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# Using Yeast to Study Glucocorticoid Receptor Phosphorylation

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**The glucocorticoid receptor (GR) is a phosphoprotein and a member of the steroid/thyroid receptor superfamily of ligand dependent transcription factors. When the glucocorticoid receptor is expressed in yeast (*Saccharomyces cerevisiae*), it is competent for signal transduction and transcriptional regulation. We have studied the glucocorticoid receptor phosphorylation in yeast and demonstrated that the receptor is phosphorylated in both the absence and presence of hormone, on serine and threonine residues. This phosphorylation occurs within 15 min upon addition of radioactivity in both hormone treated and untreated cells. As reported for mammalian cells, additional phosphorylation occurs upon hormone binding and this phosphorylation is dependent on the type of the ligand. We have followed the hormone dependent receptor phosphorylation by electrophoretic mobility shift assay, and have shown that this mobility change is sensitive to phosphatase treatment. In addition, the appearance of hormone dependent phosphoisoforms of the receptor depends on the potency of the agonist used. Using this method we show that the residues contributing to the hormone dependent mobility shift are localized in one of the transcriptional activation domains, between amino acids 130–247. We altered the phosphorylation sites within this domain that correspond to the amino acids phosphorylated in mouse hormone treated cells. Using phosphopeptide maps we show that hormone changes the peptide pattern of metabolically labelled receptor, and we identify peptides which are phosphorylated in hormone dependent manner. Then we determine that phosphorylation of residues S224 and S232 is increased in the presence of hormone, whereas phosphorylation of residues T171 and S246 is constitutive. Finally, we show that in both yeast and mammalian cells the same residues on the glucocorticoid receptor are phosphorylated. Our results suggest that yeast cells would be a suitable system to study glucocorticoid receptor phosphorylation. The genetic manipulability of yeast cells, together with conservation of the phosphorylation of GR in yeast and mammalian cells and identification of hormone dependent phosphorylation, would facilitate the isolation of molecules involved in the glucocorticoid receptor phosphorylation pathway and further our understanding of this process.** © **1998 Elsevier Science Ltd. All rights reserved.**

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## INTRODUCTION

The glucocorticoid receptor is a member of the steroid-thyroid hormone receptor family of transcription factors (reviewed in [1–3]). The GR is a cytosolic protein that upon hormone binding translocates to the nucleus, binds DNA and increases or decreases the rate of transcriptional initiation [4–7]. Most members of this family are phosphoproteins in mammalian cells and hormone binding increases the phosphorylation of some of these proteins [8–18]. Sites of phosphorylation have been identified for several members of the family including the glucocorticoid receptor from mouse cells [19], rat glucocorticoid receptor in hepatoma cells and in yeast [20], chicken and human progesterone receptor [15, 21–24], and oestrogen receptor [8, 13, 25]. The phosphorylation sites are

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mainly localised within or in the vicinity of the transcriptional activation domains of the receptor proteins suggesting that transcriptional enhancement may be regulated by phosphorylation [8, 13, 17, 19, 25–27]. Sites of ligand dependent hyperphosphorylation have been reported for the progesterone and the oestrogen receptors [8, 13, 15, 21, 25]. Phosphorylation of steroid receptors may be important for their function since replacement of target serine residues by alanine that can not be phosphorylated can lead to a reduction in receptor mediated transcriptional activation. For example, substitution of individual serines with alanines in the oestrogen and progesterone receptors leads to decreased activity of these proteins [8, 26, 27]. However, there are also several reports showing that alanine substitution of phosphorylation sites in the glucocorticoid, progesterone and oestrogen receptors had subtle effects, if any on their activity [22, 28, 29].

It has been shown that phosphorylation of the mouse glucocorticoid receptor occurs on both serine and threonine residues, and seven sites of phosphorylation have been mapped in hormone treated mouse and hamster cells [19]. However, it is not known which of these sites are preferentially phosphorylated in response to hormone in mammalian or yeast cells. In addition, the exact role of constitutive and hormone dependent phosphorylation in receptor function remains controversial. Inspection of the phosphorylation sites in several steroid receptors revealed that most of the residues localise within consensus sequences for S/T–P kinases, including MAP kinases and CDK kinases. In addition, it has been reported that several signalling pathways can affect steroid receptor activity. For example, in cells treated with okadaic acid that inhibits PP1 and PP2A, DeFranco *et al.* observed changes in GR nucleocytoplasmic distribution [30]. On the other hand, activators of protein kinase A can modulate the activity of progesterone, oestrogen and glucocorticoid receptors [31–34]. Furthermore, MAP kinase can phosphorylate oestrogen receptor derivatives *in vitro*, and the phosphorylation of oestrogen receptor increases *in vivo* after EGF treatment [35, 36]. In addition, it has been shown that cyclin dependent kinases and mitogen-activated protein kinase phosphorylate glucocorticoid receptor *in vitro* whereas mutations in these kinases have opposite effects on receptor dependent transcriptional enhancement in yeast [20, 37]. Similarly, several reports indicate that cyclin/CDK pathways play a role in phosphorylation and regulation of steroid receptors [38–40].

In order to facilitate studies of glucocorticoid receptor phosphorylation *in vivo* and to utilise the genetic potential of yeast, we have investigated whether the GR when expressed in yeast can be phosphorylated in similar fashion to mammalian cells. The GR is competent for both transcriptional activation and signal transduction in yeast [41, 42] despite the fact that the nuclear receptor family is absent from the yeast genome. We determined that receptor phosphorylation in yeast occurred on serine and threonine residues in both the presence and absence of hormone. Then we developed a simple electrophoretic mobility shift assay to show that receptor phosphorylation is modulated by hormone addition. This ligand dependent phosphorylation is sensitive to phosphatase treatment, is ligand specific, and is localised within the N-terminal transcriptional activation domain of the receptor, between residues 130 and 237. This simple method of detecting hormone dependent phosphorylation should allow easier studies of changes in receptor phosphorylation *in vivo* in response to the activation or inactivation of different signalling pathways.

Finally, using phosphotryptic mapping of *in vivo* labelled receptor we show that phosphorylation of residues S224 and S232 increases upon addition of hormone, whereas phosphorylation of amino acids T171 and S246 does not change significantly with the addition of ligand. Moreover, phosphorylation of glucocorticoid receptor expressed in yeast and mammalian cells is virtually identical, suggesting evolutionary conservation and importance of receptor phosphorylation.

## MATERIAL AND METHODS

### *Yeast strains and plasmids*

The triple protease deficient yeast strain, BJ2168 (a, pep 4–3, prc 1–417, prb 5–1122, ura 3–52, trp 1, leu 2) [43] served as the parent for derivative strains containing various 2$\mu$-origin-based expression and reporter vectors as described below. Yeast cultures were propagated at 30°C in minimal yeast medium with amino acids and 2% glucose. Transformations were performed by the lithium acetate procedure [44].

The yeast expression plasmid pG-N795 [41, 45] contains the rat glucocorticoid receptor cDNA driven by the yeast glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter, residing on a 2$\mu$ vector (10–40 copies per cell) bearing the TRP1 selectable marker.

Reporter plasmid pΔs26x contains three tandem 26 base pair oligonucleotides from the tyrosine aminotransferase GRE 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* $\beta$-galactosidase (Lac Z) coding sequences. The 2$\mu$ vector carries the URA3 selectable marker, a bacterial origin of replication and the bacterial ampicillin resistance gene [45].

### *Mammalian cells culture*

The mammalian cell line, GRH2, is a rat hepatoma HTC cell derivative transfected stably with rat glucocorticoid receptor cDNA; these cells express the

receptor at elevated levels [46]. Cells were grown at 37°C in Dulbecco's modified Eagle's medium H-16, supplemented with 10% fetal bovine serum (Hyclone, Logan, UT).

*Metabolic labelling of glucocorticoid receptor expressed in yeast and mammalian cells with $^{32}$P orthophosphate*

The yeast strain BJ2168 containing the wild type or mutant 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 collected by centrifugation, 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 ligand indicated in the text. After 2 h 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 (45 mM HEPES, pH 7.5, 400 mM NaCl, 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 1 μg/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 12000 × *g* for 10 min at 4°C. The supernatant was placed in a fresh tube for immunoprecipitation of the receptor.

The GRH2 cells were grown in 10 cm dishes to near confluency in DMEMH-16 medium (1 g/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 two hours 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 described above. Mammalian cell lysates were prepared for immunoprecipitation as described below.

*Metabolic labelling of glucocorticoid receptor expressed in yeast with $^{35}$S methionine*

Yeast cells were grown to O.D.$_{600}$ 0.4–0.7 and treated as described for metabolic labelling with $^{32}$P orthophosphate, except that 100 μCi/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.

*Immunoprecipitation and immunoblotting*

To immunoprecipitate the glucocorticoid receptor, an equal volume of 2× RIPA buffer (1× RIPA = 10 mM HEPES pH 7.5, 150 mM NaCl, 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 [47]. After 1–2 h at 4°C, 100 μl of 50 mg/ml protein A Sepharose (Sigma) equilibrated in RIPA buffer was added and incubated for an additional 2 h with gentle agitation at 4°C. Protein A sepharose was collected by centrifugation, washed 4 times with ice cold RIPA buffer, and once with PBS. Bound protein was released in 2× 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 with the BUGR2 monoclonal antibody, and developed by using an alkaline phosphatase detection system.

*Phosphopeptide mapping and phosphoamino acid analysis*

Polyacrylamide gels containing the labelled 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 h at 37°C, an additional 50 μg of fresh trypsin (1 mg/ml in aforementioned buffer), was added and incubation was continued for 10 h at 37°C. Samples were centrifuged for 5 min at 12000 × *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 two-dimensional analysis. Peptides were electrophoresed in 15% acetic acid, 5% formic acid on cellulose plates (microcrystalline cellulose adsorbent without fluorescent indicator; Kodak) at 1000 V for 50 min. Plates were then dried and subjected to ascending chromatography in the second dimension for 3 h with 37.5% butanol, 25% pyridine, 7.5% acetic acid, air dried, and exposed to film [48].

For phosphoamino acid analysis, [$^{32}$P] labelled receptor was treated as described above for peptide mapping, and polyacrylamide gels containing the labelled receptor were transferred to the Immobilon-P (Millipore). Following autoradiography, the glucocor-

ticoid receptor band was excised and placed in 400 μl of 6 M HCl (Pierce) and receptor hydrolyzed by heating to 110°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 cellulose plate, along with phosphoamino acid standards (1 μl of mixture of phosphoserine, phosphothreonine and phosphotyrosine, 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°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 can not 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.

*Phosphatase treatment*

Receptor was immunoprecipitated from yeast cells as described above, incubated with or without 10 μM deoxycorticosterone (DOC) and treated with 20 units of calf intestine phosphatase (Boehringer Mannheim) for 30 min at 25°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 BUGR2 [47].

*β-Galactosidase assay in yeast*

β-galactosidase activity was assayed as previously described [42]. Briefly, yeast cells were collected by centrifugation from 1.5 ml cultures and washed with Lac Z buffer (100 mM $NaPO_4$, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol). After recentrifugation, cells were resuspended in 50 μl of Lac Z buffer and permeabilized with 50 μl of chloroform and 20 μl of 0.1% SDS; β-galactosidase substrate (*o*-nitro-phenyl-β-galactosidase, 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

As a first step towards developing a genetic approach to study glucocorticoid receptor phosphorylation, we have expressed the rat glucocorticoid receptor in *Saccharomyces cereviasiae* and investigated its phosphorylation.

*Glucocorticoid receptor expressed in yeast changes electrophoretic mobility upon hormone binding*

We introduced a plasmid expressing the glucocorticoid receptor into *Saccharomyces cerevisiae* and studied the expression and phosphorylation of the receptor protein in the absence and presence of hormone. Yeast cells were treated with 10 μM deoxycorticosterone (DOC), which is a strong agonist in yeast, for 6 h [42]. When cells reached $O.D._{600}$ 0.4–0.7 whole cell extract was prepared and the GR was isolated and detected by western blot analysis using BUGR2 monoclonal antibody [47]. A hormone dependent change in the electrophoretic mobility of GR was detected (Fig. 1A); this mobility change was observed independently of the amount of total cell protein loaded (25–200 μg of protein concentration range was tested; data not shown). This decrease in electrophoretic mobility in SDS gels has been reported for other steroid receptors as well as for numerous transcription factors, and is commonly associated with additional phosphorylation.

In the subsequent experiment we immunoprecipitated the GR with the BUGR2 monoclonal antibody and analyzed its electrophoretic mobility by western blot using the same antibody. A similar mobility shift was also observed in these experiments (Fig. 1B). The appearance of receptor isoforms that are present only after hormone treatment could be due to phosphorylation or other posttranslational modifications. To distinguish between these possibilities, the receptor was immunoprecipitated from yeast cells, and treated with calf intestine alkaline phosphatase, as described in Material and Methods (Fig. 1B). It was observed that phosphatase treatment eliminated the slower migrating receptor isoforms, suggesting that they originated from hormone dependent phosphorylation events (compare lane 4 with the lane 2).

Finally, we analyzed the ligand dependent mobility change of the GR by using metabolic labelling with $^{35}S$ methionine *in vivo*, which detects newly synthesized potentially phosphorylated and unphosphorylated receptor isoforms. Cells were metabolically labelled with radioactive methionine and the GR was immunoprecipitated as described in Material and Methods. Figure 1C (lanes 3 and 4) shows the appearance of hormone dependent receptor isoforms consistent with different phosphorylation forms. Cells

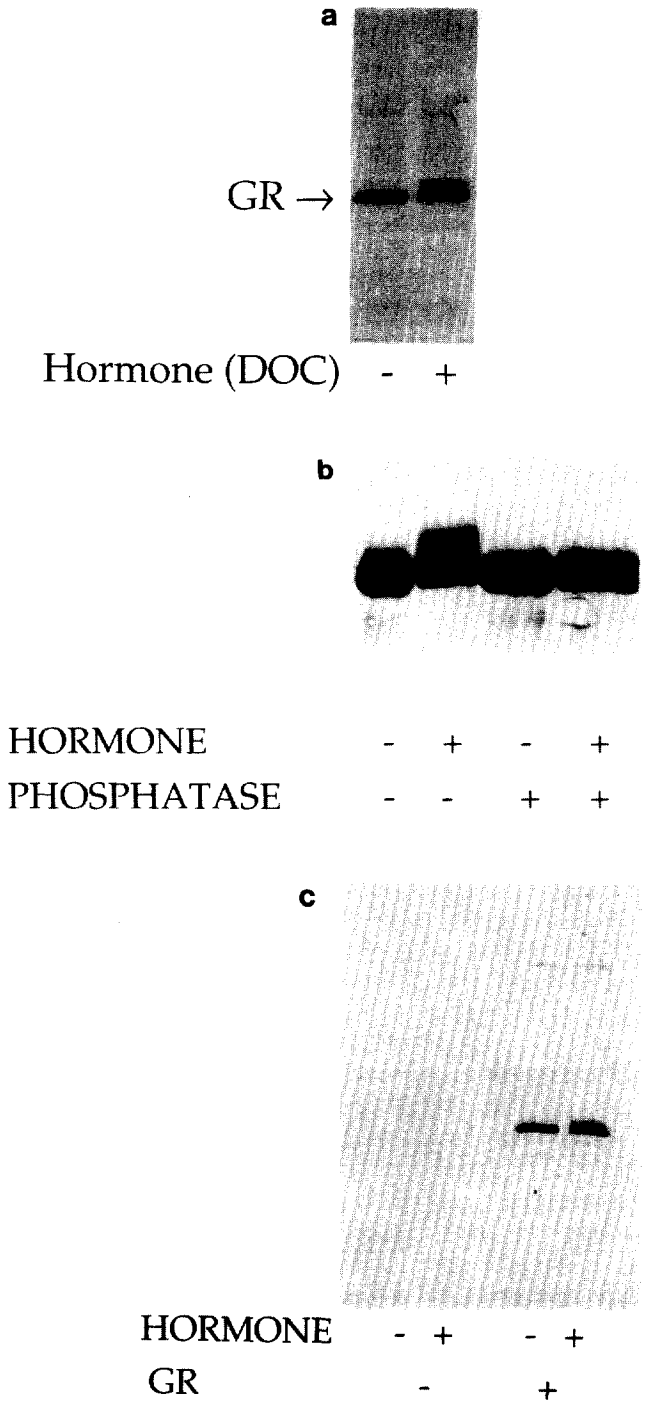


**Fig. 1. Glucocorticoid receptor expressed in yeast is hyper-phosphorylated in the presence of hormone. (A) Detection of a ligand dependent mobility shift by western blot analysis. Extracts from yeast cells treated or not treated with 10 $\mu$M DOC were made in high salt lysis buffer and mixed with 2 × SDS sample buffer. Samples were resolved on 7.5% SDS polyacrylamide gel, transferred to Immobilon-P and membrane was developed by BUGR2 monoclonal antibody using alkaline phosphatase detection system as described in Material and Methods. (B) Phosphatase treatment of the glucocorticoid receptor in yeast. Immunoprecipitates of the receptor obtained from yeast cells, incubated with or without 10 $\mu$M DOC were treated with 20 units of calf intestine phosphatase for 30 min at 25°C. Receptor was subsequently fractionated on SDS PAGE, transferred to Immobilon-P and membrane was developed by BUGR2 antibodies as described above. (C) $^{35}$S methionine labelling of the glucocorticoid receptor expressed in yeast. Yeast cells carrying receptor were metabolically labelled with $^{35}$S methionine in the absence and presence of 10 $\mu$M DOC for 90 min as described in Material and Methods. Receptor was isolated by immunoprecipitation, resolved on SDS PAGE and after drying, gel was exposed to film.**

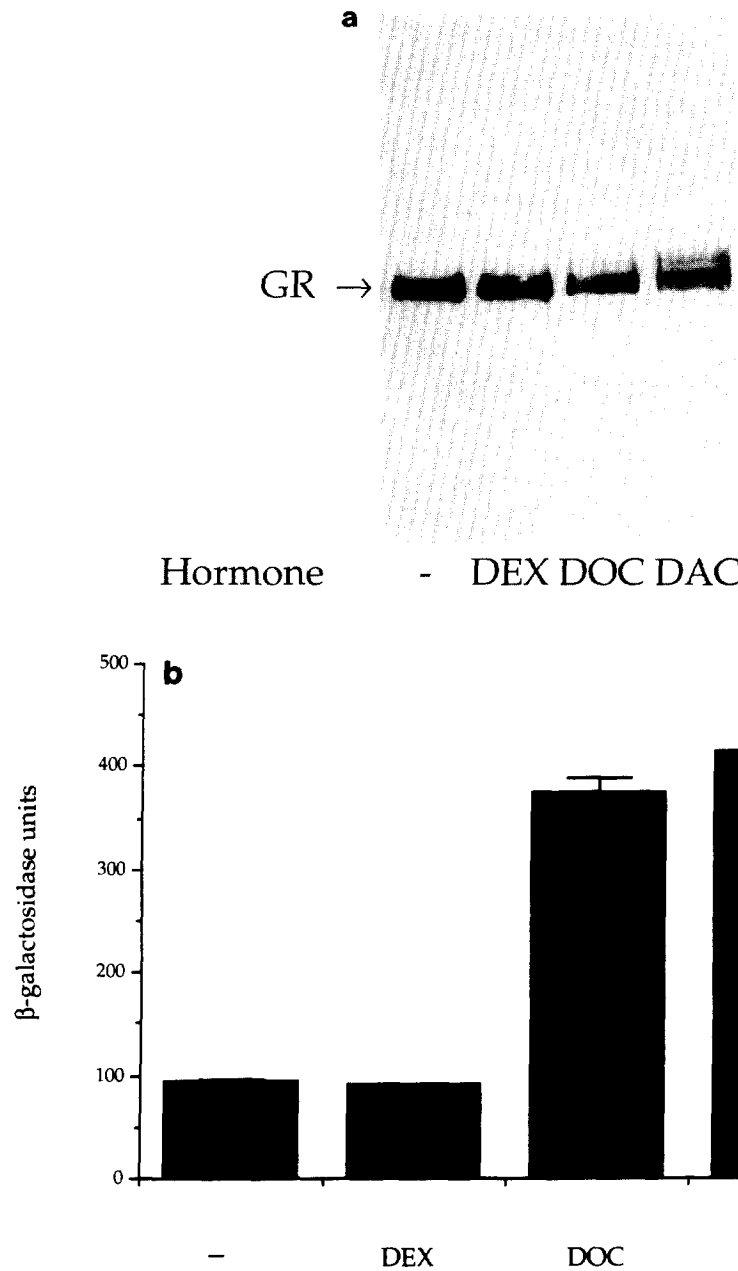


**Fig. 2. Electrophoretic mobility shift of the glucocorticoid receptor depends on the potency of the agonist. (A) Immunoblot of receptor expressed in yeast. Yeast cells were incubated in phosphate free medium as described in Material and Methods, except that $^{32}P$ orthophosphate was omitted. Hormones were used at 10 μM: DEX, dexamethasone; DOC, deoxycorticosterone; DAC, deacylcortivasol. Receptor was immunoprecipitated, fractionated by SDS polyacrylamide electrophoresis, transferred to Immobilon-P membrane (Millipore), and visualised by immunoblotting. (B) Gene activation by the receptor under conditions of metabolic labelling. Yeast cells were treated as in (A) 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.**

that have not been transformed with the receptor plasmid do not have endogenous receptor (compare lanes 1 and 2 with lanes 3 and 4 in Fig. 1C).

Taken together, these results suggest that the glucocorticoid receptor expressed in yeast is phosphorylated in a hormone dependent manner, and that yeast cells can provide a useful system to characterize the ligand dependency of the glucocorticoid receptor phosphorylation.

*Receptor phosphorylation in yeast: ligand specificity and time course of phosphorylation*

To test the effect of different ligands on this mobility shift, the glucocorticoid receptor expression plasmid was introduced into yeast cells together with a reporter plasmid carrying a GRE fused to the CYC β-galactosidase gene (as described in Material and Methods). Yeast cultures were treated for 90 min with several agonists. We used glucocorticoid ligands

that display a range of agonist potencies. The results revealed that the reduction in receptor mobility (Fig. 2A) parallels roughly the relative efficacies of the ligands to stimulate transcriptional activation by the receptor (Fig. 2B, see also Ref. [42]). Thus, dexamethasone (DEX) which is a weak agonist in *S. cerevisiae* under these conditions induced little or no shift in receptor electrophoretic mobility, whereas deacylcortivasol (DAC) and deoxycorticosterone (DOC) each conferred strong transcriptional enhancement activity on the receptor, and produced more pronounced effects on receptor mobility (Fig. 2).

To examine if the receptor was phosphorylated when expressed in *Saccharomyces cerevisiae* in both the absence and presence of hormone we first determined the time of optimal incorporation of radioactivity into the receptor. Yeast cells were incubated with $^{32}P$ orthophosphate in the presence and absence of hormone for various time periods (Fig. 3). Cell lysates were prepared after 15, 30 and 90 min and receptor was isolated by immunoprecipitation using the receptor specific monoclonal antibody BUGR2 [47]. Optimal $^{32}P$ incorporation was observed after 90 min incubation (Fig. 3, lanes 5 and 6), and this time was used in all further experiments, since longer incubation times did not change this pattern significantly (data not shown). The receptor was phosphorylated in yeast in both the presence and absence of hormone; incorporation of phosphate into the receptor protein was observed as soon as 15 min after addition of radioactivity, in both hormone treated and untreated cells, and increased further with time. In addition, a modest hormone dependent mobility shift in SDS gels was observed at all time points after addition of the agonist DOC (Fig. 3). To confirm that the ligand dependent receptor isoforms described in Fig. 2A are due to phosphorylation, we performed *in vivo* labelling of yeast cells treated with DAC and DEX. Deacylcortivazol (DAC) is the most potent agonist in yeast and induced striking hormone dependent phosphorylation (Figs 2 and 3). In parallel experiments, we tested whether dexamethasone (DEX), which is a weak agonist in yeast, will induce this mobility change. As shown in Figs 2 and 3, the effect of dexamethasone was negligible (compare lanes 1 and 2 in Fig. 2A, and lanes 7 and 8 in Fig. 3). Finally, we analyzed receptor mediated transcriptional activation from the reporter gene by primer extension analysis of CYC1-LacZ transcripts and detected receptor activated transcription within 15 min after hormone addition (data not shown).

Taken together, these experiments imply that the rat glucocorticoid receptor is phosphorylated when it is produced in yeast within 15 min of addition of radioactivity and ligand, and that the extent of hormone dependent phosphorylation correlates with agonist potency.

*Mapping the domain that confers hormone dependent mobility shift*

In order to analyze in more detail the phosphorylation state of the receptor in the absence and presence of hormone, $^{32}P$ labelling studies were performed *in vivo* with various receptor deletion mutants. The receptor derivative Δ1 (Fig. 4A) that lacks amino acids 70–130 was labelled *in vivo* with $^{32}P$ orthophosphate in the absence and presence of hormone, immunoprecipitated and resolved on SDS-PAGE. As shown in Fig. 4B this derivative shows a hormone dependent shift identical to the wild type

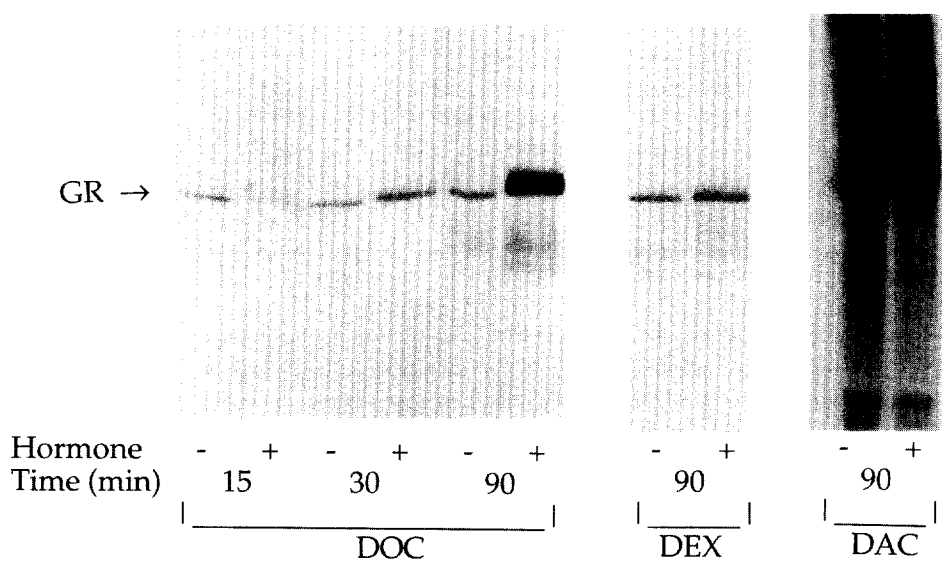


**Fig. 3. Kinetics and ligand specificity of the glucocorticoid receptor phosphorylation in yeast. Yeast cells carrying glucocorticoid receptor were incubated with $^{32}P$ orthophosphate in the absence and presence of the indicated 10 μM ligand, for indicated times. Receptor was isolated by immunoprecipitation, fractionated by SDS electrophoresis and gel was exposed to film.**

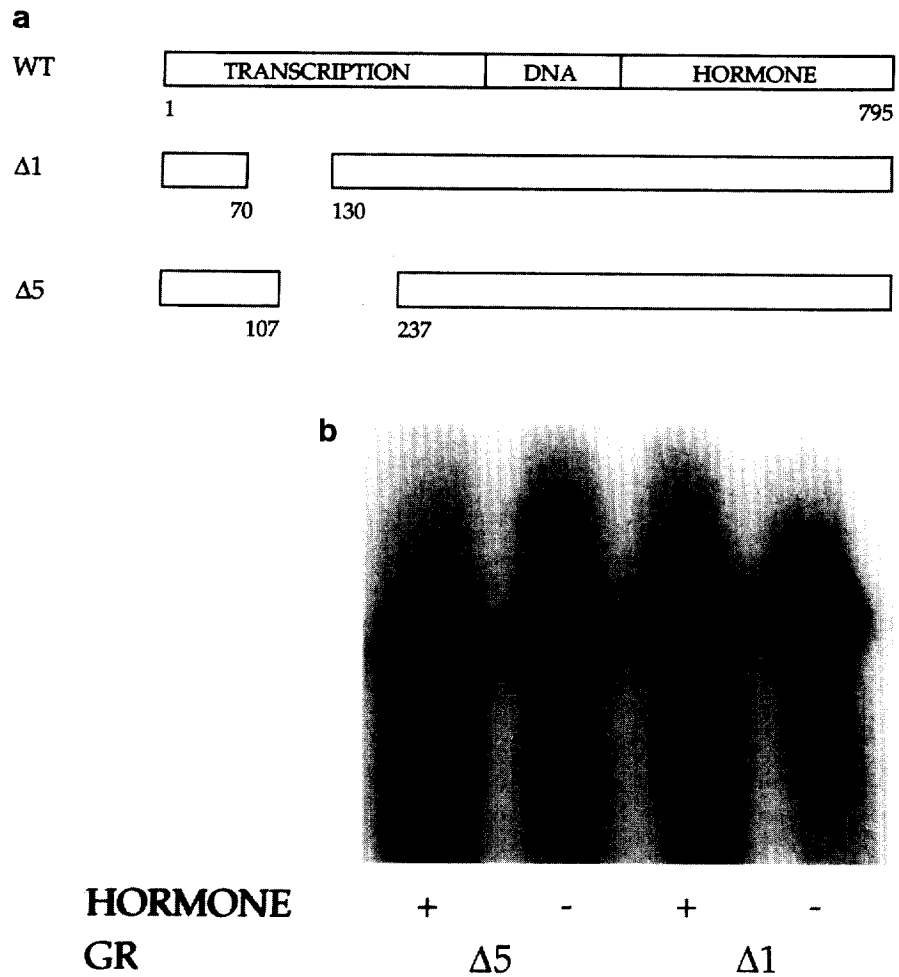


**Fig. 4. Domain conferring hormone dependent shift is localised between amino acids 130–237 of glucocorticoid receptor. (A) Schematic representation of the glucocorticoid receptor mutant derivatives. (B) *In vivo* $^{32}P$ orthophosphate labelling of receptor derivatives Δ1 and Δ5 in yeast. Yeast cells expressing the wild type and mutant receptor derivatives were incubated with $^{32}P$ orthophosphate in the absence and presence of hormone, receptor was immunoprecipitated and resolved using SDS PAGE, gel was dried and exposed to film. Receptor derivative Δ1 (lacks amino acids 70–130) shows hormone dependent mobility shift (compare lanes 3 and 4); receptor derivative Δ5 (lacks amino acids 107–237) does not show a hormone dependent shift (compare lanes 1 and 2).**

receptor. On the contrary, analysis of the hormone dependent phosphorylation of the receptor derivative Δ5 (Fig. 4A) that carries a deletion of amino acids 107–237, revealed that this mutant did not show any hormone dependent mobility shift (Fig. 4B). Both Δ1 and Δ5 were expressed at similar levels as detected by western blot analysis (data not shown). Finally, receptor mutants isolated from cells labelled *in vivo* with $^{32}P$ orthophosphate, containing deletions in the N terminal part of the protein (such as Δ3 that contains a deletion of residues 70–300, or derivative X556 containing only amino acids 407–556), showed negligible phosphate incorporation (data not shown).

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

To examine whether serine, threonine or tyrosine residues of GR expressed in yeast are phosphorylated, in the presence and absence of hormone, phosphoamino acid analysis was performed (Fig. 5). GR was expressed in both yeast and mammalian cells in the presence and absence of hormone and the protein was labelled *in vivo* with $^{32}P$ orthophosphate as described. Two dimensional electrophoresis was used to resolve amino acids and their identity was confirmed by comigration with ninhydrin stainable markers. Receptor expressed in both yeast (Fig. 5, panels

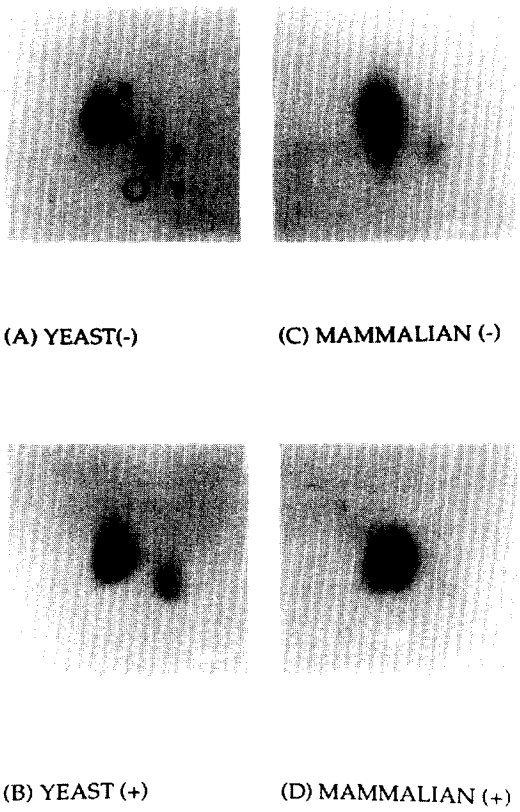


**Fig. 5. Phosphoamino acid analysis of the receptor expressed in yeast and mammalian cells. Yeast (A, B) and mammalian cells (C, D) expressing receptor protein were labelled with $^{32}P$ orthophosphate in the presence (B, D) and absence (A, C) of 10 μM DOC. Phosphoamino acid analysis was performed as described in Material and Methods. S, Serine; T, Threonine; Y, Tyrosine.**

A and B) and mammalian cells (Fig. 5, panels C and D) was phosphorylated mostly on serine and less on threonine in both the absence (panels A and C) and presence (panels B and D) of hormone. Tyrosine phosphorylation was not observed.

*Glucocorticoid receptor phosphorylation sites are similar in yeast and mammalian cells*

To determine if the same amino acids are phosphorylated when the receptor is expressed in yeast and mammalian cells, we performed phosphopeptide mapping in hormone-treated yeast and rat hepatoma cells, each expressing the receptor. These cells were metabolically labelled 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 [48]. As seen in Fig. 6 labelling of the receptor in either species yielded qualitatively similar patterns of tryptic phosphopeptides. Importantly, a similar phosphopeptide pattern was observed upon mixing the digested receptor from yeast and mammalian cells prior to two-dimensional separation (Fig. 6C; shown schematically in Fig. 6D). 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. 6A and B, the relative intensities of certain phosphopeptides differed between the two species. For example, peptides 2, 4 and 5 were more intense in mammalian cells, whereas peptides 9, 10 and 11 were more strongly labelled 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 yeast cells (data not shown). Hence, it is premature to ascribe the variations to species differences.

*Identification of the hormone dependent phosphotryptic peptides*

In order to analyze in more detail the phosphorylation state of receptor in the absence and presence of hormone, two-dimensional phosphopeptide mapping was performed. Receptor was labelled *in vivo*, 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 (Fig. 7). The time course of trypsin digestion suggested that 18 h 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 B) or without (panel A) hormone revealed 11 major and 8 minor peptides represented schematically on Fig. 7, panel D. Certain peptides (4, 5, 6) were phosphorylated to the same extent regardless of hormone treatment. On the contrary, phosphorylation of the peptide 8 was fully inducible by hormone whereas phosphorylation of peptides 2, 7 and 9 partially depended on the presence of hormone. 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. 7).

*Phosphotryptic mapping of glucocorticoid receptor point mutants*

The above mentioned studies indicate 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 localised within 130–237 region of the receptor. Inspection of this region of the rat glucocor-

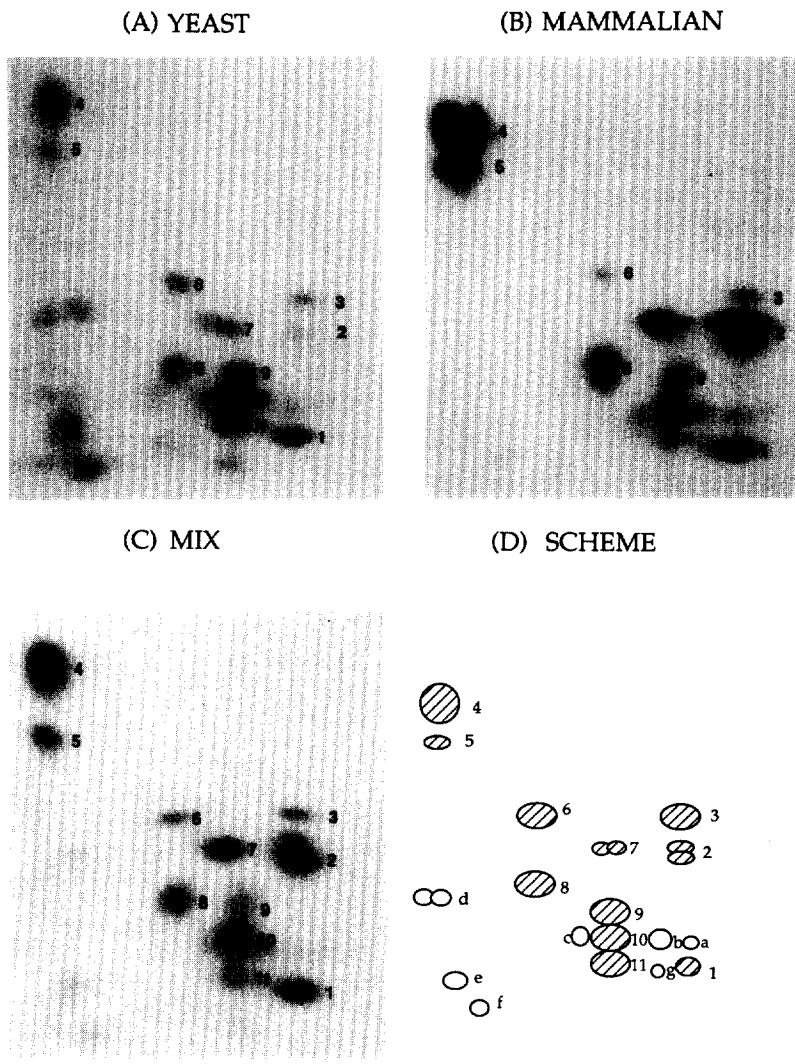


**Fig. 6. Tryptic phosphopeptides of receptor expressed in yeast and mammalian cells. Yeast and mammalian cells were incubated with $^{32}P$ orthophosphate and in the presence of hormone. Receptor was fractionated by gel electrophoresis, extracted from the gel and digested with trypsin. Phosphopeptides 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 labelled peptides (1–11; cross hatched circles) and seven minor peptides (a–g; open circles) are displayed.**

ticoid 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 [19]. In order to determine the localization of phosphorylated residues, *in vivo* $^{32}P$ labelling studies with various receptor point mutants expressed in yeast were performed. We altered four of the corresponding rat receptor residues from serine or threonine to unphosphorylatable residues (T171A, S224A S232G and S246A), and characterized the phosphorylation state of these receptor derivatives to identify the sites of phosphorylation in yeast. Metabolic labelling with $^{32}P$ orthophosphate and phosphotryptic peptide analysis of the receptor point mutants in the presence and absence of hormone is shown in Fig. 8. Peptide 10 is missing from the maps derived from the receptor mutation T171A (Fig. 8, panels 1 and 2) regardless of hormone treatment. The derivative S246A lacks peptides 4 and 5 in both absence and presence of hormone (Fig. 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 S232G. Phosphotryptic map of these mutant derivatives shows that there are changes in intensities of peptides 2 and 7 that are partially inducible by hormone (Fig. 8, panels 3–6). Moreover, in the presence of hormone, these single amino-acid substitutions result in the loss of the major hormone inducible phospho-

peptide (Fig. 8, panels 4 and 6, peptide number 8). This disappearance of multiple peptides resulting from alteration of S224A 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 S232 are consistent with their actual positions on the two-dimensional map. Similarly, Bodwell *et al.* [19] have identified by microsequencing several peptides that contain residue S224 and are result of inefficient cleavage by trypsin. In addition, these results were confirmed by phosphopeptide mapping with V8 protease, further mutagenesis and using chemically phosphorylated peptides that correspond to the receptor phosphopeptides [20]. Taken together, these results indicate that these mutations eliminate particular phosphopeptides and argue that the residues T171, S224, S232 and S246 are indeed sites of the glucocorticoid receptor phosphorylation in yeast, and that residues S224 and

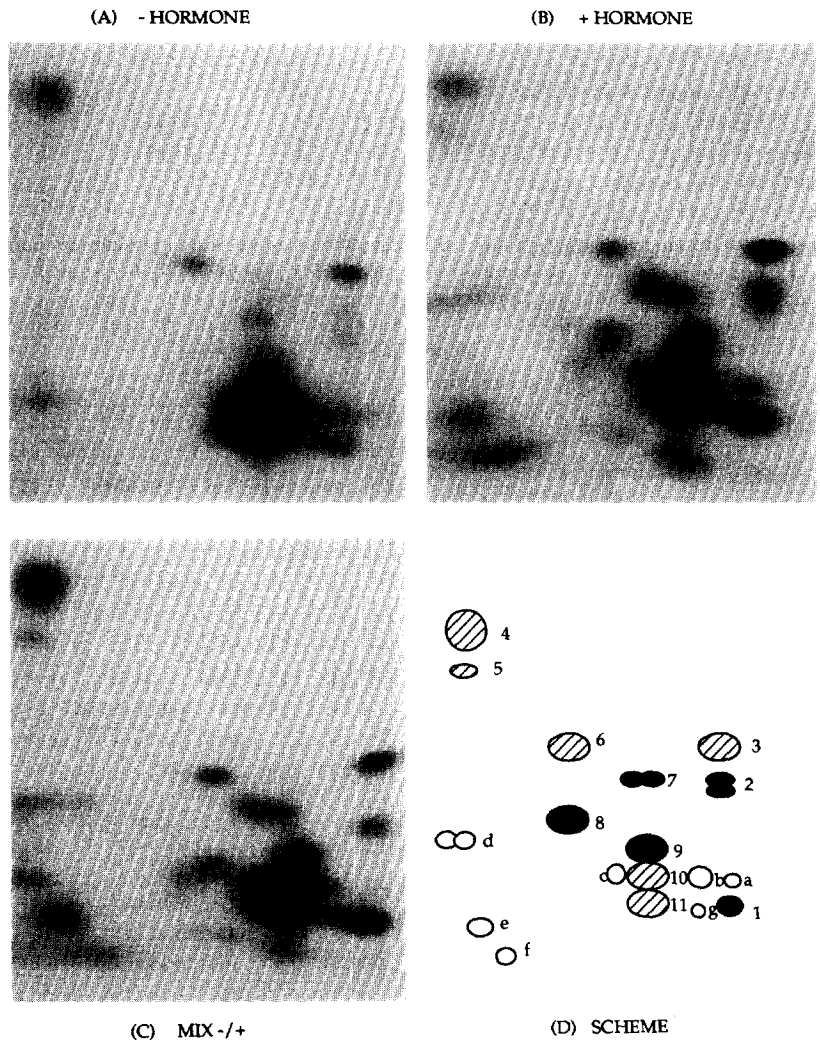


**Fig. 7. Phosphotryptic pattern of receptor expressed in yeast incubated with or without hormone. Yeast cells expressing receptor were incubated with $^{32}P$ orthophosphate and in the presence and absence of hormone. Receptor was isolated by electrophoresis, extracted from gel and treated with trypsin. 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 cells. There are 11 major labelled 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 1, 2, 7, and 9 are moderately hormone induced (filled circles).**

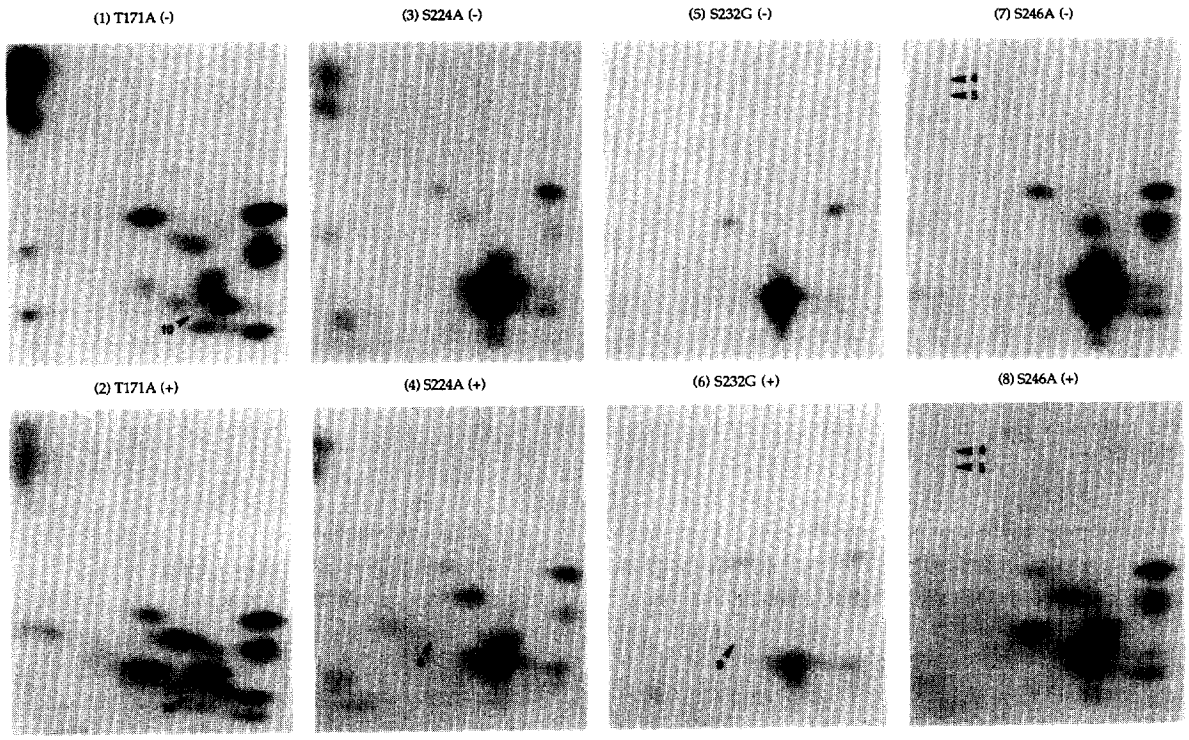


**Fig. 8. Phosphotryptic pattern of receptor point mutants. Yeast cells bearing receptor point mutants were metabolically labelled *in vivo* with $^{32}P$ orthophosphate. Receptor was isolated by immunoprecipitation and fractionated on SDS PAGE. Upper panels show tryptic phosphopeptide patterns from cells incubated with the $^{32}P$ orthophosphate in the absence of hormone and lower panels show cells that have been treated with the 10 $\mu$M DOC.**

S232 are phosphorylated in response to hormone treatment.

## DISCUSSION

Our results demonstrate that the glucocorticoid receptor expressed in yeast is phosphorylated in a similar fashion as in mammalian cells. This phosphorylation occurs on serine and threonine residues within 15 min upon addition of radioactivity in both the absence and presence of hormone. We have shown that receptor phosphorylation increases upon addition of hormone, and that this ligand dependent phosphorylation can be followed by a simple change in electrophoretic mobility of the receptor that has not been observed previously. This mobility shift is sensitive to phosphatase treatment and is dependent on the agonist strength. Various mutant derivatives of receptor were used to characterize this mobility change and residues contributing to this shift were localized within residues 130–247. This assay provides an important tool to follow ligand dependent phosphorylation *in vivo*, and to investigate the influences that different signalling pathways may have on this process.

In addition, we used phosphopeptide mapping of *in vivo* labelled receptor to show that the receptor residues that are phosphorylated are the same in both yeast and mammalian cells. Finally, we mutated receptor residues in the rat glucocorticoid receptor that correspond to the phosphorylated amino acids in the mouse glucocorticoid receptor and used these receptor derivatives to study its phosphorylation. We have labelled these receptor mutants *in vivo* with $^{32}P$ orthophosphate and determined that phosphorylation of S224 and S232 increases upon addition of hormone, whereas phosphorylation of T171 and S246 does not change significantly in the presence of the ligand.

Most steroid receptors are phosphoproteins but the functional significance of phosphorylation is still not fully understood. In this study we show that the rat glucocorticoid receptor expressed in yeast is phosphorylated and that hormone treatment of cells leads to additional phosphorylation events, which can be observed as a change in the electrophoretic mobility of the glucocorticoid receptor (Fig. 1). The shift in mobility we observed has been reported for several 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 of phosphorylated proteins is that SDS inefficiently coats the protein due to the negative phosphate charge, thus altering its mobility in the electrical field. This mobility shift caused by phosphorylation has also been observed for some steroid receptors and transcription factors. For example the progesterone receptor appears as a doub-

let on SDS gels after hormone binding [31], and the transcription factor Elk1 involved in the regulation of the c-fos promoter undergoes a phosphorylation-induced conformational change detected as a decrease in its electrophoretic mobility [49].

We have characterized this change in mobility of the GR using multiple detection assays. Yeast cells were labelled *in vivo* with $^{32}P$ orthophosphate to detect all phosphorylated receptor isoforms (Fig. 3). Then, in order to examine the whole population of receptor molecules, two types of experiments were performed. First, the receptor was labelled with $^{35}S$ methionine *in vivo*, which detects newly synthesized potentially phosphorylated and unphosphorylated receptor isoforms (Fig. 1C). Finally, immunoblotting was used to visualize the receptor, and this simple and nonradioactive approach detected the whole population of receptor molecules including several hormone dependent receptor isoforms (Figs 1 and 2). The latter two procedures visualise the entire cellular complement of receptor molecules, whereas the $^{32}P$ metabolic labelling experiment monitors the receptor molecules that acquire phosphate adducts during the 90 min labelling period. Interestingly, it appears that most or all of the receptor is phosphorylated constitutively, and that majority of it receives a net increase in phosphate accompanying hormone addition.

Proteins can change electrophoretic mobility due to different post-translational modifications such as phosphorylation, glycosylation [50] or acetylation [51]. The mobility shift we have demonstrated is a consequence of additional phosphorylation as shown by phosphatase treatment experiment (Fig. 1B). Calf intestinal phosphatase eliminated the slower migrating receptor forms observed in the hormone treated cells, suggesting that the decreased receptor mobility is due to phosphorylation. 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.

We have determined the kinetics of phosphorylation of the glucocorticoid receptor expressed in yeast (Fig. 3). Kinetic studies of receptor phosphorylation in mammalian cells suggested that the increase in receptor phosphorylation upon hormone addition occurs within 15 min, which is consistent with the results from yeast cells shown on Fig. 3. For example, Hoeck *et al.* [52] have reported an increase in GR phosphorylation in NIH 3T3 cells after 20 min of hormone treatment which increased further with time. Similarly, Orti *et al.* [53] have observed that the hormone induced increase in phosphorylation was seen as early as 5 min after hormone addition to WEHI-7 mouse thymoma cells. Thus, the kinetics of glucocorticoid receptor phosphorylation seems to be similar in both yeast and mammalian cells, in both the absence and the presence of a ligand.

We show that the GR is phosphorylated on serine and threonine residues in both yeast and mammalian cells and in the absence and presence of hormone (Fig. 5). Bodwell *et al.* [19] have reported the phosphorylation of the mouse glucocorticoid receptor in hormone treated mammalian cells on both serine and threonine residues. We did not observe any phosphorylation on tyrosine, although the glucocorticoid receptor reportedly interacts with phosphotyrosine specific antibodies [54]. Taken together, our results show that in both yeast and mammalian cells, phosphorylation of the GR occurs on serine and threonine residues in both the absence and the presence of hormone. Moreover, phosphotryptic mapping of receptor expressed in yeast and mammalian cells revealed that in both species hormone dependent and constitutive phosphorylation are virtually identical (Fig. 6). These findings suggest that phosphorylation may play an important role in receptor actions and that kinases involved in modifying the receptor are highly conserved. Interestingly, the progesterone receptor when expressed in yeast is phosphorylated in a similar fashion as in mammalian cells, suggesting that phosphorylation of the steroid receptor family may be carried out by kinases that are highly conserved between yeast and mammalian cells [55]. Finally, using receptor derivatives carrying point mutations in the putative phosphorylation sites we show that hormone dependent phosphorylation localizes to the residues S224 and S232 whereas constitutive phosphorylation is due to the modification of T171 and S246 (Fig. 8).

Several steroid receptors are phosphorylated in a hormone dependent fashion. Sites of ligand dependent phosphorylation have been identified for chicken progesterone receptor and are located in the vicinity of the DNA binding domain of this protein [15, 21]. Regarding the human progesterone receptor, hormone induced phosphorylation is located in the N terminus of the DNA binding domain [22]. It has been described that the residues which are phosphorylated in a hormone dependent manner for the oestrogen receptor are located within the transcriptional activation region of this protein [8, 13, 25]. In addition, GR has been reported to exist as a phosphoprotein in both 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.* [14] 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, Hoeck *et al.* reported that phosphorylation of the N-terminal region of the receptor was increased 2–3 fold by hormone treatment [12]. Subsequently, Bodwell *et al.* [19] have reported seven sites of phosphorylation on receptor isolated from hormone treated mouse cells, but this study did not address the question whether the hormone addition influenced the modification of these

residues. In this report we show that the glucocorticoid receptor from yeast and GRH2 cells is phosphorylated on four residues, two of which (S224 and S232) are phosphorylated in a hormone dependent fashion. Inspection of this region revealed that these residues are within the consensus sequences for proline directed kinases such as MAP kinases and CDK kinases.

The physiological significance of glucocorticoid receptor phosphorylation is not clear at present, and evidence for any 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 [56, 57]. These observations led to speculation that phosphorylation may be important for receptor function, and that receptor molecules may cycle between the nucleus and the cytoplasm in an ATP-dependent manner (Ref. [58], reviewed in Ref. [59]). In addition, in cells treated with okadaic acid, an inhibitor of protein phosphatases PP1 and PP2A, the glucocorticoid receptor is inefficiently retained in the nucleus and located predominantly in the cytoplasm [30]. As the phosphorylation sites reside close to the N-terminal transcriptional regulatory domain, the transcriptional regulation by the glucocorticoid receptor was proposed to be modulated by phosphorylation. Initially, however, Mason and Housley [29] suggested that mutagenesis of all seven residues in the mouse GR did not have significant effect on GR function. Subsequently, modest functional effects have been measured after mutation of some of the phosphorylation sites in GR [28], PR [16, 27] and ER [8, 25].

In yeast strains lacking certain genes of the CDK family, transcriptional activity of the GR is compromised, implying that phosphorylation by this class of kinases may be required for the full activity of the receptor. Indeed, CDK2 complexed with cyclin A or E phosphorylates residues S224 and S232 *in vitro*, which we show to be phosphorylated in a hormone dependent manner *in vivo* [20, 37]. In contrast, MAP kinase influences receptor function in a negative manner, and phosphorylates T171 and S246 *in vitro*, which are constitutively phosphorylated *in vivo*. It seems likely that through hormone dependent and independent phosphorylation events the glucocorticoid receptor responds to and integrates multiple signal transduction pathways. Consistent with this notion is the fact that the residues which are phosphorylated in a ligand dependent fashion in the progesterone receptor may affect ligand dependent transcriptional activity, but have no effect on the ligand independent activation of this receptor [21].

It is known that stimulation or inhibition of certain signalling pathways can influence receptor function. For example, protein kinase A can potentiate glucocorticoid receptor function in developing retina [60] and in F9 embryonal carcinoma cells [34]. A link between the steroid hormone pathway and other signalling pathways has also been hypothesized on the basis of the modulation of GR dependent transcription by the activation or expression of certain oncogenes [61]. As mentioned above, glucocorticoid receptor nuclear localization may be affected by the treatment of cells 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 these phosphatases or the above mentioned kinases directly affect GR phosphorylation and function *in vivo*.

In this report, we have shown that hormone dependent phosphorylation can be studied in yeast using an electrophoretic mobility shift assay and phosphotryptic mapping. The heterogeneous GR species observed in DOC and DAC treated cells indicate multiple phosphorylated isoforms. These ligands are potent agonists in yeast and the extent of the mobility change correlates with the potency of the ligand used to activate the receptor (Fig. 2). Thus, it appears that hormone dependent phosphorylation may be an important aspect of receptor function. A GR derivative lacking hormone dependent phosphorylation sites 107–237(Δ5) does not show this mobility shift whereas a derivative (Δ1) lacking residues 70–130 but still containing amino acids phosphorylated *in vivo* becomes phosphorylated upon hormone addition (Fig. 4). These derivatives may prove useful for future studies as hormone dependent phosphorylation can readily be monitored by western blot analysis in certain kinase deficient strains.

Our observation that the same residues are phosphorylated in yeast and mammalian cells justifies using yeast to study phosphorylation of the glucocorticoid receptor. The availability of numerous kinase deficient mutants and powerful genetic approaches available in yeast should allow further dissection of the receptor phosphorylation pathway. Furthermore, identification of the residues S224 and S232, which reside in consensus sites for the CDK kinases, as sites of hormone dependent phosphorylation, should bring us closer to understanding of the physiological significance of phosphorylation of steroid receptors.

The receptor interacts with several regulatory proteins that modulate its action in a hormone dependent, cell specific and promoter specific fashion. 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, the GR as well as other steroid receptors are covalently modified by phosphorylation, suggesting another level of regulation of receptor function. Studying the effects of phosphorylation on GR function could give an insight into the paradox of how one ubiquitous transcription factor can effect cell-specific gene regulation. Considering the complexity of the potential functions of steroid receptors that

could be affected by phosphorylation and the possible kinases involved in mammalian cells, a simple system like yeast should facilitate our understanding of the physiological role of receptor phosphorylation. Yeast cells provide several technical advantages. Numerous mutations in kinase genes have been described in yeast and it is relatively simple to test the effects of these mutations on receptor function, and then assay the effects of activation or inhibition of these kinases on GR in mammalian cells [20, 37–40].

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## REFERENCES

1. Beato M., Herrlich P. and Schutz G., Steroid hormone receptors: many actors in search of a plot. *Cell* **83** (1995) 851–857.
2. Mangelsdorf D. J., Thummel C., Beato M., Herrlich P., Schutz G., Umesono K., Blumberg B., Kastner P., Mark M., Chambon P. and Evans R. M., The nuclear receptor superfamily: the second decade. *Cell* **83** (1995) 835–839.
3. Perlmann T. and Evans R. M., Nuclear receptors in Sicily: All in famiglia. *Cell* **90** (1997) 391–397.
4. Payvar F., DeFranco D., Firestone G. L., Edgar B., Wrange O., Okret S., Gustafsson J. A. and Yamamoto K. R., Sequence-specific binding of glucocorticoid receptor to MTV DNA at sites within and upstream of the transcribed region. *Cell* **35** (1983) 381–392.
5. Scheidereit C., Geisse S., Westphal H. M. and Beato M., The glucocorticoid receptor binds to defined nucleotide sequences near the promoter of mouse mammary tumor virus. *Nature* **304** (1983) 749–752.
6. Yamamoto K. R., Steroid receptor regulated transcription of specific genes and gene networks. *Ann. Rev. Genet.* **19** (1985) 209–252.
7. Yamamoto, K. R., Pearce, D. L., Thomas, J. and Miner, K. N. In *Transcriptional regulation*, eds. S. L. McKnight and K. R. Yamamoto. Cold Spring Harbor Press, 1992, pp. 1169–1189.
8. Ali S., Metzger D., Bornert J.-M. and Chambon P., Modulation of transcriptional activation by ligand-dependent phosphorylation of the human estrogen receptor A/B region. *EMBO J.* **12** (1993) 1153–1160.
9. Chauchereau A., Cohensobal K., Jovilet A., Bailly A. and Milgrom E., Phosphorylation sites in ligand induced and ligand independent activation of the progesterone receptor. *Biochemistry* **33** (1994) 13295–13303.
10. Denton R. R., Koszewski N. J. and Notides A. C., Estrogen receptor phosphorylation. Hormonal dependence and consequence on specific DNA binding. *J. Biol. Chem.* **267** (1992) 7263–7268.
11. Denner L. A., Weigel N. L., Maxwell B. L., Shrader W. T. and O'Malley B. W., Regulation of progesterone receptor-mediated transcription by phosphorylation. *Science* **250** (1990) 1740–1743.
12. Hoeck W. and Groner B., Hormone dependent phosphorylation of the glucocorticoid receptor occurs mainly in the amino-terminal transactivation domain. *J. Biol. Chem.* **265** (1990) 5403–5408.
13. Lahooti H., White R., Danielian P. S. and Parker M. G., Characterization of ligand dependent phosphorylation of the estrogen receptor. *Mol. Endocrinol.* **8** (1994) 182–188.
14. Orti E., Mendel D. B., Smith L. I. and Munck A., Agonist-dependent phosphorylation and nuclear dephosphorylation of glucocorticoid receptors in intact cells. *J. Biol. Chem.* **264** (1989) 9728–9731.
15. Poletti A. and Weigel N. L., Identification of a hormone dependent phosphorylation site adjacent to the DNA-binding domain of the chicken progesterone receptor. *Mol. Endocrinol.* **7** (1993) 241–246.
16. Takimoto G. S., Tasset D. M., Eppert A. C. and Horwitz K. B., Hormone-induced progesterone receptor phosphorylation consists of sequential DNA-independent and DNA-dependent stages-analysis with zinc finger mutants and the progesterone antagonist ZK98299. *Proc. Natl. Acad. Sci. USA* **89** (1992) 3050–3054.
17. Sheridan P. L., Evans R. M. and Horwitz K. B., Phosphotryptic peptide analysis of human progesterone receptors – new phosphorylated sites formed in nuclei after hormone treatment. *J. Biol. Chem.* **264** (1989) 6520–6528.
18. Washburn T., Hocutt A., Brautigan D. L. and Korach K. S., Uterine estrogen receptor *in vivo*: phosphorylation of nuclear specific forms on serine residues. *Mol. Endocrinol.* **5** (1991) 235–242.
19. Bodwell J. E., Orti E., Coull J. M., Pappin D. J. C., Smith L. I. and Swift F., Identification of the phosphorylated sites in the mouse glucocorticoid receptor. *J. Biol. Chem.* **266** (1991) 7549–7555.
20. Krstic, M. D., Functional analysis of glucocorticoid receptor phosphorylation. Ph.D. thesis, University of California, San Francisco, CA, 1995.
21. Denner L. A., Schrader W. T., O'Malley B. W. and Weigel N. L., Hormonal regulation and identification of chicken progesterone receptor phosphorylation sites. *J. Biol. Chem.* **265** (1990) 16548–16555.
22. Takimoto G. S., Hovland A. R., Tasset D. M., Melville M. Y., Tung L. and Horwitz K. B., Role of phosphorylation on DNA binding and transcriptional functions of human progesterone receptors. *J. Biol. Chem.* **271** (1996) 13308–13316.
23. Zhang H. Y., Beck C. A., Poletti A., Edwards D. P. and Weigel N. L., Identification of phosphorylation sites unique to the B form of human progesterone receptor. *J. Biol. Chem.* **269** (1994) 31034–31040.
24. Zhang Y. X., Beck C. A., Poletti A., Edwards D. P. and Weigel N. L., Identification of group of ser-pro motif hormone-inducible phosphorylation sites in the human progesterone receptor. *Mol. Endocrinol.* **9** (1995) 1029–1040.
25. LeGoff P., Montano M. M., Schodin D. J. and Katzenellenbogen B. S., Phosphorylation of the human estrogen receptor: identification of hormone-regulated sites and examination of their influence on transcriptional activity. *J. Biol. Chem.* **269** (1994) 458–4466.
26. Bai W. L., Tullos S. and Weigel N. L., Phosphorylation of Ser(530) facilitates hormone-dependent transcriptional activation of the chicken progesterone receptor. *Mol. Endocrinol.* **8** (1994) 1465–1473.
27. Bai W. and Weigel N. L., Phosphorylation of Ser211 in the chicken progesterone receptor modulates its transcriptional activity. *J. Biol. Chem.* **271** (1996) 12801–12806.
28. Almlof T., Wright A. P. H. and Gustafsson J.-A., Role of acidic and phosphorylated residues in gene activation by the glucocorticoid receptor. *J. Biol. Chem.* **270** (1995) 17535–17540.
29. Mason S. A. and Housley P., Site-directed mutagenesis of the phosphorylation sites in the mouse glucocorticoid receptor. *J. Biol. Chem.* **268** (1993) 21501–21504.
30. DeFranco D. B., Qi M., Borror K. C., Garabedian M. J. and Brautigan D. L., Protein phosphatases types 1 and/or 2A regulate nucleocytoplasmic shuttling of glucocorticoid receptors. *Mol. Endocrinol.* **5** (1991) 1215–1228.
31. Beck C. A., Weigel N. L. and Edwards D. P., Effects of hormone and cellular modulators of protein phosphorylation on transcriptional activity. *Mol. Endocrinol.* **6** (1992) 607–620.
32. Cho H. and Katzenellenbogen B. S., Synergistic activation of estrogen receptor mediated transcription by estradiol and protein kinase activators. *Mol. Endocrinol.* **7** (1993) 441–452.
33. Fujimoto N. and Katzenellenbogen B. S., Alteration in the agonist/antagonist balance of antiestrogens by activation of protein kinase A signaling pathways in breast cancer cells antiestrogen selectivity and promoter dependence. *Mol. Endocrinol.* **8** (1994) 296–304.

34. Rangarajan P. N., Umesono K. and Evans R. M., Modulation of glucocorticoid receptor function by protein kinase A. *Mol. Endocrinol.* **6** (1992) 1451–1457.
35. Bunone G., Brieand P.-A., Miksicek R. J. and Picard D., Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *EMBO J.* **15** (1996) 2174–2183.
36. Kato S., Endoh H., Masuhiro Y., Kitamoto T., Uchiyama S., Sasaki H., Masushige S., Gotoh Y., Nishida E., Kawashima H., Metzger D. and Chambon P., Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* **270** (1995) 1491–1494.
37. Krstic M. D., Rogatsky I., Yamamoto K. R. and Garabedian M. J., Mitogen-activated and cyclin-dependent protein kinases selectively and differentially modulate transcriptional enhancement by the glucocorticoid receptor. *Mol. Cel. Biol.* **17** (1997) 3947–3954.
38. Rogatsky I., Trowbridge J. M. and Garabedian M. J., Glucocorticoid receptor-mediated cell cycle arrest is achieved through distinct cell-specific transcriptional regulatory mechanisms. *Mol. Cel. Biol.* **17** (1997) 3181–3193.
39. Zhang Y. X., Beck C. A., Poletti A., Clement J. P., Prendergast P., Yip T. T., Hutchens T. W., Edwards D. P. and Weigel N. L., Phosphorylation of human progesterone receptor by cyclin-dependent kinase 2 on three sites that are authentic basal phosphorylation sites *in vivo*. *Mol. Endocrinol.* **11** (1997) 823–832.
40. Zwijsen R. M. L., Wientjens E., Klompmaker R., Van der Sman J., Bernards R. and Michalides R. J. A. M., CDK-independent activation of estrogen receptor by cyclin D1. *Cell* **88** (1997) 405–415.
41. Schena M. and Yamamoto K. R., Mammalian glucocorticoid receptor derivatives enhance transcription in yeast. *Science* **241** (1988) 965–967.
42. Garabedian M. J. and Yamamoto K. R., Genetic dissection of the signaling domain of a mammalian steroid receptor in yeast. *Mol. Biol. Cell* **3** (1992) 1245–1257.
43. Jones E. W., Proteinase mutants of *Saccharomyces cerevisiae*. *Genetics* **85** (1977) 23–33.
44. Ito H., Fukuda Y., Murata K. and Kimura A., Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153** (1983) 163–168.
45. Schena M., Freedman L. P. and Yamamoto K. R., Mutations in the glucocorticoid receptor zing finger region that distinguish interdigitated DNA-binding and transcriptional enhancement activities. *Genes Dev.* **3** (1989) 1590–1601.
46. Vanderbilt J. N., Miesfeld R., Maler B. A. and Yamamoto K. R., Intracellular receptor concentration limits glucocorticoid dependent enhancer activity. *Mol. Endocrinol.* **1** (1987) 68–74.
47. Gametchu B. and Harrison R. W., Characterization of a monoclonal antibody to the rat liver glucocorticoid receptor. *Endocrinol.* **114** (1984) 274–288.
48. Ward G. E. and Kirschner M. W., Identification of cell cycle regulated phosphorylation sites on nuclear lamin-C. *Cell* **61** (1990) 561–577.
49. Marais R., Wynne J. and Treisman R., The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell* **73** (1993) 381–393.
50. Jackson S. P. and Tjian R., *O*-glycosylation of eukaryotic transcription factors: Implications for mechanisms of transcriptional regulation. *Cell* **55** (1988) 125–133.
51. Bartsch J., Truss M., Bode J. and Beato M., Moderate increase in histone acetylation activates the mouse mammary tumor virus promoter and remodels its nucleosome structure. *Proc. Natl. Acad. Sci. USA* **93** (1996) 10741–10746.
52. Hoeck W., Rusconi S. and Groner B., Down-regulation and phosphorylation of glucocorticoid receptors in cultured cells. *J. Biol. Chem.* **264** (1989) 14396–14402.
53. Orti E., Hu L. M. and Munck A., Kinetics of glucocorticoid receptor phosphorylation in intact cells – evidence for hormone-induced hyperphosphorylation after activation and recycling of hyperphosphorylated receptors. *J. Biol. Chem.* **268** (1993) 7779–7784.
54. Auricchio F., Migliaccio A., Castoria G., Rotondi A., DiDomenico M., Pagano M. and Nola E., Phosphorylation of tyrosine of oestradiol-17-beta receptor and glucocorticoid receptors with antiphosphotyrosine antibodies. *J. Steroid Biochem.* **27** (1987) 245–253.
55. Polleti A., Conneely O. M., McDonnell D. P., Schrader W. T., O'Malley B. W. and Weigel N. L., Chicken progesterone receptor expressed in *Saccharomyces cerevisiae* is correctly phosphorylated at all four ser-pro phosphorylation sites. *Biochemistry* **32** (1993) 9563–9569.
56. Bell P. A. and Munck A., Steroid-binding properties and stabilization of cytoplasmic glucocorticoid receptors from rat thymus cells. *Biochemical J.* **136** (1973) 97–107.
57. Munck A. and Holbrook N. J., Glucocorticoid receptor complexes in rat thymus cells – rapid kinetic behavior and a cyclic model. *J. Biol. Chem.* **259** (1984) 820–831.
58. Mendel D. B., Bodwell J. E. and Munck A., Glucocorticoid receptors lacking hormone-binding activity are bound in nuclei of ATP-depleted cells. *Nature* **324** (1986) 478–480.
59. Orti E., Bodwell J. E. and Munck A., Phosphorylation of steroid hormone receptors. *Endorine Rev.* **13** (1992) 105–128.
60. Zhang H. Y., Li Y. C. and Young A. P., Protein kinase-A activation of glucocorticoid mediated signaling in the developing retina. *Proc. Natl. Acad. Sci. USA* **90** (1993) 3880–3884.
61. Jaggi R., Salmons B., Muellener D. and Groner B., The V-Mos and H-Ras oncogene expression represses glucocorticoid hormone-dependent transcription from the mouse mammary-tumor virus LTR. *EMBO J.* **5** (1986) 2609–2616.