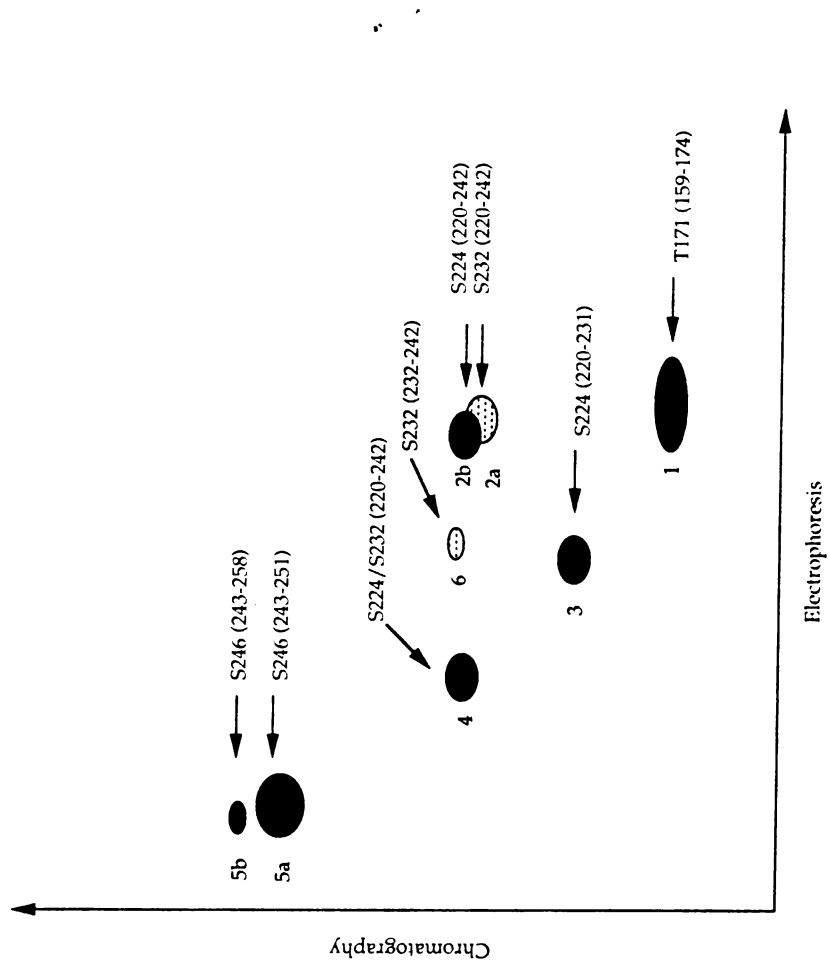

T171 (159-174)
S224 (220-231)
S224 (220-242)
S232 (220-242)
S232 (232-242)
S224/S232 (220-242)
S246 (243-258)
S246 (243-251)
1
2b
2a
3
6
4
5b
5a
Electrophoresis
Chromatography

Fig. 6. Phosphoamino acid analysis of point mutant receptor derivatives. Yeast cultures expressing receptor protein were labeled with [^{32}P] orthophosphate in the presence of 10 μM DOC. Receptor was immunoprecipitated, fractionated by SDS polyacrylamide gel electrophoresis, transferred to Immobilon-P, and visualized by autoradiography; receptor specific band was excised and phosphoamino acid analysis was performed as described in Material and Methods. (A, B) Wild type receptor, (C, D) T171S, (E, F) T171S/S224T, (G, H) T171S/S232T (I, J) T171S/S246T. Upper panels, absence of hormone; lower panels, presence of hormone; S, Serine; T, Threonine; Y, Tyrosine.

Figure 6

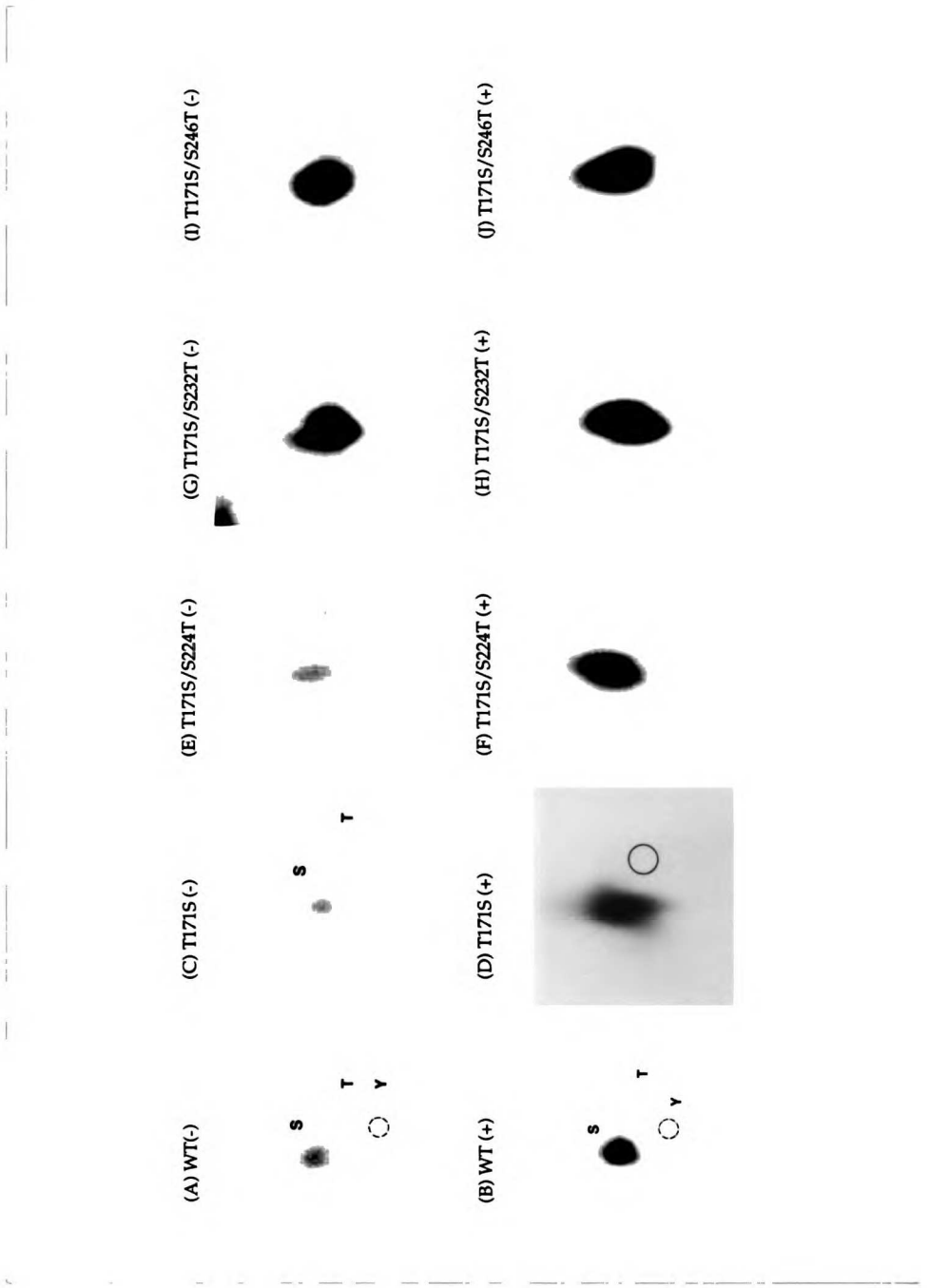

(A) WT(-)
(B) WT (+)
(C) T171S (-)
(D) T171S (+)
(E) T171S/S224T (-)
(F) T171S/S224T (+)
(G) T171S/S232T (-)
(H) T171S/S232T (+)
(I) T171S/S246T (-)
(J) T171S/S246T (+)
S
T
Y

Fig. 7. Transcriptional regulation mediated by phosphorylation deficient receptor derivatives (A) Repression by phosphorylation defective receptor derivatives in mammalian cells. Subconfluent monolayers of F9 cells were cotransfected with 2 μg of rat glucocorticoid receptor expression vector (6RGR), 0.1 μg each of cJun (pRSV cJun) and cFos (pRSV cFos) expression vectors, 2 μg of plfG3 CAT reporter vector, and 50 ng of β-galactosidase expression plasmid (6RZ), as an internal control of transfection efficiency. After transfection, cells were washed with PBS, then incubated 24 hr in medium containing or lacking 0.1 μM dexamethasone. Cell extracts were prepared by freeze thaw in dry ice-ethanol. CAT and β-galactosidase activities were measured as described in Material and Methods. (B) Enhancement by phosphorylation defective receptor derivatives in mammalian cells. Subconfluent COS7 cells were cotransfected with 10 ng of rat glucocorticoid receptor expression vector, 2μg of ΔGTCO reporter vector, and 1 μg of carrier DNA. Cells were treated with hormone as described above. (C) Enhancement by phosphorylation defective receptor derivatives in yeast. Yeast strain W303 was transformed with the receptor expression plasmid PRS 314GR and receptor reporter plasmid pΔs1Gs3. Cultures were incubated for 16 hr in the presence and absence of 1 μM DOC; β-galactosidase activity was measured as described in Material and Methods. For panels A-C, values represent percent of wild type activity, showing the average and range of two transfections, each carried out in duplicate. (D) Phosphorylation defective receptor derivatives are expressed at the same level as the wild type receptor. Lysates from yeast cells were prepared as described in Material and Methods. Cell extracts were mixed with 2xSDS sample buffer and equal amount of protein was loaded, fractionated by SDS polyacrylamide electrophoresis, transferred to Immobilon-P membrane (Millipore), and visualized by immunoblotting.

Figure 7A

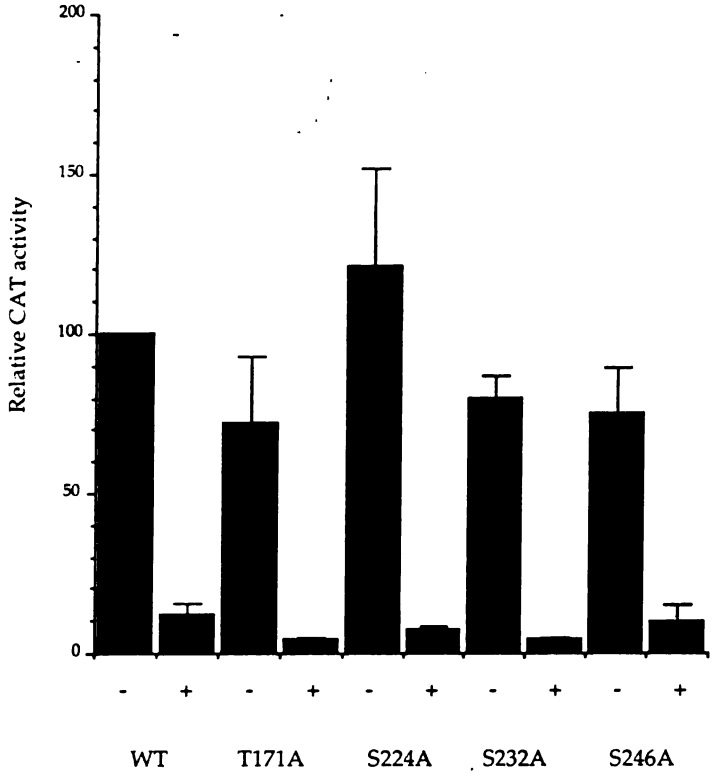


Figure 7B

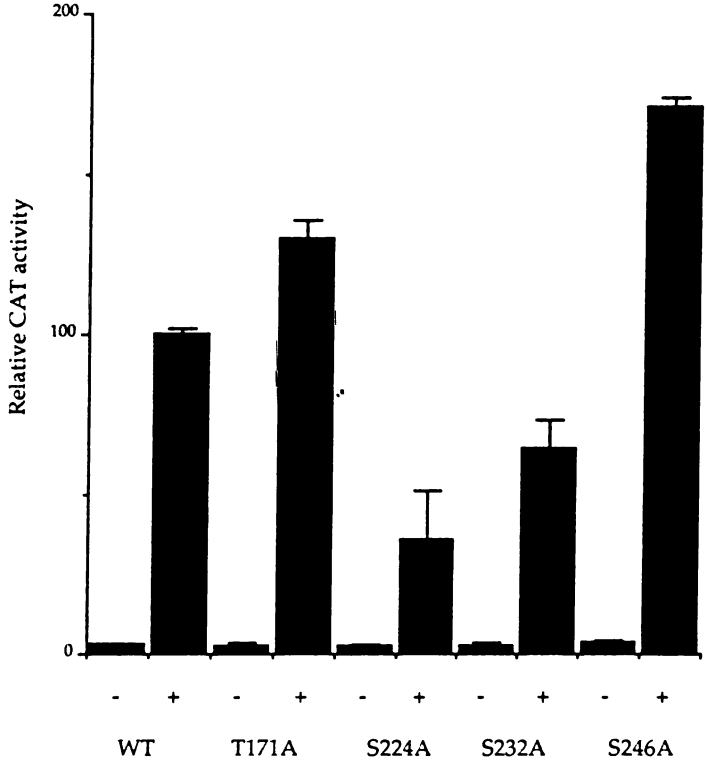


Figure 7C

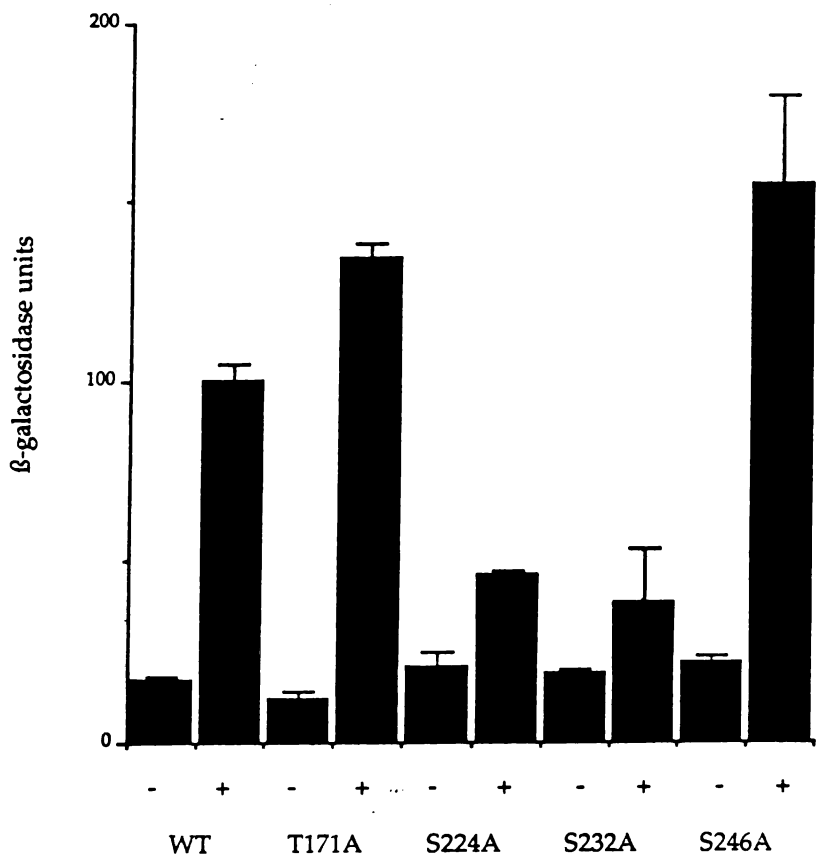
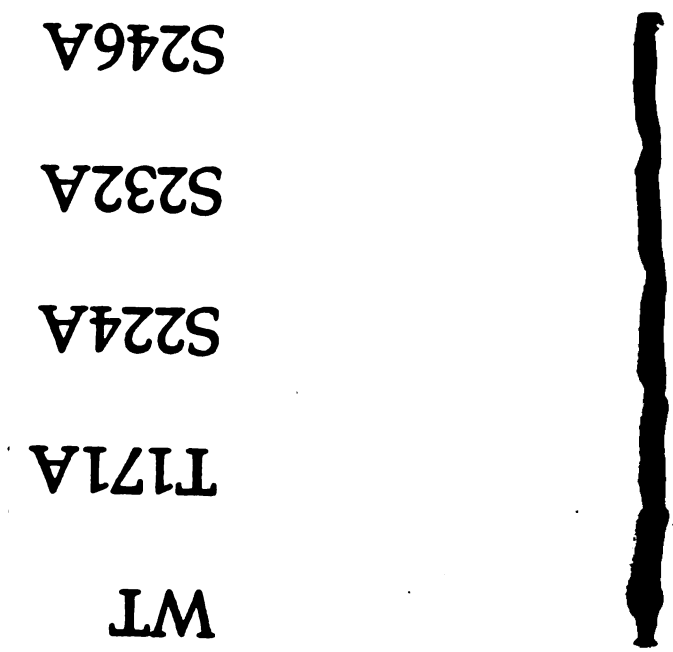


Figure 7D



CHAPTER II: Functional analysis of glucocorticoid receptor phosphorylation
Part B (appendix)

Receptor activity and receptor phosphorylation in yeast: kinetic experiments

To examine if receptor was phosphorylated when expressed in *Saccharomyces cerevisiae*, a glucocorticoid receptor expression plasmid was introduced into yeast cells together with reporter plasmid carrying GRE fused to the CYC-β-galactosidase gene (as described in Material and Methods in Chapter IIA). In order to determine the time of optimal incorporation of radioactivity into the receptor, cells were incubated with ^{32}P orthophosphate in the presence and absence of hormone, for various time periods (Fig. 2.1). Cell lysates were prepared after 15, 30 and 90 minutes and receptor was isolated by immunoprecipitation using the receptor specific monoclonal antibody BUGR2 (Gametchu and Harrison, 1984). Immunoprecipitates were separated by SDS polyacrylamide gel electrophoresis and the gel was dried and exposed to film. Optimal ^{32}P incorporation was observed after 90 min of incubation of cells with radioactivity and hormone, and this time was used in all further experiments. Receptor was phosphorylated in yeast both in the presence and absence of hormone; in addition, hormone dependent phosphorylation of receptor was detected by mobility shift in SDS gels; the extent of the mobility change depended on the strength of the agonist used (Fig. 2.1, compare DOC with DEX; see chapter IIA, Fig. 1.B). This shift in mobility was often observed in phosphorylated proteins and is believed to be the result of a conformational change caused by the addition of phosphate that is preserved under conditions of electrophoresis; one potential explanation for the altered electrophoretic

mobility is that SDS can not efficiently coat the protein due to the negative phosphate charge, thus altering its mobility in the electrical field. This mobility shift caused by phosphorylation is also observed in other steroid receptors and transcription factors. For example the progesterone receptor appears as a doublet on SDS gels after hormone binding (Beck et al., 1992). Moreover, the transcription factor Elk1 involved in the regulation of the c-fos promoter undergoes a phosphorylation-induced conformational change detected as electrophoretic decrease in its mobility (Marais et al, 1993).

The hormone dependent phosphorylation can be detected as early as 15 minutes after hormone treatment (Fig. 2.1). Kinetic studies of receptor phosphorylation in mammalian cells suggested that the increase in receptor phosphorylation upon hormone addition occurs rapidly, which is consistent with the results from yeast cells shown at Figure 2.1. For example, Hoeck et al. (1989) have reported an increase in GR phosphorylation in NIH 3T3 cells after 20 minutes of hormone treatment that was further increased by 40 and 60 minutes. Similarly, Orti et al. (1993) have observed that hormone induced increase in phosphorylation was seen as early as 5 minutes after hormone addition to WEHI-7 mouse thymoma cells.

In the subsequent experiment, receptor mediated transcriptional activation was analyzed at several time points and compared with receptor phosphorylation. Transcription from the reporter gene was followed by primer extension analysis of CYC1-lacZ transcripts (as described in Material and Methods, chapter IIB), after incubation of yeast cells for 15, 30, 90 and 120 minutes in the absence and presence of hormone (Fig. 2.2); receptor activates transcription from this reporter gene within 15 minutes of hormone addition; it is interesting that the hormone dependent phosphorylation was first observed after 15 minutes of agonist treatment as well, suggesting a potential

functional significance of this modification. Similar kinetics of receptor mediated transcriptional activation was reported in mammalian cells. Ringold et al. (1975) have observed that hormone increases the accumulation of mouse mammary tumor virus RNA with the lag period of 15 minute and a half life of 2.5 hrs.

Taken together, these results suggest that yeast cells may be similar to mammalian cells with respect to receptor phosphorylation, as receptor expressed in these cells is a phosphoprotein in both the absence and presence of hormone and additional phosphorylation accompanies agonist binding; furthermore, phosphorylation could be an important modulator of receptor function, since the time of appearance of hormone dependent phosphorylation in yeast cells correlated with the the time of appearance of receptor mediated transcriptional enhancement.

The experiments described above identified only phosphorylated receptor isoforms. In order to examine the whole population of receptor molecules, two type of experiments were performed. First, receptor protein was labeled with ^{35}S methionine *in vivo*, which detects newly synthesized potential phosphorylated and unphosphorylated receptor isoforms. Receptor was treated as described in metabolic labeling experiments with ^{32}P (Material and Methods, Chapter IIA), except that ^{35}S methionine was used (Material and Methods, Chapter IIB). Figure 2A.3. (lanes 3 and 4) shows a hormone dependent mobility shift of receptor from yeast cells that have been metabolically labeled with radioactive methionine. Yeast cells that have not been transformed with the receptor plasmid do not have endogenous receptor (compare lines 1 and 2 with lines 3 and 4 in Fig. 2.3).Finally, immunoblotting was used to visualize receptor, and this simpler and nonradioactive approach detected the whole population of receptor molecules, including several

hormone dependent receptor isoforms (Fig. 2.4, lines 1 and 2 and Chapter IIA, Fig.1B). Thus, the majority of glucocorticoid receptor expressed in yeast is phosphorylated and receives at least one additional phosphate after addition of hormone (see also Chapter IIA).

Phosphatase treatment of glucocorticoid receptor in yeast

The appearance of receptor isoforms that are present only after hormone treatment can be due to phosphorylation or other posttranslational modifications such as glycosylation. To distinguish between these possibilities, receptor immunoprecipitated from yeast cells was treated with calf intestine alkaline phosphatase and subsequently visualized by immunoblotting as described in Materials and Methods in Chapter IIB (Fig. 2.4). Phosphatase treatment eliminated slower migrating receptor isoforms, suggesting that this shift was indeed due to phosphorylation. Similarly, the progesterone receptor that appears as a doublet on SDS gels, changes its mobility into more homogenious, faster migrating form after phosphatase treatment (Bagchi et al., 1992).

Phosphoamino acid analysis of GR expressed in yeast and mammalian cells.

To examine whether serine, threonine or tyrosine residues are phosphorylated, phosphoamino acid analysis was performed. Cells were labeled with ^{32}P orthophosphate as described, and receptor was isolated from yeast and mammalian cells incubated in the presence and absence of hormone. Receptor was immunoprecipitated, transferred to Immobilon-P and hydrolyzed with 6 M HCl for 60 minutes at 110^{o}C as described in Material

and Methods in Chapter IIA. Two dimensional electrophoresis was used to resolve amino acids and their identity was confirmed by comigration with ninhydrine stainable markers. Receptor expressed in both yeast (panels A and B) and mammalian cells (panles C and D) was phosphorylated mostly on serine and less on threonine in both the absence (panels A and C) and presence (panels C and D) of hormone. Phosphotyrosine phosphorylation was not observed (Fig. 2.5). Similarly, several groups have reported that GR in mammalian cells is phosphorylated on serine and threonine residues (Bodwell et al., 1991). Phosphorylation on tyrosine was not observed, although glucocorticoid receptor reportedly interacts with tyrosine specific antibodies (Auricchio et al., 1987). Taken together, these results confirmed that both in yeast and mammalian cells, phosphorylation of GR occurs on serine and threonine residues.

Mapping the regions of hormone dependent phosphorylation

In order to analyze in more details phosphorylation state of receptor in the absence and presence of hormone, two-dimensional phosphopeptide mapping was performed. Receptor was labeled *in vivo* as described in Material and Methods in Chapter IIA, the receptor specific band was isolated from the gel slice, and subsequently digested with trypsin. The resulting phosphopeptides were resolved in two dimensions by electrophoresis and chromatography. The time course of trypsin digestion suggested that 18 hours was the optimal time for cleavage, since phosphopeptide maps did not change after longer periods of incubation (data not shown). The tryptic phosphopeptide pattern of receptor isolated from yeast cells incubated with (panel A) or without (panel B) hormone revealed 11 major and 8 minor

peptides represented schematically on Figure 2.6, panel D. Certain peptides (4,5,6) were phosphorylated to the same extent regardless of hormone treatment and others (2,7,8,9) were phosphorylated in a hormone dependent fashion. Finally, receptor specific peptides isolated from yeast cells incubated with or without hormone were mixed and loaded on a TLC plate (panel C). This experiment suggested that GR specific peptides obtained from cells not treated with the hormone comigrated with peptides obtained from cells treated with the hormone (Fig. 2.6).

In order to determine the localization of phosphorylated residues, in vivo ^{32}P labeling studies with various receptor deletion mutants expressed in yeast were performed. These studies indicated that the hormone binding domain of the receptor is not phosphorylated since the N556 derivative contains all the same phosphopeptides as the wild type receptor (data not shown). Likewise, phosphopeptide mapping of the receptor derivative Δ 1 that lacks amino acids 70-130, also revealed the wild type tryptic pattern (data not shown and Fig. 2.7). On the contrary, various receptor derivatives with deletions in the N terminal part of the receptor (such as Δ70-300 that contains deletion of indicated residues, or derivative containing only amino acids 407-557, named X556), were phosphorylated very weakly. However, a receptor derivative Δ5 that carries deletion of amino acids 107-237 lacked several peptides (data not shown), suggesting that at least some of the phosphorylation sites must be localized in this region. Analysis of the hormone dependent phosphorylation of this mutant derivative revealed that Δ5 mutant did not show hormone dependent mobility shift, while Δ1 mutant derivative retained wild type hormone dependent mobility change (Fig. 2.7). These studies indicated that most of the receptor phosphates, lie N terminally to the receptor DNA binding domain, which encompasses the part of the

receptor involved in transcriptional regulation, and that hormone dependent phosphorylation is most likely localized within 130-237 region of the receptor.

Phosphotryptic mapping of glucocorticoid receptor point mutants

Inspection of this region of the rat glucocorticoid receptor (amino acids 130-237) revealed several consensus sequences for MAP kinase and CDK kinase. In addition, seven sites of phosphorylation have been identified on the mouse glucocorticoid receptor from hormone treated mammalian cells (Bodwell et al. 1991). We altered four of the corresponding rat receptor residues from serine or threonine to alanine or glycine (residues T171A, S224A S232G and S246A), and characterized the phosphorylation state of these receptor derivatives to identify the sites of phosphorylation in yeast. Metabolic labeling with ^{32}P orthophosphate and phosphotryptic peptide analysis of the receptor point mutants in the presence and absence of hormone is shown in Figure 2.8. Peptide 10 is missing from the maps derived from the receptor mutation T171A (Figure 2.8, panels 1 and 2) regardless of hormone treatment. The derivative S246A lacks peptides 4 and 5 in both absence and presence of hormone (Figure 2.8, panels 7 and 8). These results suggest that T171 and S246 are phosphorylated in both untreated and hormone treated yeast cells. However, a more complicated result arises with receptor point mutants S224A and S323G. Without hormone, there are changes in intensities of peptides 2 and 7 on the phosphotryptic map of these mutant derivatives (Fig. 2.8, panels 3 and 5). However, in the presence of hormone, these single amino-acid substitutions results in the loss of the major hormone inducible phosphopeptide (Fig. 2.8, panels 3-6, peptide number 8) and several other weakly hormone stimulated phosphopeptides (peptides 2 and 7). This

disappearance of multiple peptides resulting from alteration of S224 or S232G can be explained by the loss of partial trypsin digestion products. Indeed, the protease recognition sites flanking S224 and S232 are poor cleavage sites for trypsin (lysine followed by acidic amino-acids). This partial tryptic cleavage in the case of wild-type receptor will generate many of distinct phosphopeptides, all containing the same phosphorylated amino acid S224, but differing in their charge and length. Thus the alteration of S224 or S232 to a non-phosphorylatable residue might result in the disappearance of not one peptide, but multiple phosphotryptic peptides. Furthermore, the predicted mobilities of the partial tryptic-phosphopeptides surrounding residues S224 and S232G are consistent with their actual positions on the two-dimensional map. Similarly, Bodwell and colleagues (1991) have identified by microsequencing several peptides that contain residue S224 and are result of inefficient leavage by trypsin. All of these mutations, therefore, eliminate particular phosphopeptides and argue that these residues (T171, S224, S232 and S246) are indeed sites of the glucocorticoid receptor phosphorylation in yeast.

MATERIAL AND METHODS

Metabolic labeling of glucocorticoid receptor expressed in yeast with ^{35}S methionine

Yeast cells were grown at OD_{600} 0.6 and treated as described for metabolic lebeling with ^{32}P orthophosphate, except that 100μCi/ml of ^{35}S methionine was added instead of the radioactive phosphate; in addition, cells were incubated in methionine free media instead in phosphate free media.

Following receptor dependent transcriptional activation by primer extension analysis

Primer extension analysis was performed as described in Schena et al. (1989). Briefly, total RNA was isolated from yeast cells carrying receptor and reporter plasmids and subjected to primer extension with a 20 nucleotide primer complementary to the LacZ sense strand sequences 17 bp downstream of the BamH1 site. Extension products from 25μg of RNA were fractionated on a sequencing gel adjacent to ^{32}P labeled HaeIII fragments from PBR 322.

Phosphatase treatment

Receptor was immunoprecipitated from yeast cells as described in Material and Methods, Chapter IIB, incubated with or without 10μM DOC and treated with 20 units of calf intestine phosphatase (Boerhinger Manneheim,) for 30 minutes at 25 ^{o}C. The phosphatase reaction was performed in the phosphatase buffer (50 mM Tris pH 8.8 and 1 mM $ZnCl_2$). Immunoprecipitates

were washed with 100 µl of the phosphatase buffer, resuspended in equal volume of 2XSDS sample buffer, fractionated on SDS PAGE and transferred to the Immobilon-P (Millipore). Membrane was developed with GR specific monoclonal antibody BUGR (Gametchu and Harrison, 1984).

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Schena, M., and Yamamoto, K., R., 1989, Science, 241, 965-967

Figure 2.1 Time course of glucocorticoid receptor phosphorylation in yeast Yeast cells carrying glucocorticoid receptor were incubated with ^{32}P orthophosphate in the absence and presence of 10μM DOC, for indicated times. Two different hormones were used: deoxycorticosterone (DOC), a strong agonist and dexamethasone (DEX) weak agonist in yeast. Receptor was isolated by immunoprecipitation, fractionated by SDS electrophoresis and gel was exposed to film as described in Chapter IIA.

Figure 2.1

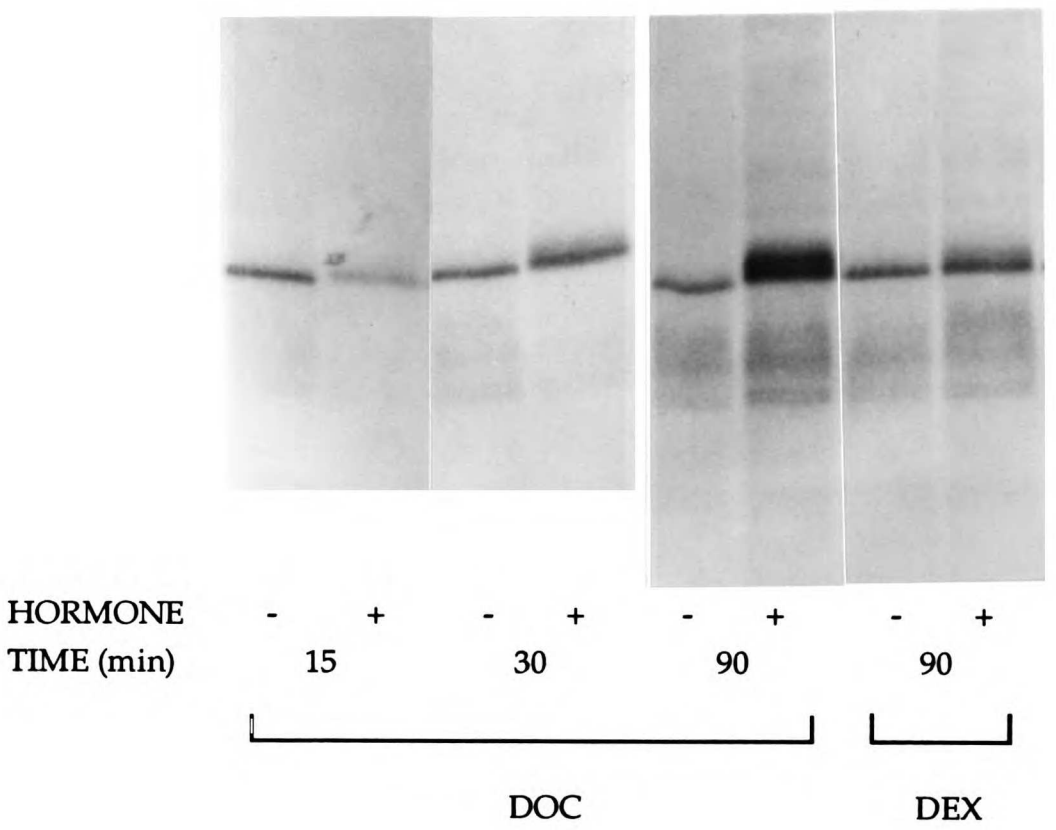


Figure 2.2 Time course of glucocorticoid receptor mediated transcriptional enhancement in yeast

Yeast cells carrying receptor were incubated with or without 10μM DOC for 15, 30, 90 and 120 minutes as described in Material and Methods. Total RNA was isolated from yeast cells and subjected to primer extension with a 20-nucleotide primer complementary to lac Z sense strand sequences 17 bp downstream of the Bam H1 site. Extension products from 25 μg of RNA were fractionated on a sequencing gel adjacent to ^{32}P-labeled Hae II fragments from pBR322.

Figure 2.2

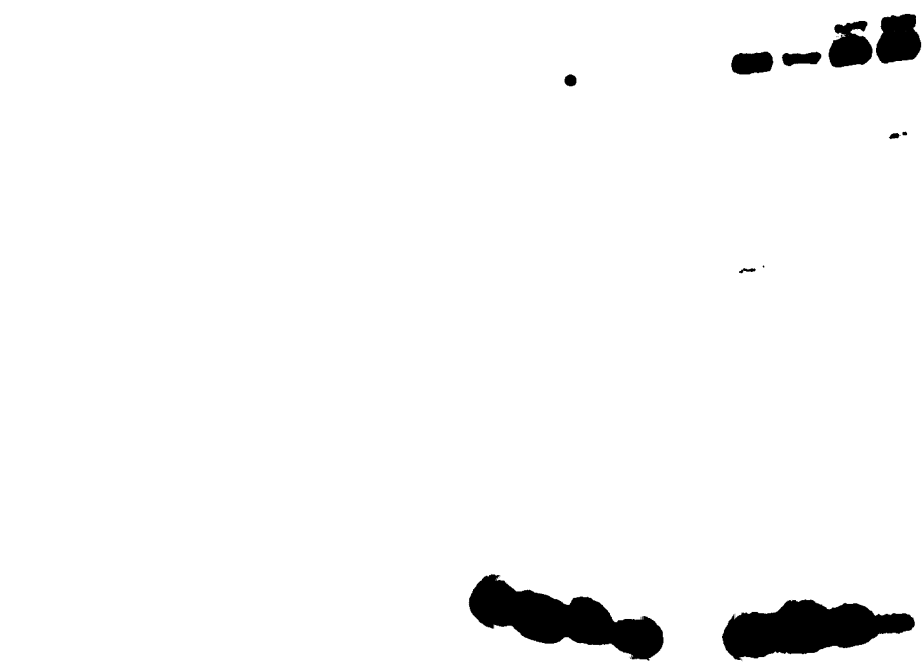


Figure 2.3 ^{35}S methionine labeling of glucocorticoid receptor expressed in yeast

Yeast cells carrying receptor were incubated with ^{35}S methionine in the absence and presence of 10μM DOC for 2 hrs as described in Material and Methods, Chapter IIB. Receptor was further treated as described in Figure 2A.1, except that methionine free yeast media was used instead of phosphate free media for metabolic labeling.

Figure 2.3

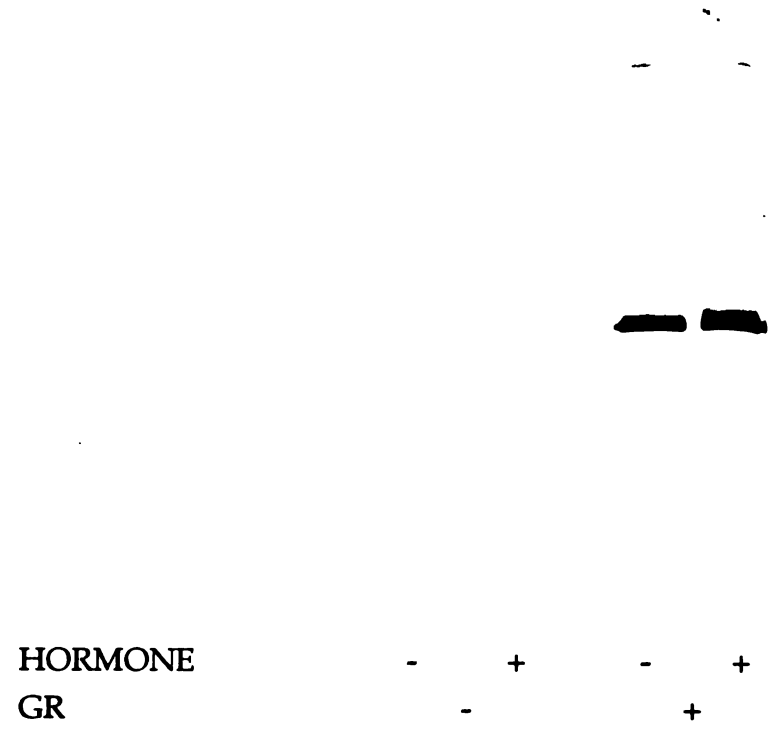
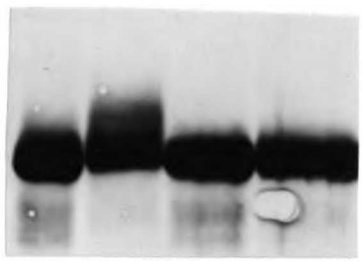


Figure 2.4 Phosphatase treatment of the GR in yeast

Immunoprecipitates of the receptor obtained from yeast cells, incubated with or without 10μM DOC were treated with 20 units of calf intestine phosphatase (Boerhinger Manneheim) for 30 minutes at 25 ^{o}C as described in Material and Methods. Receptor was subsequently fractionated on SDS PAGE, transferred to Immobilon-P and membrane was developed by BUGR antibodies.

Figure 2.4



HORMONE	-	+	-	+
PHOSPHATASE	-	-	+	+

Figure 2.5 Phosphoamino acid analysis of the receptor expressed in yeast and mammalian cells

Yeast (panels A and B) and mammalian cells (panels C and D) expressing receptor protein were labeled with ^{32}P orthophosphate in the presence (panels B and D) and absence (panels A and C) of hormone. Receptor was immunoprecipitated, fractionated by SDS polyacrylamide gel electrophoresis, transferred to Immobilon-P, washed three times in wather and visualized by autoradiography. Receptor specific band was excised and phosphoamino acid analysis was performed as described in Material and Methods in Chapter IIA. S, Serine; T, Threonine; Y, Tyrosine;

Figure 2.5

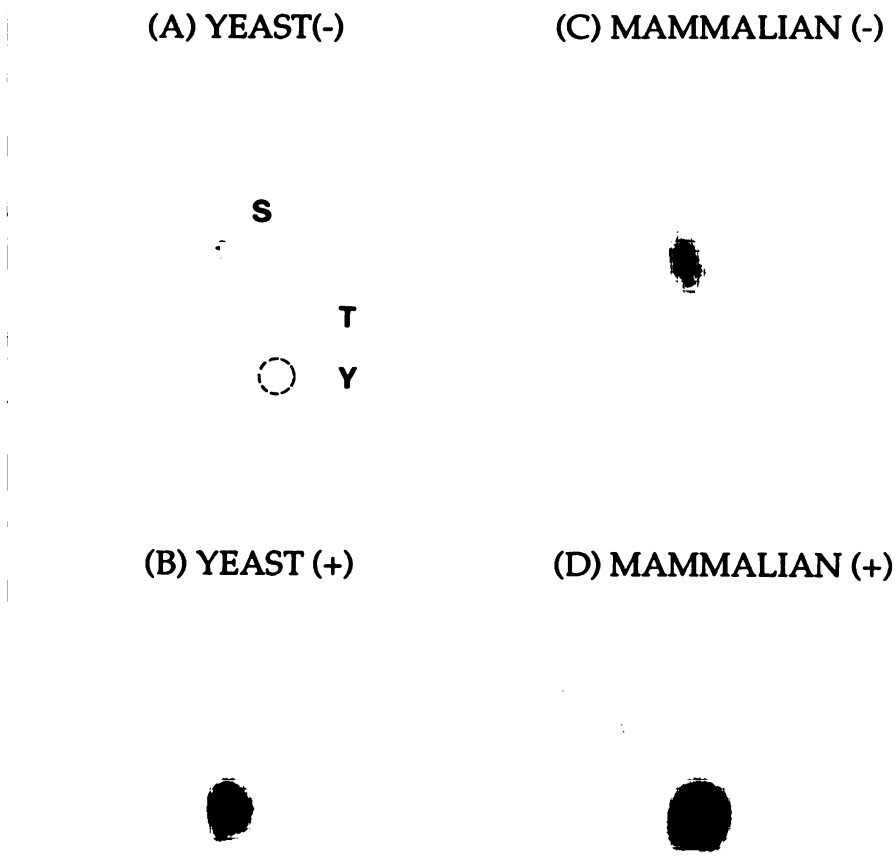


Figure 2.6 Phosphotryptic peptide pattern of receptor expressed in yeast cells incubated with or without hormone

Yeast cells expressing receptor were incubated with ^{32}P orthophosphate and in the presence and absence of hormone. Receptor was isolated by electrophoresis, extracted from gel and treated with trypsin. Phosphopeptides were separated in the first dimension by electrophoresis and in the second dimension by chromatography (A) Receptor phosphopeptides from cells not treated with hormone (B) Receptor phosphopeptides from cells treated with hormone (C) Phosphopeptides from cells incubated with or without hormone were mixed and subjected together to two-dimensional separation (D) Schematic representation of the phosphopeptides obtained from hormone treated and untreated yeast cells. There are 11 major labeled peptides (1-11; cross hatched circles) and 7 minor peptides (a-g; open circles); peptide number 8 is hormone inducible to the highest degree and peptides number 2, 7 and 9 are moderately hormone induced (filled circles).

Figure 2.6

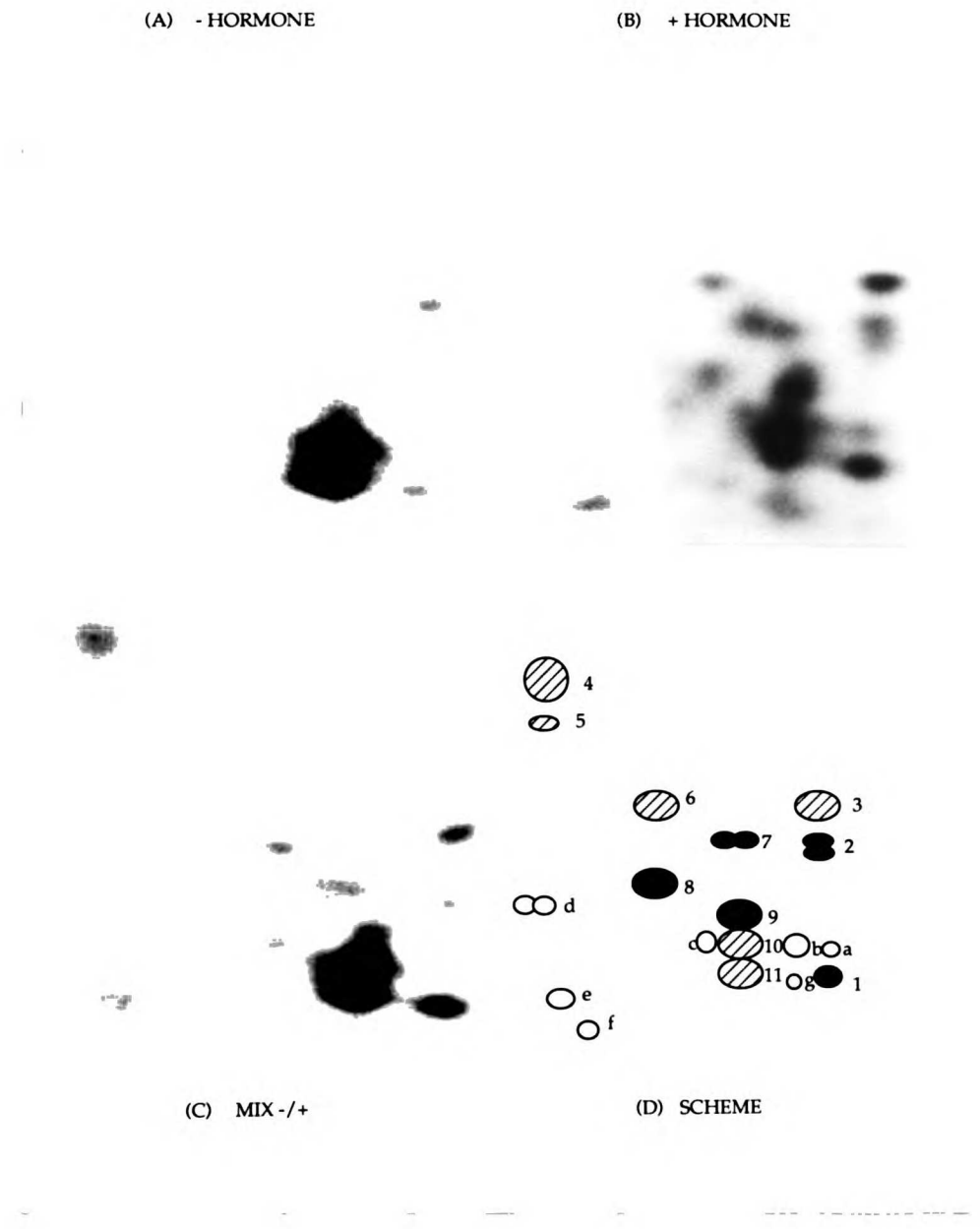
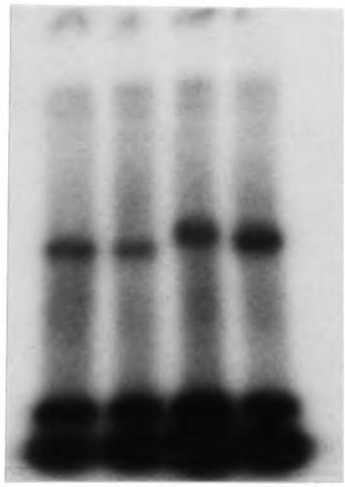


Figure 2.7 Hormone dependent phosphorylation is localized between amino acids 130-237 of glucocorticoid receptor expressed in yeast

Yeast cells expressing the wild type and mutant receptor derivatives were incubated with ^{32}P orthophosphate in the absence and presence of hormone. Receptor derivative Δ1 (lacks amino acids 70-130) shows hormone dependent mobility shift (compare lines 1 and 2); receptor derivative Δ5 (lacks amino acids 107-237) does not show hormone dependent shift (compare lines 3 and 4); for immunoblot receptor was immunoprecipitated, fractionated on SDS gel electrophoresis, transferred to Immobilon-P membrane (Millipore) and visualized by immunoblotting. (A) In vivo ^{32}P orthophosphate labeling of receptor derivatives Δ1 and Δ5 in yeast. (B) Immunoblot of receptor derivatives Δ1 and Δ5.

Figure 2.7A



HORMONE + - + -

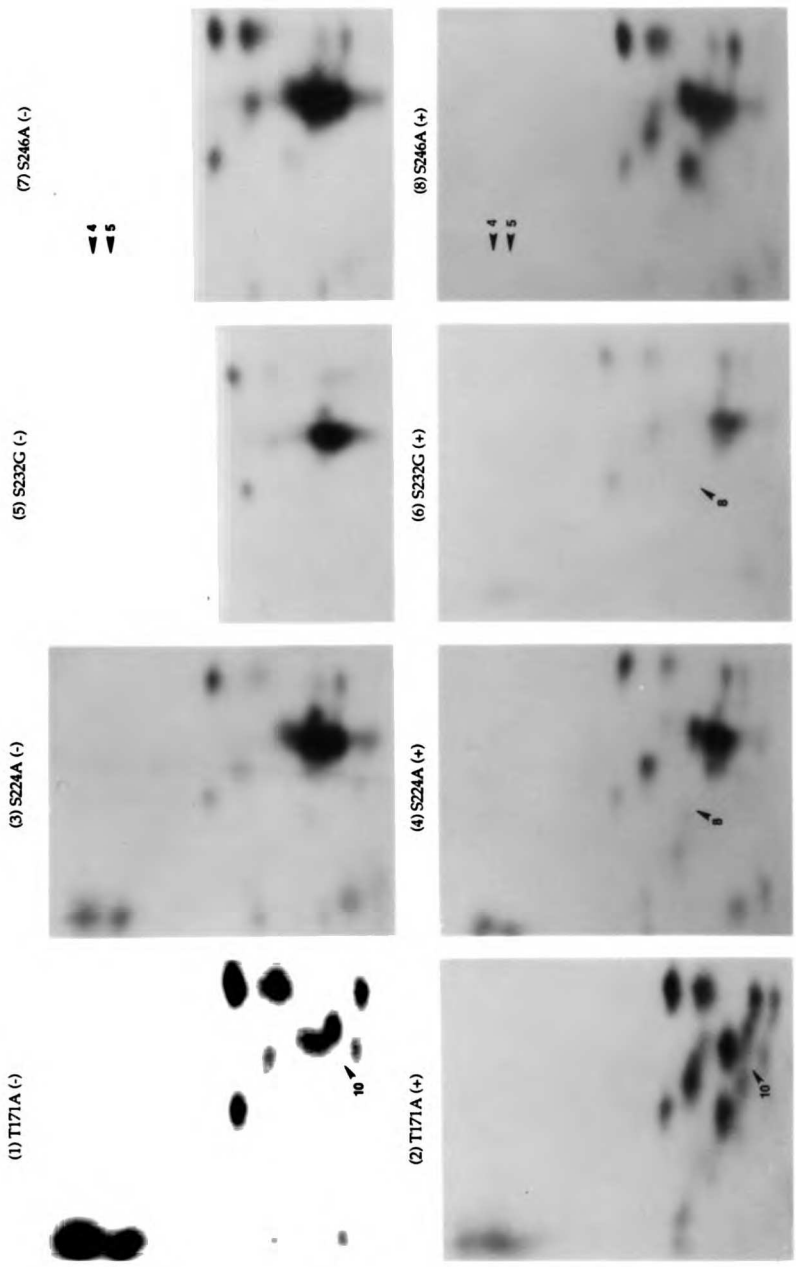
GR Δ5 Δ1

Figure 2.7B

Figure 2.8 Phosphotryptic pattern of receptor point mutants

Yeast cells bearing receptor point mutants were metabolically labeled in vivo with ^{32}P orthophosphate. Receptor was isolated by immuniprecipitation and fractionated on SDS PAGE as described in Material and Methods, Chapter IIA. Upper panels show tryptic phosphopeptide patterns from cells incubated with the ^{32}P orthophosphate in the absence of hormone and lower panels show cells that have been treated with the 10μM DOC.

Figure 2.8


(7) S246A (-)
(8) S246A (+)
(5) S232G (-)
(6) S232G (+)
(3) S224A (-)
(4) S224A (+)
(1) T171A (-)
(2) T171A (+)
10
8

CHAPTER III:

Mitogen-Activated and Cyclin-Dependent Protein Kinases Selectively and Differentially Modulate Transcriptional Enhancement by the Glucocorticoid Receptor

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ABSTRACT

Cyclin-dependent kinase (CDK) and mitogen-activated protein kinase (MAPK) phosphorylate the rat glucocorticoid receptor *in vitro* at distinct sites that together correspond to all of the phosphorylated receptor residues observed *in vivo;* MAP kinase phosphorylates receptor residues threonine 171 and serine 246, whereas cyclin-dependent kinase modifies serines 224 and 232. Mutations in these kinases have opposite effects on receptor transcriptional activity *in vivo.* Receptor-dependent transcriptional enhancement is reduced in yeast strains deficient in the catalytic ($p34^{CDC28}$) or regulatory subunits (cyclins) of the cyclin-dependent kinase and is increased in a strain of yeast devoid of the MAP kinases FUS3 and KSS1. These findings indicate that the glucocorticoid receptor is a target for multiple kinases *in vivo,* which either positively or negatively regulate receptor transcriptional enhancement. The control of receptor transcriptional activity via phosphorylation permits an increased array of regulatory inputs, in addition to steroids, to influence receptor function by altering protein kinase activity.

INTRODUCTION

The glucocorticoid receptor is a member of a steroid receptor family of hormone-dependent transcriptional regulatory proteins. The hormone-receptor complex binds specific DNA sequences and modulates transcription from nearby promoters, whereas the unliganded aporeceptor is transcriptionally inactive (Yamamoto, 1985; Yamamoto et al., 1992). Recent studies indicate that steroid hormone alone is not the sole determinant of receptor activity. Rather, these proteins receive and process information through interaction with various cellular proteins (Picard et al., 1990;

Diamond et al., 1990; Yoshinaga et al., 1992) and by phosphorylation (Krstic et al., 1995).

Indeed, like other steroid hormone receptors and transcriptional regulatory proteins, GR is a phosphoprotein (Orti et al., 1992). The aporeceptor is phosphorylated, with additional phosphorylation events occurring in conjunction with ligand binding. Receptor phosphorylation occurs on serine and threonine residues in both the presence and absence of hormone and is reportedly subject to cell-cycle-related changes (Hsu et al., 1992; Hu et al., 1994). The hormone-dependent increase in receptor phosphorylation fueled speculation that phosphorylation may modulate subcellular localization, DNA binding or protein-protein interactions important for transcriptional regulation.

The majority of the phosphorylated residues lie within the N-terminal region of the receptor, a domain involved in transcriptional enhancement and repression (Pearce and Yamamoto, 1993; Godowski et al., 1988). Krstic et al. (1995) identified and characterized sites on the rat glucocorticoid receptor that are phosphorylated both in mammalian cells and in yeast. They showed that phosphorylation of S224 and S232 increases in the presence of hormone agonists, whereas residues T171 and S246 are phosphorylated constitutively. Interestingly, S224 and S232 reside in consensus sequences for cyclin-dependent kinase, CDK, and T171 and S246 in consensus sequences for mitogen-activated protein kinase, MAPK. Moreover, mutations of certain sites have opposite phenotypic consequences on transcriptional enhancement by the receptor *in vivo*. For example, phosphorylation of serine 224 has a stimulatory effect on receptor activity: a derivative carrying a mutation of this serine into alanine activates transcription less efficiently than the wild type receptor (Krstic et al., 1995). In contrast, phosphorylation of S246 reduces

receptor transcriptional activity, suggesting that phosphorylation can influence receptor activity in opposite fashion (Krstic et al., 1995).

To determine what enzymes are involved in phosphorylating the receptor, we first surveyed a battery of serine/threonine kinases for their capacity to phosphorylate the receptor *in vitro* at sites identified by *in vivo* studies. We then measured the transcriptional activity of the receptor in yeast strains bearing genetic defects in candidate kinases to assess their effects on receptor function *in vivo*.

MATERIAL AND METHODS

Yeast strains

The following yeast strains were used as receptor hosts. Strains carrying altered alleles of G1 cyclins were in S288C background (Ogas et al., 1991; unpublished data): (MATa trp1 ura3 his3 ade2 lys2 leu2 cln1::TRP1; cln2::LEU2; cln3::URA3). Strains carying altered alleles of G2 cyclin were in 15DUa background (Richardson et al., 1992): (MATa ura3 leu2 trp1 his2 arg4 ade1 clb1::URA3; clb2::LEU2; clb3::TRP1; clb4::HIS2). Strains deficient in the cyclin-dependent kinase (cdc28-4, cdc28-1N) were in W303 background (MATa ade2, trp1, cal1, leu2, his3, ura3, ho) (Moll et al., 1991). The same wild type isogenic strain was also compared with strains carrying cdc-7 mutation which affects a non cyclin-dependent kinase. Yeast strains carying deletions of MAP kinases were in the EG123 background (MATa trp1 leu2 ura3 his4 can1 fus3::URA3, kss1:: URA3) (Neiman et al., 1993).

These strains were transformed using the lithium acetate and polyethylene glycol method (Ito et al., 1983) with a glucocorticoid receptor expression plasmid and a reporter plasmid containing a glucocorticoid

response element (GRE) placed upstream of the CYC1-LACZ gene as described below. Yeast cultures were propagated at 30°C (unless otherwise indicated) in minimal medium with amino acids and 2% glucose (Jones, 1977)

Plasmids

The yeast glucocorticoid receptor expression plasmids, such as PRS 314GR, are vectors with centromeric CEN/ARS origins, that maintain a copy number of 1-2 per cell. These vectors contain the rat glucocorticoid receptor cDNA downstream of the yeast glyceraldehyde-3-phosphate dehydrogenase promoter (Schena et al., 1989). Reporter plasmids pΔs26x and pΔs1Gs3 contain three tandem 26 bp oligonucleotide from tyrosine amino transferase GRE or a single palindromic GRE, respectively, inserted upstream of a minimal yeast cytochrome c1 promoter, retaining only a TATA box and transcription start site, fused to the *E. coli* b-galactosidase (Lac Z) coding sequences. These plasmids also contain a selectable marker, a bacterial origin of replication, and the ampicillin resistance gene (Schena et al., 1989; Luisi et al., 1991).

β-galactosidase assay

For liquid assay, overnight yeast cultures were diluted 1: 5 in the selective minimal medium and treated with 1μM hormone for indicated times. β-galactosidase activity was assayed as substrate hydrolysis; units were defined as previously described (Schena et al. 1989). Briefly, 1.5 ml of yeast cells was pelleted and washed with LAC Z buffer (10mM KCl, 1mM $MgSO_4$, 50 mM β-mercaptoethanol, and 100 mM $NaPO_4$, pH 7.0). Cells were pelleted again, resuspended in 50 μl of LAC Z buffer and treated with 50 μl $CHCl_3$ and 20 μl 0.1% SDS. 0.7 ml of 2 mg/ml of the β-galactosidase substrate (o-nitro phenyl-b-

galactoside) was added to the mix. After 1-10 min incubation, reaction was stopped with 0.5 ml 1 M Na_2CO_3.

In vitro kinase assay

Receptor derivative EX556 and its expression and purification have been described previously (Freedman et al, 1989). Briefly, this derivative was constructed by fusing in-frame amino acids 407-556 downstream of amino acids 106-318. This construct was then cloned in T7 RNA polymerase-dependent vector pAR3040 and overexpressed and purified from *E. coli.* MAP kinase $p44^{mapk}$, has been obtained from Upstate Biotechnology Incorporated, is active purified kinase from starfish oocytes. CDK2/cyclinA and CDK4/cyclinD obtained in Baculovirus expression system were used as source of kinase activity as described by Desai and colleagues (1992). Briefly, kinase activity was assayed in 30 µl reaction mix containing 10mM MgCl2, 50µM ATP, 1mMDTT, 2.5 µCi ^{32}P ATP (3000Ci/mmol, Amersham, Arlington Height, IL). After 5 min at 24°C, reactions were boiled in 2XSDS sample buffer and fractionated on polyacrylamide gels. The receptor specific band was excised from gels, and receptor was eluted for phosphopeptide mapping as described below.

Phosphopeptide mapping

Phosphopeptide mapping was performed as described by Krstic et al. (1995). Briefly, polyacrylamide gels containing the labeled receptor were washed in 500 ml water, 3x10 min, and dried between cellophane sheets. Following autoradiography, the glucocorticoid receptor band was excised and the rehydrated gel slice was placed into a microfuge tube at room temperature in

50 mM ammonium acetate, 1 mM DTT, and 50 μg/ml of V8 protease (Endoproteinase Glu-C, Boehringer Mannheim), adjusted to pH 4. Samples were centrifuged for 5 min at 12000 x g and the supernatant containing the digested peptides was evaporated to dryness in a Speedvac (Savant, Farmingdale, NY). Peptides were resuspended in 500 μl of water, dried and washed once more. Finally, peptides were dissolved in 10 μl of 15% acetic acid, 5% formic acid and >1000 cpm was loaded on a TLC plate. Peptides were electrophoresed in 15 % acetic acid, 5% formic acid on cellulose plates (microcrystalline cellulose adsorbent without fluorescent indicator; Kodak) at 1000 volts for 50 min. Plates were then dried and subjected to ascending chromatography in the second dimension for 3 hr with 37.5% butanol, 25% pyridine, 7.5% acetic acid, air dried, and exposed to film (Ward and Kirschner, 1990).

RESULTS

The phosphorylated residues on the unliganded and hormone-bound rat glucocorticoid receptor from rat hepatoma cells and from the receptor expressed in yeast have been recently identified (Fig. 1A). The receptor is phosphorylated on residues T171, S224 , S232 and S246, with agonist binding increasing phosphorylation of residues S224 and S232. Inspection of the sequence context of these receptor phosphorylation sites (Ser/Thr-Pro) suggests that they maybe phosphorylated by any of several kinases, including the cyclin-dependent family of kinases (CDKs) and the mitogen-activated protein kinases (MAPK). To determine whether receptor is a substrate for these and other kinases, a purified receptor derivative produced in *E.coli* (EX556) and containing the zinc-finger region of the receptor linked to a 213 amino acid segment from the N-terminus (Freedman et al., 1989) was tested *in vitro* with various kinases. This constitutive receptor derivative contains all the major receptor phosphorylation sites identified *in vivo* and is active for DNA binding and transcriptional enhancement *in vitro*. Importantly, this receptor derivative is not phosphorylated in *E.coli* as determined by mass spectroscopy (Garabedian et al., unpublished).

We have tested eleven different kinases, including cAMP dependent protein kinase (PKA), protein kinase C (PKC), casein kinases I and II (CK I & II), S6 kinase 2, protease-activated kinases I and II (PAK I & II), Mos and double-stranded DNA-dependent protein kinase (dsDNAk) (Fig. 2). These enzymes either failed to phosphorylate receptor or phosphorylated the receptor at sites not phosphorylated *in vivo* (Fig. 2). In contrast, the human cyclin A/cdk2 complex produced in baculovirus and activated p44 MAP-kinase from starfish oocytes phosphorylated receptor at sites that are phosphorylated *in vivo*. Lysates of baculovirus-infected Sf9 insect cells containing cdk2 were

mixed with lysates containing cyclin A, and incubated with the receptor derivative EX556 and histone H1. Lysates containing either cdk2 or cyclin A alone exhibited negligible kinase activity toward either substrate, but lysates containing both cyclin A and cdk2 exhibited similar increases in kinase activity towards both substrates. Other cyclin/cdk combinations were less effective at phosphorylating the receptor: the rank order potency *in vitro* was: cycA/cdk2> cycB/cdk2>>cycA/cdc2 or cycB/cdc2), relative to their vigorous activity on histone H1. We have also analyzed the ability of p44 map kinase (MAPK) to phosphorylate the receptor as a substrate *in vitro*. In separate reactions, equimolar amounts of the receptor derivative EX556 or myelin basic protein (MBP), a standard MAP kinase substrate, were mixed with MAPK purified from starfish oocytes and kinase activity measured. In general, MAPK phosphorylated the receptor and MBP to similar extents and with parallel kinetics. Taken together, these results suggest that, as with cyclinA/cdk2 , MAPK utilizes the receptor efficiently as a substrate *in vitro*.

We next employed two-dimensional peptide mapping to monitor the receptor phosphopeptides generated by MAPK and CDK *in vitro*, and thus deduce the amino acid residues phosphorylated by these kinases. Two-dimensional phosphopeptide maps of receptor phosphorylated *in vivo* in mammalian cells were directly compared to those generated *in vitro*. As the relationship between individual phosphopeptides and their positions on the 2D peptide map is known (Krstic et al. 1995), we were able to assign particular receptor phosphopeptides to individual sites (Fig. 3). A similar receptor phosphopeptide pattern indicates that the receptor is likely to be phosphorylated at identical amino acid residues *in vitro* and *in vivo* and implicates the receptor as a substrate for the kinase. Phosphorylation of the receptor derivative EX556 *in vitro* by cyclin A/CDK2 and subsequent cleavage

by V8 protease yielded two phosphopeptides (Fig. 3). These peptides comigrate with a pair of receptor phosphopeptides generated *in vivo* corresponding to phosphopeptides V. 2 and V. 3. Previous mapping and mutagenesis studies revealed that peptide V. 3 encompasses receptor residues 220-231 and contains a single CDK consensus phosphorylation site at residue S224. Phosphopeptide number V.2 corresponds to the region spanning residues 220 and 242 and includes CDK phosphorylation sites at S232 and S224; this peptide caries phosphorylated S232 residue (V.2a) or phosphorylated S224 residue (V.2b). Surprisingly, we failed to observe the doubly phosphorylated version of peptide V.4 (S232 and S224). This may imply that cyclinA/CDK2 complex phosphorylates S224 but not S232 (Fig. 4). The lack of sequential phosphorylation of neighboring sites could conceivably be caused by conformational or electrostatic changes resulting from the initial phosphorylation event; alternatively, additional members of this family or different kinases may be involved in adding phosphates consecutively to the receptor *in vivo*.

We have also used another member of this kinase family, cyclin D/CDK4 as a source for the kinase activity. Indeed, this complex phosphorylated receptor derivative EX556 in vitro at peptides 2, 3 and 4, suggesting that both S224 and S232 residues are phosphorylated. In any case, the simplest explanation of these mapping data is that serine 232 is a target for cyclin D/CDK4 and that S224 and S232 are targets for the cyclinA/CDK2 and cyclinD/CDK4 (Fig. 4).

Two phosphopeptides appear on 2D peptide maps from the V8 cleavage of the MAPK-phosphorylated receptor (Fig. 3 and 4). These peptides comigrate with a subset of receptor phosphopeptides that correspond to positions V. 1 and V. 5 (Fig. 3). Previous mapping and mutagenesis experiments (Krstic et al.,

1995) demonstrate that V. 5 contains a single site of receptor phosphorylation at serine 246, whereas peptide V. 1 contains the site of phosphorylation at T171. We conclude that S246 and T171 are phosphorylated by MAPK. Interestingly, the residues phosphorylated by MAPK are distinct from those modified by the cyclin-dependent kinase. Together MAP and cyclin-dependent kinases account for all major peptides of receptor peptides phosphorylated *in vivo* (Fig. 3).

To correlate the activity of individual kinases with receptor function *in vivo*, we examined the transcriptional activity of the receptor in yeast strains with defects in particular kinases. Since equivalent protein kinases in many signal transduction pathways are maintained throughout evolution, and since receptor phosphorylation is conserved between yeast and mammalian cells (Krstic et al. 1995), mutations of candidate kinases in yeast should inform parallel studies in mammalian contexts. In *S. cerevisiae,* CDC28 controls both the G1/S and the G2/M phase transitions by associating with two distinct classes of positive regulatory subunits known as cyclins (Murray, 1994). At least three G_1 cyclin proteins (CLN1, CLN2 and CLN3) and six G_2 cyclins (CLB 1-6) bind to and activate the CDC28 protein kinase. All possible single and double CLN deletions are viable, but the CLN1 CLN2 CLN3 triple deletion is lethal. Likewise, single and certain double clb deletions are viable, whereas combinations of any three CLB deletions (except clb1 3 4) is lethal (Richardson et al., 1989; Richardson et al., 1992).

We began by measuring receptor dependent transcriptional enhancement in yeast strains with defects in CDC28. The following experiments are designed to test effects of mutations in kinase alleles on receptor-mediated enhancement under experimental conditions that preserve normal cell cycling, therefore uncoupling effects of kinase mutations from

perturbations of the cell cycle. The glucocorticoid receptor and a GRE-linked reporter gene were introduced into a wild-type strain and isogenic derivatives bearing two distinct temperature-sensitive alleles of CDC28, cdc28-4 and cdc28-1N (Moll et al., 1991). As a control, the receptor reporter plasmids were also introduced into a strain deficient in a non-cyclin-dependent kinase, cdc 7-1. The CDC28 mutants have single amino-acid changes in the CDC28 protein that lead to arrest of the cell cycle at the non-permissive temperature (37°C) in strains carrying these alleles (LoRincz and Reed, 1986). In the presence of 1 mM deoxycorticosterone (DOC), a receptor agonist in yeast, receptor-dependent transcriptional enhancement was decreased 4-5 fold in the CDC28 strains relative to wild-type; these cells were grown at permissive temperature and cycled normally (Fig. 5). In contrast, hormone-dependent transcriptional enhancement of the receptor was virtually unaffected in the cdc7-1 strain. To test whether differences in response were a result of alterations in receptor concentration among strains, an immunoblot was performed using a receptor-specific monoclonal antibody BUGR2 (Gametchu and Harrison, 1984). The blot revealed that the level and integrity of the receptor in each strain were identical (data not shown). These results indicate that receptor transcriptional enhancement is reduced in cells harboring defective CDC28.

Receptor-dependent transcriptional enhancement was next monitored in yeast cells deficient in particular G_1 (CLN) and G_2 (CLN) cyclins. The glucocorticoid receptor and a GRE-linked reporter gene were introduced into an isogenic set of strains deleted of various CLN genes and receptor activity was monitored. As observed for the CDC28 mutants, receptor-dependent transcriptional activity was sharply reduced in some of the CLN mutants, such as cln1 and cln3; these cells cycled normally in spite of these cln mutations. In

contrast, single deletions of CLN1, CLN2 or CLN3 had no effect on receptor transcriptional activity (Fig. 6).

A substantial reduction in receptor transcriptional activity was also observed in strains deleted for particular CLB genes. Receptor-dependent transcription enhancement was reduced up to 10 fold in cells deleted for CLB1 and CLB2, and was decreased 4 fold in CLB3 deficient strains compared with the wild-type isogenic strains (Fig. 7). In contrast, in strains devoid of CLB4, receptor-dependent transcriptional activity was only modestly affected relative to wild-type. In all cases, the level and integrity of the receptor in each strain was identical to wild-type. In addition, we backsrossed the CLB2 mutant strain to the isogenic wild type strain, 15DUa and followed receptor activity versus CLB2 genotype in segregants. These genetic crosses indicated that reduction in receptor activity segregated 2:2 and cosegregated with the deletion of CLB2 gene, indicating that CLB2 gene is indeed genetically linked to receptor function (MK unpublished). Taken together, these results suggest that both G1 and G2 cyclins participate in receptor-dependent transcriptional enhancement and that only certain cyclin-CDK complexes are required for full receptor-dependent transcriptional enhancement *in vivo*.

To begin to assess the effect of MAPK on receptor activity, receptor and reporter plasmids were introduced into a yeast strain deleted of the MAPKs FUS3 and KSS1 (Neiman et al., 1993). Receptor-dependent transcriptional activity was measured and compared to a wild-type isogenic strain containing the same receptor and reporter genes in the absence and presence of 1 μM DOC. In contrast to the decreased activity observed in the cdc28 strains, the receptor displayed increased transcriptional activity in the absence of FUS3 and KSS1 (Fig. 8). Together, these results lend strong genetic support for cyclin-

dependent and mitogen activated protein kinases in receptor function, potentiating and attenuating transcriptional enhancement, respectively (Fig. 9)

DISCUSSION

Our experiments demonstrate that members of two distinct protein kinase families, cyclin-dependent kinase and mitogen-activated kinase, are capable of phosphorylating the glucocorticoid receptor efficiently *in vitro*. These kinases phosphorylate distinct receptor residues and together phosphorylate all of the phosphorylated receptor amino acids observed in mammalian hepatoma cells *in vivo*. Mutations in certain cdk catalytic or regulatory subunits decrease receptor-dependent transcriptional activation *in vivo* and demonstrate that CDC28 function is necessary for full receptor dependent-transcriptional enhancement *in vivo*. In contrast, deletion of MAP kinases FUS3 and KSS1 in yeast increases transcriptional activation by the receptor and indicates that one or both of these enzymes attenuate receptor-mediated enhancement. Consistent with the contrasting effects of MAPK and CDK on receptor function are the phenotypic consequences of receptor phosphorylation site mutations. Indeed, receptor transcriptional enhancement is potentiated when the MAP kinase phosphorylation site, S246, is mutated to alanine and attenuated when the cyclin-dependent kinase target, S224, is mutated to alanine. Together, these findings suggest that the receptor is a common target for signaling by multiple kinases that affect its transcriptional activity oppositely.

Phosphorylation at multiple sites by different protein kinases can result in regulation at several distinct levels. In principle, phosphorylation may control receptor activities such as dimerization, DNA binding, subcellular

localization or interaction with other proteins, including those in the transcriptional machinery. Each of these functions may be affected either positively or negatively, thereby adjusting the receptor response to changing physiological conditions. For instance, insulin counteracts the physiological actions of glucocorticoids and blunts receptor-dependent transcriptional responses in certain cells (Cheatham et al., 1992). The attenuation of receptor transcriptional enhancement by the MAPKs provides one explanation for the opposite physiological effects of glucocorticoids and insulin. Upstream regulation of MAPK involves an array of signals that ultimately converge on Raf, which phosphorylates and activates MAP-kinase-kinase (MEK), leading to MAPK stimulation (Cooper, 1994). It is tempting to speculate that the activation of MAPK initiated by the insulin signal at the cell surface can antagonize receptor activity by altering receptor phosphorylation. Similarly, GR mediated enhancement is diminished in cells transformed with H-ras and v-Mos (Jaggi et al., 1986), which stimulate MAPK activity via Raf and MEK respectively. In contrast, inhibitors of the MAPK pathway, such as cAMP-dependent protein kinase A, potentiate receptor-dependent transcriptional enhancement (Rangarajan et al., 1992; Zhang et al., 1993). As multiple signaling pathways converge upon MAPK, it is conceivable that regulatory molecules that increase or decrease MAPK activity, such cytokines, growth factors and cAMP, will ultimately have an impact on receptor function (Fig. 9).

Multiple MAP kinases have been identified. For example, overlapping sets of MAP kinases have been recently cloned in mammalian cells and genetic studies in yeast have shown that MAP kinases participate in at least three distinct signaling pathways (Cooper, 1994). The FUS3 and KSS1 pathway is regulated by G protein-linked pheromone receptors; another MAP kinase, HOG1 is regulated by a two-component system that may be involved in

measuring osmolarity; MPK1 is regulated by a PKC homologue and is implicated in cell wall synthesis. In mammalian cells, ERK1 and ERK2 are coordinately regulated by mitogenic stimuli; stress-activated MAP kinases SAPk and JNK are activated by stress signals such as heat shock, or cytokine TNF, and UV radiation; the osmoregulated MAPK p38 is regulated in response to osmolarity and by lipopolysaccharide treatment. These different family members likely have distinct target specificies; one implication of this complexity is that modulation of receptor function can be accompanied by multiple routes in a manner highly dependent on cell type and the physiological status of the organism.

The cyclin-dependent kinases also respond to various extracellular stimuli as well as to temporal changes in cell-cycle positioning (Murray, 1994). All CDKs are activated by association with cyclin regulatory subunits and by phosphorylation via CDK activating enzymes known as CAKs. In addition, several inhibitors of CDK activity have been recently identified whose level and activity are also modulated by extracellular stimuli. As with the MAPK family, the CDK family is large. For example, CDC2/cyclin B induces mitosis; CDK2/cyclin E controls DNA replication (Murray, 1994); cyclin D is synthesized in response to growth factors (Xiong et al., 1991; Ajchenbaum et al., 1993); CDK 5 is expressed in postmitotic neurons (Tsai et al., 1994). Conceivably, each cyclin-CDK complex phosphorylates a distinct set of specific target proteins. Evidence presented here suggests that cyclin A/CDK2 and cyclin D/CDK4 efficiently phosphorylate the receptor at S224, whereas cyclin D/CDK4 phosphorylates S232; these two residues are sites of hormone-dependent phosphorylation *in vivo* (Fig. 4). Indeed, our genetic evidence suggests that both G1 and G2 cyclin-cdk complexes affect receptor transcriptional enhancement to different extents *in vivo*. Thus,

phosphorylation of the receptor appears to be influenced by multiple cyclin-cdk complexes and may provide a mechanistic basis for reported cell-cycle induced changes in receptor phosphorylation and activity (Hsu et al., 1992; Hu et al., 1994). Importantly, CDK enzymes regulate processes other than the cell cycle. In *S. cerevisiae* the PHO85/PHO80 CDK-cyclin complexis activated by high phosphate levels, and phosphorylates and inactivates the transcriptional regulator PHO4, which controls the expression of genes involved in phosphate metabolism (Kaffman et al, 1994). In mammalian brain cells, CDK5 is complexed with a protein that is not a cyclin and is probably involved in regulation other that cell cycle since these neuronal cells do not divide (Tsai et al., 1994). Therefore, phosphorylation of glucocorticoid receptor by CDK can exert regulation unrelated to the cell cycle and is consistent with the fact that in all experiments presented in this study we did not measure effects of cell cycle perturbations on receptor function, but effects of alterations in alleles of the CDK kinase.

In conjunction with the steroid signal, we propose that receptor phosphorylation helps direct and refine receptor transcriptional response in order to fit particular cellular needs (Fig. 9). The ability of the receptor to respond to extracellular signals via phosphorylation permits a plasticity in receptor action that, in conjunction with the steroid ligands, may be crucial in coordinating the various growth and metabolic processes managed by glucocorticoids.

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Figure 1: Rat glucocorticoid receptor phosphorylation. Major sites of receptor phosphorylation *in vivo* as determined by Krstic and colleagues (1995). The receptor expressed in mammalian cells or in yeast is phosphorylated on residues T171, S224, S232 and S246. Filled boxes represent transcriptional regulatory region and DNA binding region (from left to right), that are fused in frame in EX556 receptor derivative used for *in vitro* studies.

Figure 1

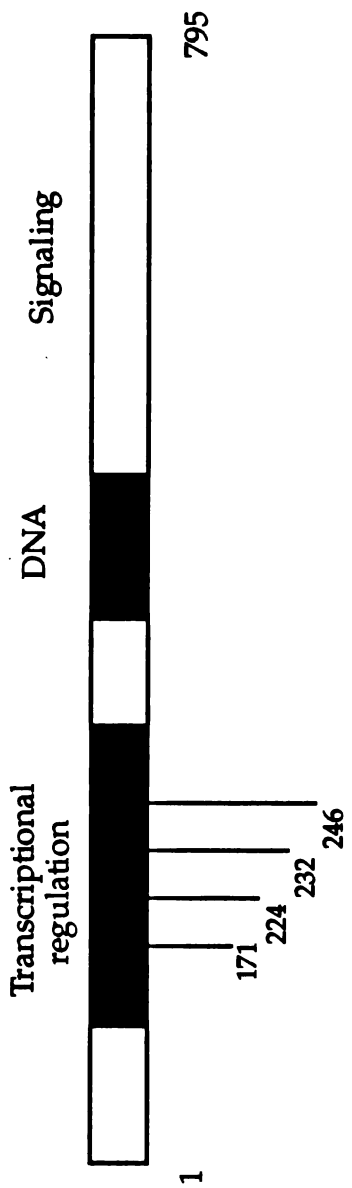
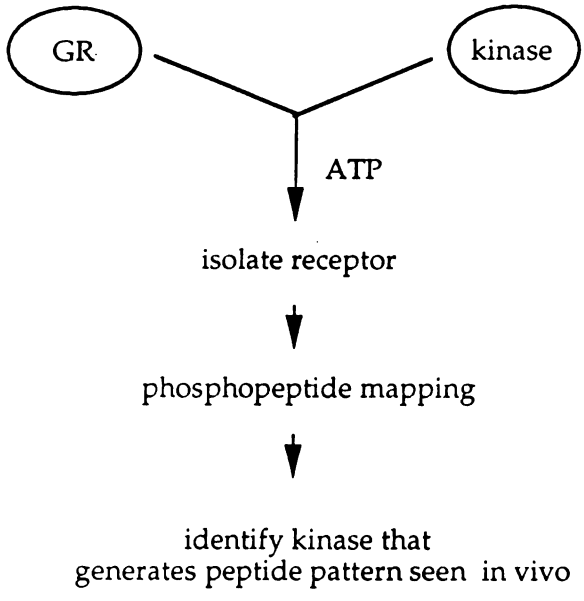

795
Signaling
DNA
Transcriptional regulation
246
232
224
171
1

Figure 2: Phosphorylation of the glucocorticoid receptor *in vitro*
Glucocorticoid receptor derivative EX556 was overexpressed and purified from *E. coli,* and used as source of substrate for screening the 11 kinases listed, as described in Materials and Methods. First column (GR phosphorylation) describes extent of phosphorylation of the receptor *in vitro* expressed relatively to the MAPK and CDK phosphorylation. Second column describes identification of kinases that *in vitro* phosphorylated receptor on peptides that were phosphorylated *in vivo.*

Figure 2



KINASE	GR PHOSPHORYLATION	IN VIVO PATTERN
PKA	+	-
PKC	-	-
CKI	+	-
CKII	+	-
S6KII	-	-
v-MOS	-	-
MAPK	+++	+
CDK	+++	+
PAKI	+/-	-
PAKII	-	-
DNAdpdtK	+	-

Figure 3: Glucocorticoid receptor is phosphorylated by mitogen-activated protein kinases and cyclin-dependent kinases on sites observed *in vivo* Identity of receptor phosphopeptides was determined by Krstic et al. (1995). Schematic representation of the relationship between V8 protease-derived receptor phosphopeptides and their positions on the two-dimensional peptide map. Phosphorylated amino acids are shown adjacent to each phosphopeptide indicated by arrows. The residues encompassing each phosphopeptide are shown in parenthesis. Phosphopeptides phosphorylated by MAP kinase and cyclin-dependent kinases are indicated.

Figure 3

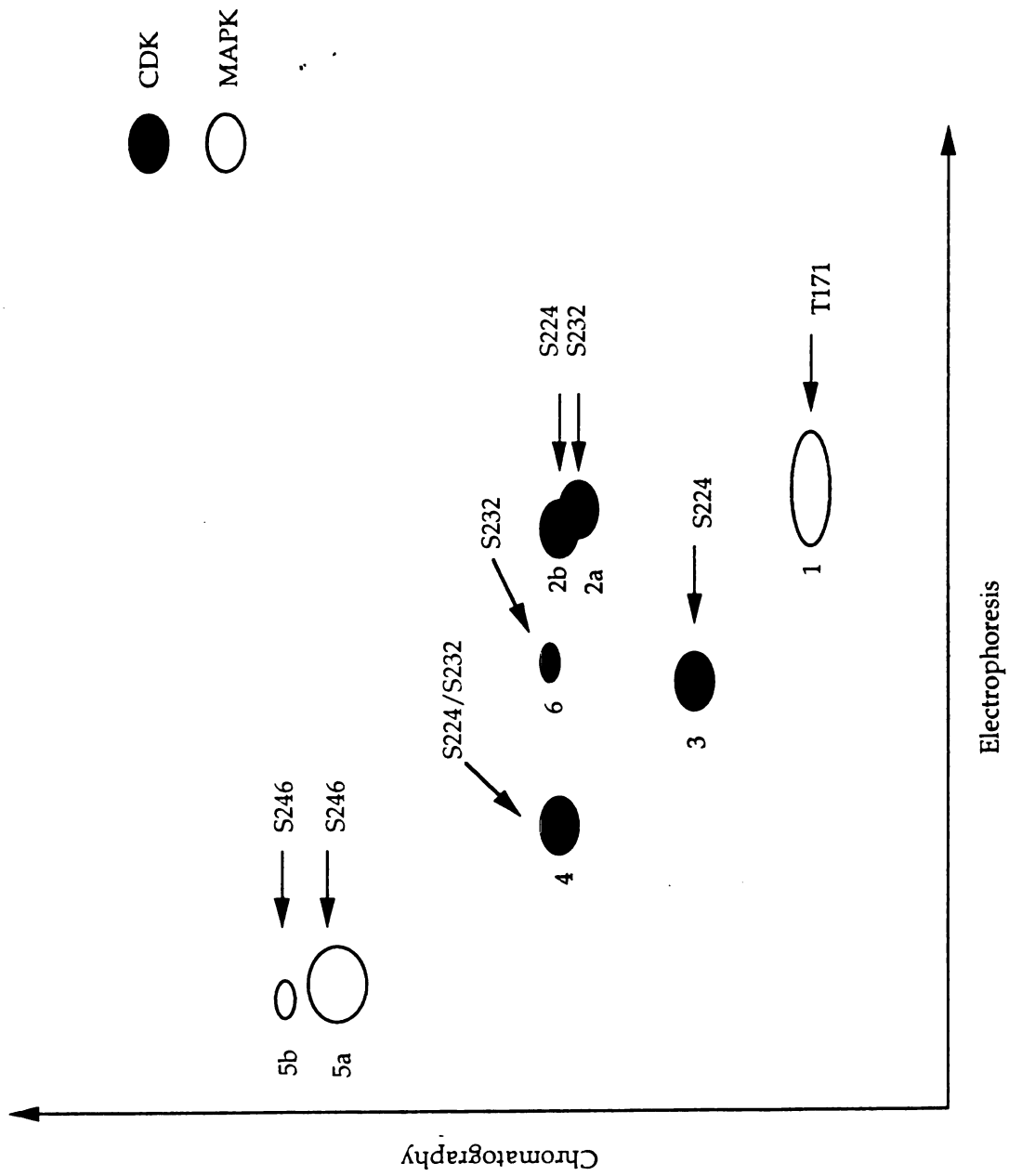

CDK
MAPK
T171
S224
S232
S224
S232
S224/S232
S246
S246
1
2b
2a
3
6
4
5b
5a
Electrophoresis
Chromatography

Figure 4: Sequence context of the glucocorticoid receptor phosphorylation sites, their hormone dependency and kinase preference

Sequence context of the receptor phosphorylation sites. Sequences surrounding the receptor phosphorylated residues T171 and S246 are potential targets for mitogen-activated kinase, MAPK, (MAPK consensus = non-polar-X-Ser/Thr-Pro), while the sequence context surrounding S224 and S232 coincide to putative sites of phosphorylation by the cyclin-dependent kinases, CDK; (CDK consensus = Ser/Thr-Pro-X-Arg/Lys). T171 and S246 are phosphorylated constitutively and S224 and S232 are phosphorylated in a hormone-dependent fashion. T171 and S246 are targets for the MAP kinase phosphorylation *in vitro*; S232 is a target for cyclin D/CDK4 complex and S224 is phosphorylated by cyclin A/CDK2 and cyclin D/CDK4 *in vitro*.

Putative kinase:

MAPK (non-polar-X-S/T-P)

CDK (S/T-P-X-K/R)

(T171) Gly Cys Ala Thr Pro Thr Glu (hormone independent) MAPK

(S224) Ser Ala Gly Ser Pro Gly Lys (hormone dependent) CYCA/CDK2 and CYCD/CDK4

(S232) Thr Asn Glu Ser Pro Trp Arg (hormone dependent) CYCD/CDK4

(S246) Asn Leu Leu Ser Pro Leu Ala (hormone independent) MAPK

Figure 4

Figure 5: Phenotypes of receptor-mediated transcriptional enhancement in strains with alterations in CDC28

Receptor-dependent transcriptional activity in yeast strains altered in the cyclin-dependent kinase, CDC 28. An isogenic set of strains with defects in the cyclin-dependent kinase cdc28-4 and cdc28-1N or in a non-cyclin dependent kinase cdc 7-1 were transformed with receptor expression vector PRS314 -GDP-GR N795 and a reporter gene containing a single consensus glucocorticoid response element. Cells were treated with the 1μM deoxycorticosterone overnight at 25°C, and receptor dependent β-galactosidase activity was measured. β-galactosidase activity is normalized to cell density and represent mean values from two or more independent assays, with less than 10% variation.

Figure 5

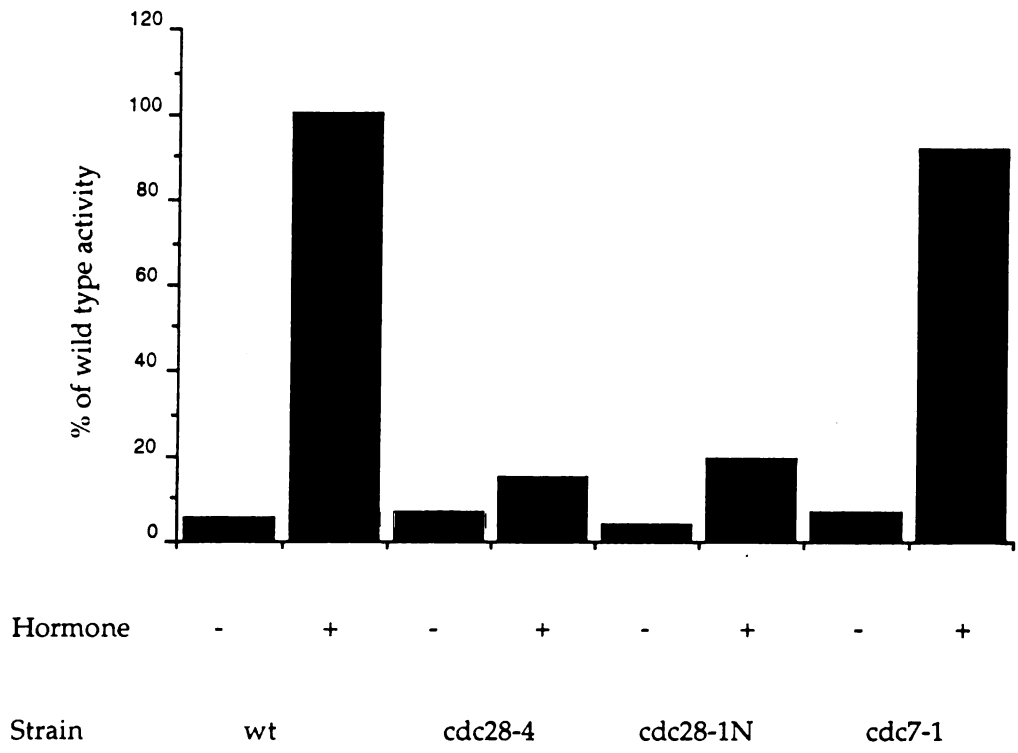

120
100
80
60
40
20
0
% of wild type activity
Hormone - + - + - + - +
Strain wt cdc28-4 cdc28-1N cdc7-1

Figure 6: Receptor dependent transcriptional enhancement in yeast strains deleted of yeast G_1 cyclins (CLNs). A wild type and an isogenic strain deleted of CLN1, CLN2, CLN3 and CLN1,3 were transformed with the paired receptor and reporter gene described above. Cells were treated with the hormone deoxycorticosterone at the 1μM overnight at 30°C and receptor-dependent β-galactosidase activity was measured.

Figure 6

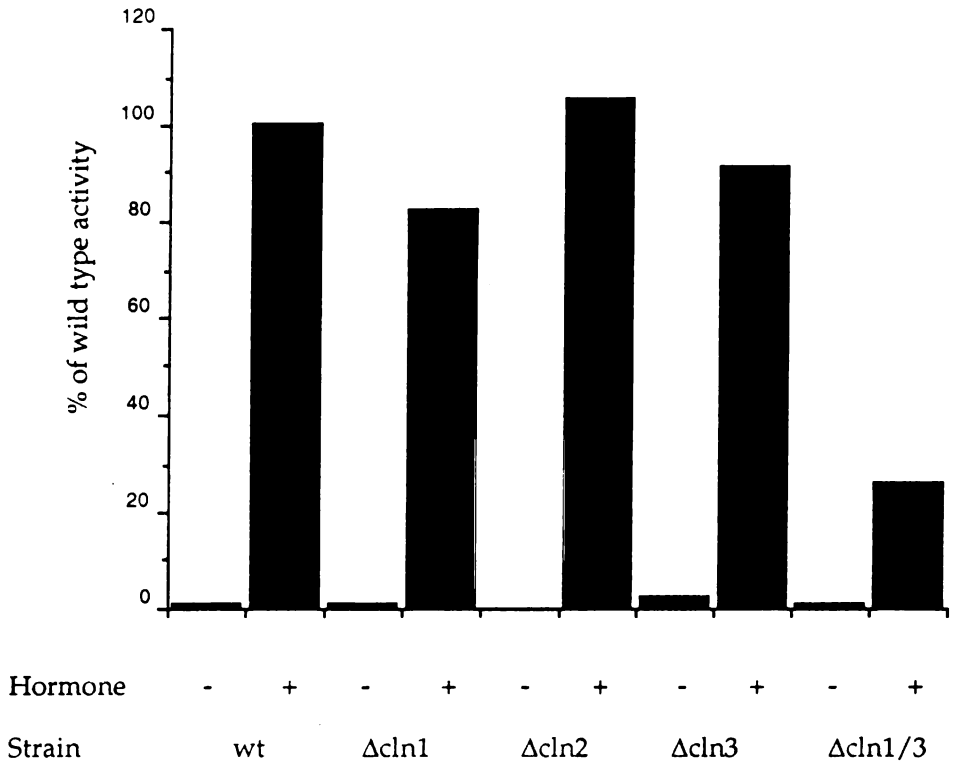

120
100
80
60
40
20
0
% of wild type activity
Hormone - + - + - + - + - +
Strain wt Δcln1 Δcln2 Δcln3 Δcln1/3

Figure 7: Receptor dependent transcriptional enhancement in yeast strains deleted of yeast G_2 cyclins (CLBs). A wild type and an isogenic set of strains deleted of CLB1, CLB2, CLB3 and CLB4 were transformed with paired receptor and reporter plasmids as described above and receptor-dependent transcriptional activity measured.

Figure 7

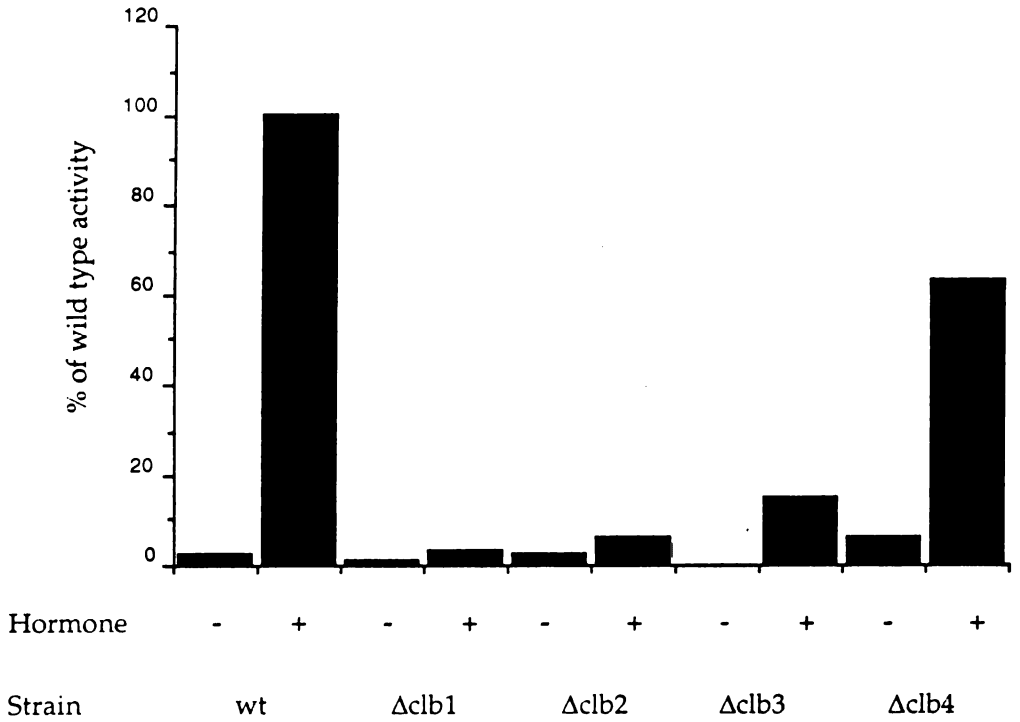

120
100
80
60
40
20
0
% of wild type activity
Hormone - + - + - + - + - +
Strain wt Δclb1 Δclb2 Δclb3 Δclb4

Figure 8: Receptor-dependent transcriptional enhancement in yeast strains deleted of MAPKs FUS3 and KSS1. A wild type and an isogenic strain deleted for FUS3 and KSS1 were transformed with the receptor on a low copy expression vector and a reporter gene containing a single consensus glucocorticoid response element. Cells were treated with the 1μM deoxycorticosterone overnight at 30°C and receptor dependent β-galactosidase activity was measured. In all cases β-galactosidase activity is normalized to cell density and is expressed as percentage of wild-type activity.

Figure 8

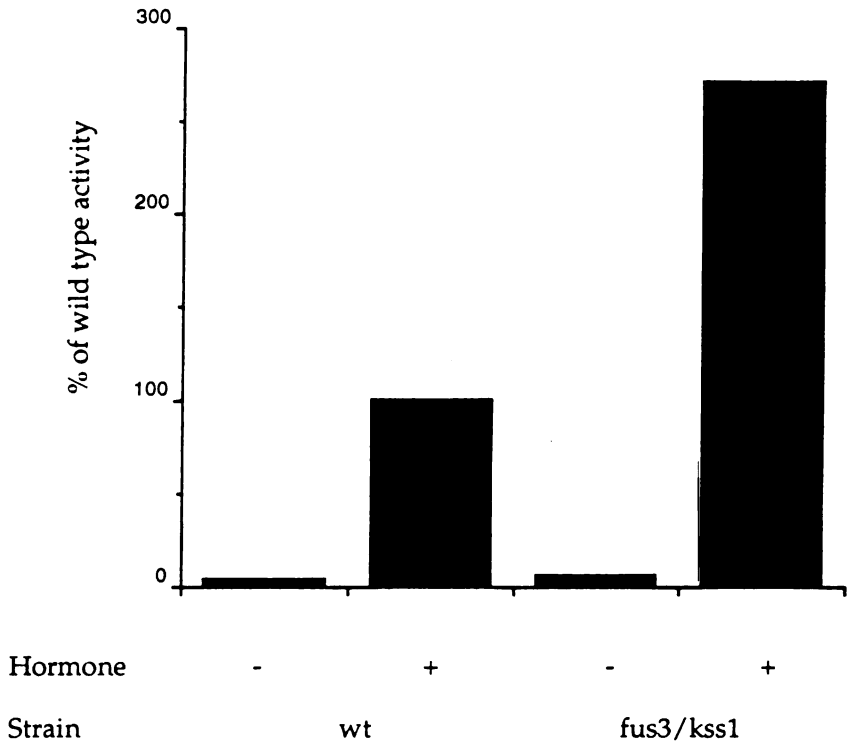
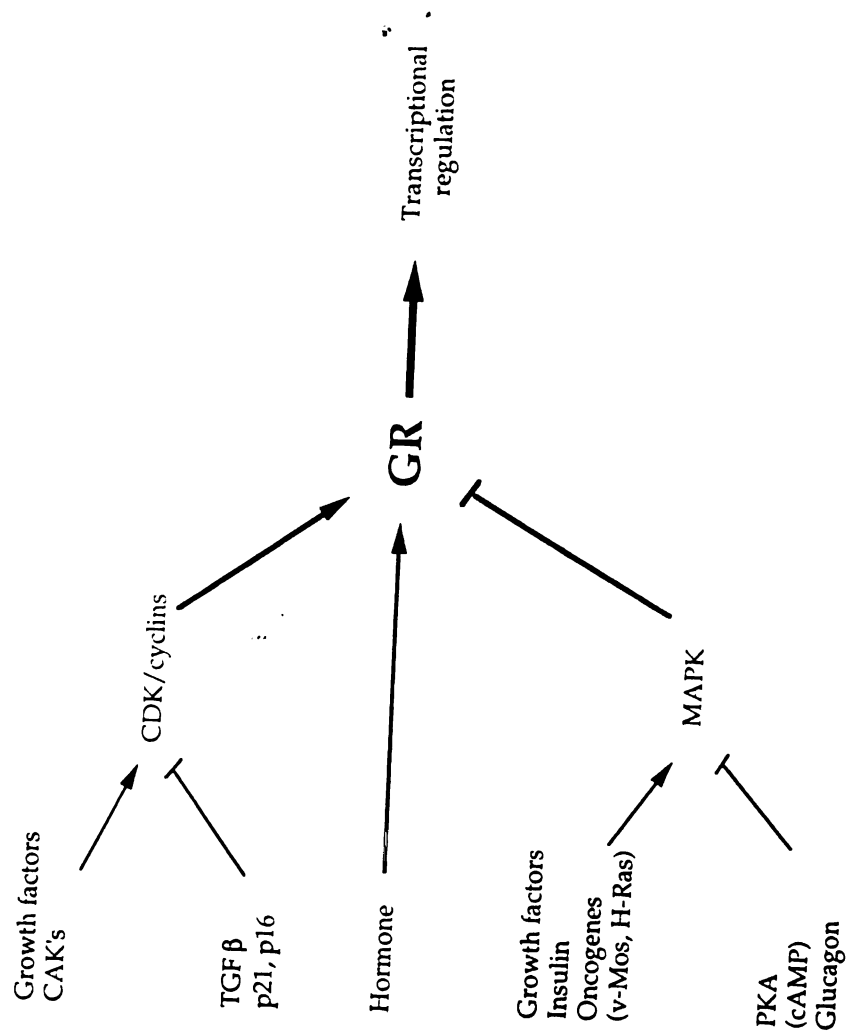

300
200
100
0
% of wild type activity
Hormone - + - +
Strain wt fus3/kss1

Figure 9: Receptor regulatory pathways: mitogen-activated protein kinases and cyclin-dependent kinases exert opposite effects on receptor transcriptional enhancement. Activators of the MAP kinase pathway, such as growth factors, insulin and certain oncoproteins, or inhibitors of CDK function, such as TGF -β, p21 and p16, shold attenuate receptor-induced transcriptional responses. In contrast, negative regulators of MAP kinase, such as PKA, as well as activators of CDK, such the cyclins or CDK activating kinases (CAKs), should potentiate receptor action.

Figure 9


Transcriptional regulation
GR
CDK/cyclins
MAPK
Growth factors
CAK's
TGF β
p21, p16
Hormone
Growth factors
Insulin
Oncogenes
(v-Mos, H-Ras)
PKA
(cAMP)
Glucagon

CHAPTER IV: Conclusions and Perspectives

The mechanisms by which phosphate additions modulate the function of most regulatory proteins are not known. I asked two questions in order to understand glucocorticoid receptor phosphorylation: (1) how does phosphorylation modulate activities of glucocorticoid receptor, and (2) what kinases target receptor *in vivo*? I will first discuss our findings concerning "downstream effects", i.e. the consequences of receptor phosphorylation and consider the following question: What is the mechanism of modulation of receptor activity by phosphorylation? What are the implications for combinatorial regulation of transcription by receptor? Second, I will consider "upstream effectors", i. e. the enzymes and potential signaling pathways converging on receptor through its phosphorylation and discuss the following question: What experiments would test our models? What is the significance of my findings for understanding control of steroid receptor activity by multiple signaling pathways? How do my results affect views of regulation of transcription by phosphorylation of regulatory factors in general?

I decided to investigate phosphorylation of the glucocorticoid receptor, a transcription regulatory factor with many defined functional domains and diverse activities easily measurable by multiple assays. I studied receptor phosphorylation in its "natural" context, mammalian cells, as well as in the more genetically tractable organism, the yeast *Saccharomyces cerevisiae*, as signal transduction and transcriptional regulation by glucocorticoid receptor are conserved in this system. Remarkably, all of the sites phosphorylated in mammalian cells appear to be phosphorylated in yeast, strongly suggesting

that glucocorticoid receptor phosphorylation is similar in both yeast and mammalian cells. This experimental approach facilitated identification of receptor phosphorylation sites and identification of receptor kinases. These studies exploited yeast strains that express high levels of receptor, display reduced levels of proteases, and carry mutations in candidate kinase genes. Furthermore, conclusions and inferences obtained in yeast could readily be tested in animal cells.

I. Downstream Effects: Consequences of Phosphorylation for Receptor Function

A. Conclusions

I determined that the phosphorylation sites of the glucocorticoid receptor expressed in yeast and mammalian cells are virtually identical and are localized within N-terminal region of the protein. Receptor derivatives possessing nonphosphorylatable amino acids at these particular sites have different transcriptional regulatory properties than the wild type protein. Phosphorylation of residues T171 and S246 inhibits transcriptional activation, whereas phosphorylation of S224 and S232 stimulates transcriptional activation. ALthough the magnitude of the effect of any of the individual point mutations was modest (2-4 fold), such weak effects may have significant impact on receptor function in particular physiological settings. In addition, the mutations might display far stronger effects in different promoter or cell contexts. Notably, the effects of multiple mutations have not yet been examined.

Remarkably, phosphorylation of these amino acids does not affect receptor- mediated transcriptional repression in mammalian cells, suggesting that effects of phosphorylation are selective for some regulatory processes and not others. In addition, the fact that receptor is functional for repression but has compromised transcriptional enhancement provides an internal control demonstrating that the effects of mutations of phosphorylation sites not on structural perturbations but rather to the lack of phosphorylation. As transcriptional repression is unaffected by these mutations, phosphorylation must affect transcriptional regulation and not signal transduction. Finally, the fact that we observed effects of phosphorylation of receptor on activation and not on repression suggests that phosphorylation sites are localized within surfaces specifically affecting transcriptional enhancement but not transcriptional repression. Alternatively, phosphorylation effects can be specific for particular combination of GRE and promoter context. Testing the activity of phosphorylation mutants using a battery of simple and composite elements should resolve this issue.

B. Future experiments

Protein domains that mediate transcriptional enhancement or repression in transcriptional regulatory factors are in general poorly defined with respect to their boundaries and structural characteristics. These transcriptional regulatory domains are commonly phosphorylated, although the function of this covalent modification is largely unknown. As described above, glucocorticoid receptor phosphorylation sites are localized within its transcriptional regulatory domain. The following experimental approaches

may facilitate further dissection of this domain and of the modulatory effect phosphorylation has on its function.

1. *In vitro* transcription

Biochemical analysis of transcriptional regulation by phosphorylation deficient receptor mutants should allow identification of exact steps in transcriptional initiation that may be modulated by phosphorylation. This question can be asked by using *in vitro* transcription system supported by Drosophila embryo nuclear extract or yeast whole-cell extract developed by Freedman et al. (1989) and Kralli et al. (unpublished) respectively. This system can be improved as described by Dynlacht and colleagues (1994), who established an E2F/DP1-dependent *in vitro* transcription assay using purified general transcription factors. Receptor derivatives carrying point mutations in phosphorylation sites can be expressed in *E.coli*, purified and used as a source of receptor in this assay. Freedman and colleagues proposed that receptor may facilitate formation of functional initiation complexes. This notion can be tested with receptor phosphorylation mutants in combination with purified general transcription factors. These experiments may lead to identification of a component of the transcriptional machinery that specifically interacts with the particular phosphorylated isoform of receptor. However, there are two potential caveats of an *in vitro* transcription system: first, the enzymatic activity of some kinases may differ *in vitro* and *in vivo;* therefore, abberant residues may be phosphorylated, with unpredictable consequences; second, *in vitro* activation by receptor derivatives by using cell-free extracts from Drosophila embryo or yeast cells may not be strong enough, making developing of an assay to study effects of phosphorylation on receptor function difficult. The first problem can be addressed by determining the sites

of phosphorylation in the *in vitro* transcription reaction. The second potential difficulty may be solved by utilizing purified general transcription factors.

Another strategy would be to use *in vitro* synthesized peptides that carry phosphorylated sequences of the receptor. These peptides can be used as competitors for the putative protein whose interaction with receptor is mediated by phosphorylation. The phosphorylated form of the peptide should compete for this protein with the receptor. Specificity of these effects can be established by studying in parallel unphosphorylated or mutant form of the same peptide. In this case, identification of the putative receptor accessory protein would be facilitated by radioactively labeling the peptide or by addition of crosslinking agents. One potential caveat of this experiment is that the whole protein-protein interaction surface has to be on peptide for it to squelch efficiently. To address this problem, different fragments of receptor sequences can be expressed in *E.coli* and used as competitors.

2. Phosphorylation-specific antisera

Another way that the *in vitro* synthesized receptor phosphopeptides can be useful is to raise antibodies specific for phosphorylated and unphosphorylated forms of particular residues. This assay seems to be a future direction for studying phosphorylation. Although originally thought that it may be difficult to make antibodies that are specific only for phosphorylated forms of a protein, examples with peptides made against phosphorylated form of a CREB protein suggest otherwise (Ginty et al., 1993). These antibodies will allow characterization of phosphorylation state of each particular residue under different conditions (in the absence and presence of

hormone, under conditions when receptor activates or represses transcription, in different cell cycle stages etc.).

3. Interacting proteins

There are several examples of modulatory effects of phosphates in protein-protein interaction. Crystallization studies by Waksman et al. (1993) have shown that phosphotyrosine interacts tightly with SH2 protein surfaces, leading to a small and highly localized conformational change. Phosphoserine and phosphothreonine are typically not involved in strong protein-protein contacts, but there is solid evidence that they indeed can mediate communication between two polypeptides. For example, the phosphorylation state of the transcriptional regulator CREB is a key determinant of its interaction with a coactivator protein, CBP, therefore regulating its transcriptional activity (Chrivia et al., 1993). It is possible that phosphorylation of glucocorticoid receptor will mediate or inhibit interaction with an accessory protein, conceivably even CBP itself. There are several experiments that can be useful in testing that hypothesis. First, the above mentioned peptides can be used to identify macromolecules that specifically interact with unphosphorylated or phosphorylated receptor proteins. One way to do this experiment is to covalently link these peptides to the carrier beads and to detect proteins that specifically interact with column carrying one form of the peptide and not the other.

4. Isolation of second site suppressors

Dissection of components involved in receptor phosphorylation can be started using a genetic approach. Receptor phosphorylation mutants can be potentially used in genetic screen to isolate second site suppressors or

enhancers of the phosphorylation defect. For this purpose, the assay conditions would have to be developed where these mutants can be distinguished from the wild type receptor to the degree that allows such a screen. This may be achieved if multiple combination of phosphorylation mutants is used and if their activities are assayed under conditions where receptor is a limiting component. That can be accomplished by using suboptimal concentrations of hormone, or using low expression level plasmids for receptor, or simple GRE versus multimerized one. If putative suppressors are isolated, they can be tested for specific effects on mutant protein and not on the wild type receptor to allow faster sorting and cloning of desired proteins.

C. Perspectives

The glucocorticoid receptor is expressed in virtually all mammalian cells, but it regulates different sets of genes in each cell type (Yamamoto, 1985). This notion raised the question how can one regulator do multiple things, such as activate or repress distinct genes to different extents in each particular cell. Our findings imply that phosphorylation may define surfaces in N-terminal region of the receptor that determine its regulatory capacity and direction of this regulation. Furthermore, phosphorylation selectively influences receptor-mediated transcriptional activation, whereas transcriptional repression is independent of its phosphorylation status. We speculate that inhibitory or stimulatory effects of phosphorylation on receptor mediated transcriptional enhancement may originate from changes in receptor conformation or receptor electrostatic charge and that these changes may affect protein-protein contacts of receptor with putative accessory

proteins. In fact, we already know several proteins that interact with receptor and influence its function. For example, the AP1 family of transcription factors interacts with receptor at composite element PlfG and either enhances or represses transcription, depending on the subunit composition of this regulator (Diamond et al., 1990); SWI proteins, which are required for regulated transcription of multiple yeast genes, are necessary for positive regulation by receptor and physically interact with receptor (Yoshinaga et al., 1992). Finally, Garabedian and colleagues (unpublished observations) found that receptor interacts *in vitro* with a regulatory subunit of cyclin dependent kinase, cyclin D. Phosphorylation may influence interaction of receptor with these or other proteins in both positive and negative ways.

Another possibility is that phosphorylation may play a role in communication between sequences in the N-terminal region and the DNA binding region of receptor (Lefstin et al., 1994). For example, phosphorylation can influence not only local receptor conformation but may in addition have an allosteric effect on DNA binding . It is possible that conformational changes in the DNA binding domain may influence receptor phosphorylation in its N-terminal region. This two- way communication can be mediated via conformational changes, electrostatic interactions or through interaction with other proteins. Alternatively, phosphorylation may define a dimerization surface other than already described one in the DNA binding domain (Thomas, 1993).

In general, transcriptional regulatory factors operate through heteromultimeric protein networks in which phosphorylation could alter dramatically the association of component factors, thus providing additional inputs for combinatorial regulation of gene expression.

II. Upstream Effectors: Kinases and Signaling Pathways

A. Conclusions

Two classes of protein kinases phosphorylate receptor *in vitro* on sites phosphorylated *in vivo* and influence receptor function in opposite ways: mitogen- activated protein kinases inhibit receptor function and cyclin dependent kinase stimulates receptor function. We found that members of the MAP kinase family phosphorylated *in vitro* residues T171 and S246, whereas members of the cyclin dependent kinase family phosphorylated S224 and S232. Mutation of these sites have differential effects on transcriptional regulation by receptor that correlate with the effects of kinase mutations, i.e., mutation of MAP kinases potentiates receptor function, whereas mutations in cyclin-dependent kinases compromises receptor function. I consider here some of the implications of the results that concern signals upstream from receptor and propose experiments to address the questions raised by our findings.

B. Future experiments

1. Genetic assays of kinase selectivity

We have started to test the selectivity of these multiple kinase family members for receptor as a target. For example, receptor function is compromised in strains carrying mutations in the CDC28 kinase, but it is unaffected in strains carrying deletions of PHO 85 (Kaffman et al., 1994), another member of the CDK family (Ming Chan, unpublished). To systematically test these effects, yeast strains deficient in individual members

of the particular kinase family could be used to identify enzymes that selectively affect receptor. Once the effects of a particular member of a kinase family are established in yeast, the effects of the mammalian homologs of these kinases can also be tested. The advantage of using yeast to start these experiments is that mutant kinase alleles are readily available, and testing the activities of receptor in these strains is straightforward. A caveat is that precise relationship between kinase family members in yeast and mammalian cells in some cases has not been established (Cooper, 1994; Sherr, 1994). In addition, the phosphorylation pattern of receptor expressed in different yeast strains with altered kinase alleles can be determined by using antibodies made to phosphorylated forms of individual receptor residues. This assay will provide rapid and nonradioactive way to determine phosphorylation status of receptor *in vivo* and correlate it with the mutations in particular kinases.

A further genetic dissection of the receptor phosphorylation pathway could be initiated by isolation of second site suppressors of receptor defect by using yeast strains with mutant kinase alleles. This assay could allow isolation of other components of this pathway that can be later characterized biochemically. For example, receptor activity is weaker up to 10fold in yeast strains with deletion in G2 cyclin CLB2, when compared to the wild type yeast strain. It is possible that overexpression of another protein may suppress this phenotype.

2. *In vivo* tests of kinase selectivity for receptor as target

There are at least three different MAP kinase pathways (Cooper, 1994; Herskowitz, 1995) and multiple cyclin/CDK complexes (Murray, 1994) described in both mammalian cells and yeast. These pathways are trigerred by distinct extracellular stimuli and converge on individual kinase family

members. For example, MAP kinase pathways in yeast include KSS1 and FUS3 kinases, which respond to pheromones, HOG1, which responds to hyperosmotic changes, and MPK1, which participates in cell wall synthesis. The cyclin-dependent family of kinases in yeast includes CDC28 and Pho85. Pho85 activity differs depending on phosphate level (Kaffman et al., 1994) and CDC28 activity varies throughout the cell cycle (Murray, 1994).

One strategy to test *in vivo* selectivity of these kinases is to follow receptor function under conditions when these pathways are activated or inhibited. This approach offers the following advantages: deletions of particular kinases would be difficult to engineer in mammalian cells; one could test effects on receptor activity when a pathway is both activated and inhibited, whereas mutations of components of the pathway usually lead to a loss of function phenotype (unless the result of mutation is a rare dominant negative phenotype). A caveat of this strategy is that it may be difficult to manipulate *in vivo* specifically one pathway without affecting the other.

3. Cell cycle effects on receptor activity

We investigated glucocorticoid receptor function under conditions where CDK activity was perturbed, but we intentionally avoided the complications associated with interrupting the cell cycle. Our results indicate that phosphorylation by cyclin-dependent kinases stimulates glucocorticoid receptor. Thus, it would now be interesting to assess whether receptor activity fluctuates during the cell cycle. In fact, a potential glucocorticoid receptor and cell cycle connection has been studied for a long time, but no simple conclusions have been reached. For example, the TAT gene was found to be inducible by addition of glucocorticoids in G1 and S phase of the cell cycle, but to be uninducible in G2 and mitosis (Martin et al., 1968). Recently, reports by

two laboratories suggest that GR function and phosphorylation and activity may vary during the cell cycle (Hsu et al., 1992; Hu et al., 1994). However, these reports conflict with respect to the specific changes in receptor phosphorylation. There are two straightforward experiments that may shed light on this issue. First, receptor phosphorylation can be followed in different phases of the cell cycle. Yeast cells offer substantial technical advantages of arresting the cell at different points in the cell cycle. Receptor phosphorylation pattern could be followed by above mention phopho-specific antibodies. Second, receptor activity can be followed by primer extension analysis of LacZ transcripts that will detect receptor specific transcripts in different cell cycle stages. Once these effects are determined in yeast, they could be tested in mammalian cells.

4. *In vitro* assays of selectivity of kinases for receptor as target

We have observed that different members within one family of kinases may phosphorylate receptor selectively *in vitro* on one residue and not the other: cyclin A/ cdk2 phosphorylates only S224 and not S232. We also found that two different members of the same kinase family can phosphorylate the same residue on the receptor (S224 is phosphorylated by both cyclin A/cdk2 and cyclinD /cdk4 kinases. One way to confirm and extend these finding is to test the enzymatic activity and selectivity of individual kinase family members for activity on particular synthetic peptides.

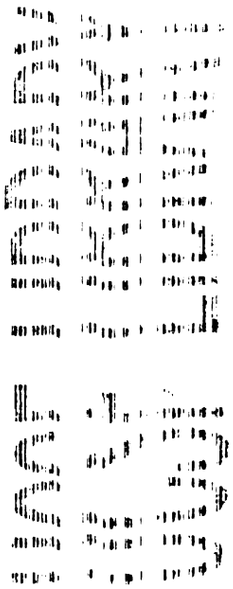
To assay effects of individual kinases on receptor-mediated transcriptional regulation, receptor can be tested in an *in vitro* transcription assay in the presence and absence of purified kinases. This experiment will allow dissection of the role phosphorylation plays in modulating receptor activity in different steps of transcriptional initiation.

Finally, it should be possible to test directly whether receptor physically interacts with kinases. This could be done *in vitro* by using proteins expressed in reticulocyte lysates and then by coimmunoprecipitation experiments. Alternatively, this interaction can be tested *in vivo* by coimmunoprecipitation or by two hybrid system.

C. Perspectives

Our model concerning signals upstream from the receptor says that receptor activity differs depending on the activities of these enzymes. We speculate that in each particular cell type, receptor function may be determined in part by the magnitude and the ratio of the activities of MAP kinases and cyclin-dependent kinases. This allows numerous possibilities for control of receptor function given the complexity of these kinase families and their effects on receptor function. This complexity is not new in processes involving kinases. For example, protein kinase A phosphorylates and activates transcriptional regulator CREB on serine 133 (Gonzalez and Montminy, 1989). However, this residue is also a target for phosphorylation *in vivo* by calmodulin-dependent protein kinase (Sheng et al., 1991). Moreover, phosphorylation of this residue creates a consensus sequence for glycogen synthase kinase, which phosphorylates serine 129 after serine 133 has been phosphorylated, leading to three-way regulatory circuit that converges on one transcriptional regulator (Fiol et al., 1994). According to our view, receptor like CREB, receives, processes and integrates information from multiple signaling pathways and not only from steroid hormones.

It is interesting to consider the possibility that MAP kinases, cyclin dependent kinases or other signals might be able to activate receptor even in



the absence of hormone. This scheme could explain observations made by O'Malley and colleagues of hormone-independent dopaminergic activation of progesterone receptors (Power et al., 1991). Dopamine action is mediated by receptors coupled to G proteins, which in turn activate adenylate cyclase and increase production of cyclic AMP. This increase in cAMP could inhibit MAP kinase that in turn could relieve inhibition of progesterone receptor activity.

The signaling pathways that converge on glucocorticoid receptor and presumably other steroid receptors provide a basis for understanding the complexity of physiological regulation by glucocorticoids and other hormones. For example, gluconeogenesis is controlled in the liver by glucocorticoids, which stimulate, and insulin, which inhibits the process. Among the best studied enzymes from this pathway are phosphoenolpyruvate carboxykinase (PEPCK) and tyrosine aminotransferase (TAT). Insulin also inhibits induction of the TAT gene by glucocorticoids (Cheatham et al., 1992). Conceivably, insulin suppression of glucocorticoid stimulation could be explained at least in part by activation of MAP kinase, which in turn would inhibit receptor by phosphorylation. Similarly, the potentiating effect of cAMP on receptor function (Rangarajan et al., 1992) could be explained by inhibition of the MAP kinase, which would reduce phosphorylation of inhibitory sites on receptor leading to increase in receptor activity .

An additional implication of our results is that certain pathological conditions that affect kinases may directly affect receptor function. Indeed in cells transformed with V-mos and H-ras oncogenes glucocorticoid hormone dependent transcription was repressed (Jaggi et al., 1986). It is possible that this effect is a consequence of activation of a MAP kinase pathway by these oncogenes.

A general goal in molecular biology is to understand how a relatively modest set of components can account for complexity of regulatory processes. Regulation of expression of most eukaryotic genes represents collaboration of multiple transcription factors at response elements. Our findings suggest that, in addition to using multiple factors to regulate one process, a single factor can receive, process and integrate multiple signaling inputs. Interestingly, this strategy is also used throughout the cell with various components. For example, T cell antigen receptor responds to foreign antigenic peptide in the context of the specific host protein; NMDA (glutamate receptor) responds to synaptically released glutamate and depolarization that is a consequence of activity at neighboring synapses (Bourne and Nicoll, 1993). We can hope that soon we will be able to understand how these "signaling machines" work, as dissection and analysis of these pathways suggest that there is limited number of families of regulatory proteins and that frequently they share common molecular mechanisms.

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