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Kinases and protein phosphorylation as regulators of steroid hormone action

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Although the primary signal for the activation of steroid hormone receptors is binding of hormone, there is increasing evidence that the activities of cell signaling pathways and the phosphorylation status of these transcription factors and their coregulators determine the overall response to the hormone. In some cases, enhanced cell signaling is sufficient to cause activation of receptors in medium depleted of steroids. Steroid receptors are targets for multiple kinases. Many of the phosphorylation sites contain Ser/Thr-Pro motifs implicating proline-directed kinases such as the cyclin-dependent kinases and the mitogen-activated kinases (MAPK) in receptor phosphorylation. Although some sites are constitutively phosphorylated, others are phosphorylated in response to hormone. Still others are only phosphorylated in response to specific cell signaling pathways. Phosphorylation of specific sites has been implicated not only in overall transcriptional activity, but also in nuclear localization, protein stability, and DNA binding. The studies of the roles of phosphorylation in coregulator function are more limited, but it is now well established that many of them are highly phosphorylated and that phosphorylation regulates their function. There is good evidence that some of the phosphorylation sites in the receptors and coregulators are targets of multiple signaling pathways. Individual sites have been associated both with functions that enhance the activity of the receptor, as well as with functions that inhibit activity. Thus, the specific combinations of phosphorylations of the steroid receptor combined with the expression levels and phosphorylation status of coregulators will determine the genes regulated and the biological response.

Received January 8th, 2007; Accepted April 27th, 2007; Published May 17th, 2007 | **Abbreviations:** **AF-1:** activation function 1; **AF-2:** activation function 2; **AIB1:** amplified in breast cancer 1; **AR:** androgen receptor; **CBP:** CREB (cyclic AMP response element binding protein) binding protein; **Cdk2:** cyclin-dependent kinase 2; **ChIP:** chromatin immunoprecipitation; **cPR:** chicken progesterone receptor; **DBD:** DNA binding domain; **DHT:** dihydrotestosterone; **EGF:** epidermal growth factor; **ER α :** estrogen receptor α ; **ER β :** estrogen receptor β ; **GR:** glucocorticoid receptor; **GRIP1:** (glucocorticoid receptor interacting protein 1); **GSK3 β :** (glycogen synthase kinase 3 β); **HER2:** human epidermal growth factor receptor 2; **HRE:** hormone response element; **I κ B:** I κ B kinase; **JNK:** jun N terminal kinase; **KGF:** keratinocyte growth factor; **LBD:** ligand binding domain; **MAPK:** mitogen-activated protein kinase; **MEKK1:** MEK kinase 1; **MMTV:** mouse mammary tumor-like virus; **MNAR:** modulator of nongenomic action of estrogen receptor; **N-CoR:** nuclear receptor corepressor; **NTD:** amino terminal domains; **Pol II:** polymerase II; **PP5:** protein phosphatase 5; **PPM1D:** p53-induced serine/threonine phosphatase, protein phosphatase 1D magnesium-dependent, delta isoforms; **PR:** progesterone receptor; **PSA:** prostate specific antigen; **SMRT:** silencing mediator of retinoid and thyroid hormone receptors; **SPBP:** stromelysin-1 platelet-derived growth factor-responsive element-binding protein; **SRC-1:** steroid receptor coactivator-1; **TIF2:** transcription intermediary factor 2; **TRAP220:** thyroid receptor associated protein 220 | Copyright © 2007, Weigel and Moore. This is an open-access article distributed under the terms of the Creative Commons Non-Commercial Attribution License, which permits unrestricted non-commercial use distribution and reproduction in any medium, provided the original work is properly cited.

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Introduction

Steroid hormone receptors are hormone-activated transcription factors whose activities are also modulated by posttranslational modifications including phosphorylation [Faus and Haendler, 2006]. In the absence of hormone, receptor monomers associate with heat shock protein complexes and as a rule are minimally phosphorylated (Figure 1). Upon binding hormone, the receptors dimerize, cytoplasmic receptors translocate to the nucleus and the receptors bind to sequence-specific hormone response elements (HRE). Typically, hormone binding and localization to specific DNA binding sites is accompanied by an increase in receptor phosphorylation. The receptors recruit a series of coactivator complexes that facilitate chromatin remodeling, recruitment of Pol II (polymerase II), and transcription of specific target genes. Phosphorylation of coactivators and Pol II is also integral to regulation of transcription. Moreover, some of the proteins recruited to the chromatin by steroid receptors are themselves kinases that can modify histones or other proteins associated with chromatin.

In some cases, upon hormone binding, a portion of the cytoplasmic receptor associates with and activates Src kinase, leading to activation of downstream signaling [Edwards, 2005; Lange, 2004]. In addition, there is evidence that a small fraction of some of the receptors is associated with the cell membrane and hormone binding induces activation of a variety of signaling pathways.

Structurally, the steroid receptors share many common features [Evans, 1988], as shown in Figure 2. The receptors all contain carboxyl terminal ligand binding domains (LBD) that include a region termed activation function 2 (AF-2), which is a site for coactivator binding and thus is important for the induction of transcriptional activity. The LBD is linked to the DNA binding domain (DBD) by a hinge region (H) that contains a nuclear localization signal. The DNA binding domains are the most highly conserved regions of the receptors and each contains two Zn⁺⁺ binding motifs. The amino terminal domains (NTD) are the least conserved regions both in sequence and in length, but all contain at least one region, termed AF-1 (activation function 1), that is required for

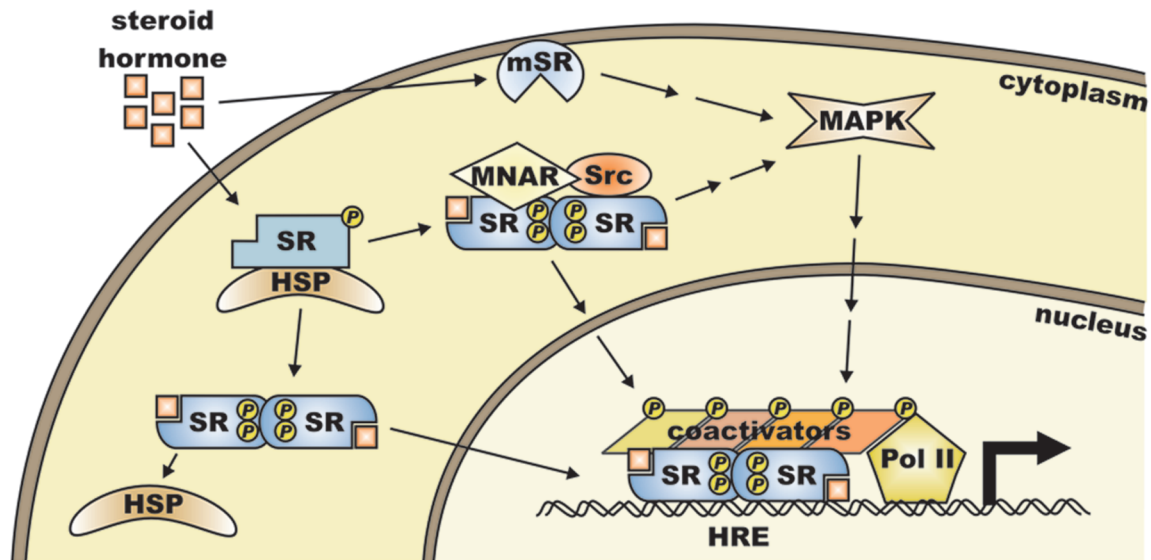


Figure 1. Mechanism of steroid hormone action. In the absence of hormone, steroid receptor monomers (SR) are associated with heat shock protein complexes (HSP) and are typically basally phosphorylated. Upon binding hormone, receptors dissociate from heat shock proteins, dimerize, bind to target gene-specific sites containing hormone response elements (HRE), and recruit a series of coactivator complexes to regulate target gene transcription. Site-specific phosphorylation of receptors increases subsequent to hormone binding, with some increases occurring rapidly, and others with delayed kinetics. Upon steroid binding, some receptors also interact with Src and MNAR, activating Src and downstream kinases including p42/p44 MAPK. Membrane-associated receptors (mSR) also bind hormone and initiate signaling cascades. While some of these are classical steroid receptors, others bear no homology to the steroid receptor superfamily.

optimal transcriptional activity. Most of the phosphorylation sites identified in steroid receptors are located in the NTDs of the receptors, although many have at least one phosphorylation site in the hinge region, and there are limited reports of phosphorylation sites in the hormone and DNA binding domains. Shown in Figure 2 are the best characterized phosphorylation sites in the human steroid receptors. Others have been proposed based on *in vitro* studies and additional sites have been identified in steroid receptors from other species. Consequently, there are likely to be additional unidentified sites in at least some of the human steroid receptors.

Several strategies have been used to elucidate the roles of cell signaling in steroid receptor action and in crosstalk between steroid receptors and growth factor signaling pathways. The first is simply to alter the activity of a signaling pathway using activators or inhibitors and to measure the effect on steroid receptor activity. Changes identified using this technique demonstrate a role for the pathway in regulating receptor action, but the target may be an associated protein rather than the receptor, or a combination of the receptor and other proteins. A second approach is to measure the activity of receptors containing alanine substitutions for one or more of the serine or threonine phosphorylation sites. This strategy requires knowledge of the location of the phosphorylation sites. While many have been located, others have yet to be identified. Finally, a number of studies in recent years

implicate steroid receptors in the direct activation of cell signaling pathways potentially modifying receptor function, as well as inducing activation of target genes sensitive to increased kinase activity.

Modulation of cell signaling pathways alters steroid receptor activity

Ligand-independent activation

The most striking evidence for the potential importance of cell signaling in steroid receptor action was the finding that some steroid receptors can be activated in the absence of measurable levels of hormones by treatments that enhance activity of kinases or inhibit phosphatase activities. Denner et al. first showed that 8-Br cAMP treatment of cells transfected with a chicken progesterone receptor (cPR) expression vector and a PR-responsive reporter caused hormone-independent, but cPR-dependent, activation of the reporter [Denner et al., 1990b]. This activation was independent of the four characterized phosphorylation sites in cPR and 8-Br cAMP did not induce phosphorylation of PR [Bai et al., 1997]. However, subsequent studies revealed that 8-Br cAMP caused activation of p42/p44 MAPK (mitogen-activated kinase) and increased phosphorylation of the p160 coactivator, SRC-1 (steroid receptor coactivator-1); these SRC-1 phosphorylation sites contributed to the ligand-independent activation [Rowan et al., 2000]. Other signaling pathways also induce

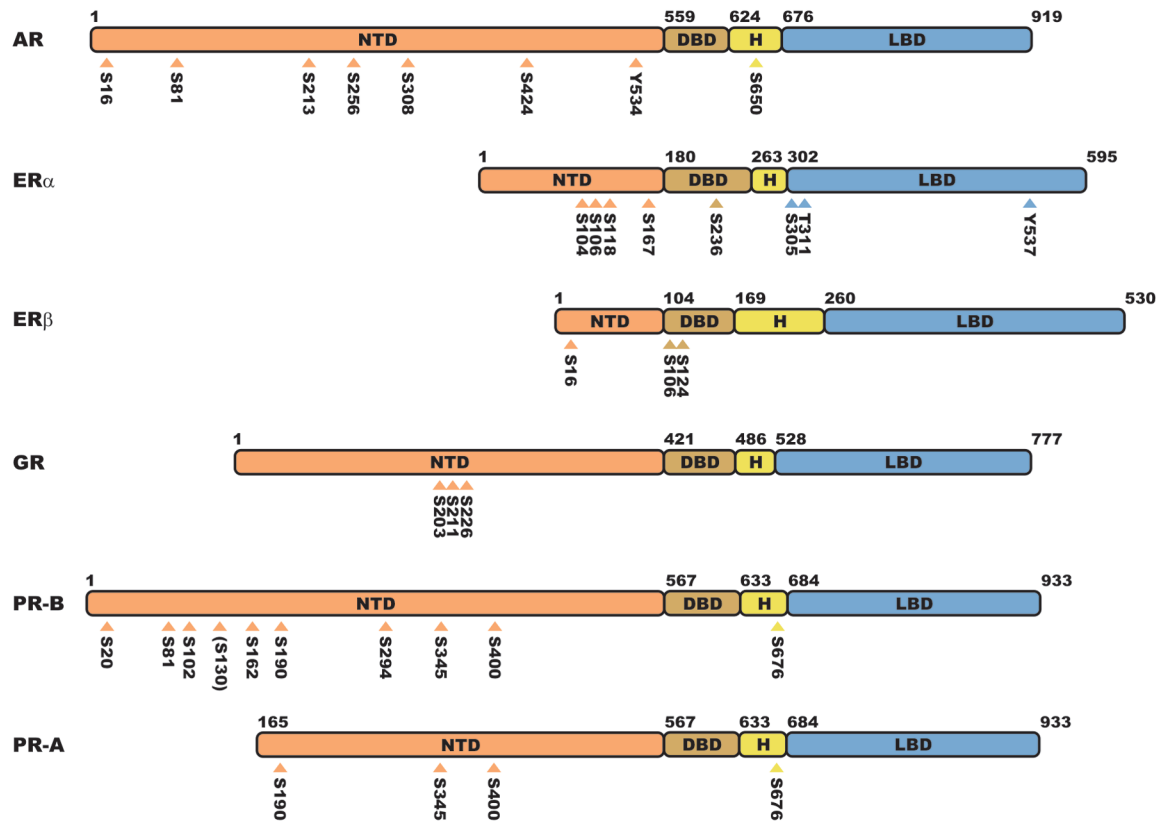


Figure 2. Domain structures of steroid receptors. The numbers of the amino acids found at the boundaries in the individual receptors between the NTD (amino-terminal domain), DBD (DNA binding domain), hinge region (H), and ligand binding domain (LBD) are indicated in the figure. Also shown are the best characterized phosphorylation sites in the human steroid receptors.

activity. Treatment of transiently transfected cells with dopamine activates a number of receptors including cPR [Power et al., 1991] and ER α (estrogen receptor α) [Smith et al., 1993] in the absence of their cognate ligands. Treatment with the phosphatase inhibitor, okadaic acid, also induces ligand-independent activation of cPR [Denner et al., 1990b]. There is evidence that these alternate activation pathways are also functional *in vivo*. For example, treatment with dopamine causes PR-dependent induction of lordosis in female rats and mice [Apostolakis et al., 1996; Mani et al., 1996; Mani et al., 1994; Mani et al., 2006]. As expected for a PR-dependent response, PR null mice are not responsive.

Estrogen receptor α

The responsiveness of receptors to cell signaling pathways in the absence of hormone differs greatly. Whereas the glucocorticoid receptor (GR) requires ligand for activation, ER α is very responsive to cell signaling pathways. Indeed, ER α in cells maintained in phenol red-free, charcoal-stripped serum, utilized to minimize/eliminate steroids, frequently displays substantial basal transcriptional activity in the absence of added ligand; this activity can be reduced by the use of a pure anti-estrogen such as ICI 182780, showing that the activity is dependent upon ER α [Smith et al., 1993].

There are multiple pathways for hormone-independent activation of ER α . One of the best characterized of the hormone-independent pathways for the activation of the

ER α is the EGF (epidermal growth factor)-dependent activation of human ER α in transfected HeLa cells. EGF-dependent activation induces phosphorylation of Ser¹¹⁸ in the amino terminus of ER [Kato et al., 1995]. Substitution of an alanine for the serine abrogates the hormone-independent activation. However, substitution of a glutamic acid, which provides a constitutive negative charge, restores responsiveness to EGF, but does not cause the receptor to become constitutively active [Bunone et al., 1996].

Thus, EGF-dependent activation requires phosphorylation of ER α at Ser¹¹⁸, as well as phosphorylation of at least one other target. One possibility is Ser¹⁶⁷ in ER α . EGF treatment typically results in activation of Rsk downstream of p42/p44 MAPK; Ser¹⁶⁷ is a substrate for Rsk [Clark et al., 2001; Joel et al., 1998]. Other pathways are independent of Ser¹¹⁸ phosphorylation (or the corresponding mouse Ser¹²²), demonstrating that there are multiple means of activating ER independent of its ligand [Patrone et al., 1998].

Androgen receptor

The androgen receptor (AR) also has the capacity to respond to cell signaling pathways in cells grown in medium depleted of hormone. Among the factors reported to induce AR activity are EGF, KGF (keratinocyte growth factor) [Culig et al., 1994], IL-6 (interleukin-6) [Hobisch et al., 1998], and forskolin [Nazareth and Weigel, 1996], an activator of protein kinase A. Overexpression of the

EGF receptor family member, HER2 (Human Epidermal Growth Factor Receptor 2), also induces AR activity [Craft et al., 1999]. Activation of AR by cell signaling pathways in the absence of normal levels of androgens likely contributes to the recurrence of prostate cancer. The recurrent tumors express high levels of AR and re-express many AR target genes including PSA (prostate specific antigen) (reviewed in [Agoulnik and Weigel, 2006]). Several investigators have demonstrated that AR-positive, androgen-independent cell lines that express PSA in medium depleted of androgens still require AR for cell growth and expression of PSA [Agoulnik et al., 2005; Zegarra-Moro et al., 2002]. Because the AR sequence does not differ from that of AR in androgen-dependent cells, it is presumed that altered cell signaling is causing aberrant receptor activation.

Potentiation of partial antagonist activity

Although some receptors show minimal responsiveness to cell signaling pathways in the absence of ligand, enhanced cell signaling often potentiates receptor activity in the presence of a partial antagonist. Treatment with 8-Br cAMP causes the mammalian PR and GR antagonist, RU486 (mifepristone), to act as a GR [Nordeen et al., 1993] and a PR-B isoform [Beck et al., 1993; Sartorius et al., 1994] specific agonist. The finding that cell signaling pathways can induce hormone-independent activation, can cause receptors to be responsive to lower levels of hormone, and can cause antagonists such as tamoxifen and RU486 to have agonist activity, is particularly relevant in hormone-sensitive cancers such as breast and prostate cancer. Signaling pathways that enhance AR activity are often activated in advanced prostate cancers [Gioeli et al., 1999; Gregory et al., 2004; Gregory et al., 2005; Mellinghoff et al., 2004] and HER2 signaling combined with overexpression of AIB1 is one contributor to tamoxifen resistance in breast cancer [Shou et al., 2004].

Modulation of agonist-dependent activity

The activities of specific kinases are also required for hormone-dependent activation of steroid receptors, although in many cases the targets of receptor action have not been fully elucidated. This is an active area of research and the studies are far from comprehensive. The response of receptors to signaling pathways can be cell-specific and it is likely that many of the responses will also be target gene-specific. Although there are some recent studies examining regulation of endogenous genes, most of the information has been obtained using transfected reporters.

Whereas cyclin-dependent kinases generally enhance the activity of steroid receptors, the actions of other kinases are receptor-specific. Surprisingly, some of the cyclins function as receptor coactivators or corepressors independent of their partner kinases. Only those actions that require kinase activity are discussed below. Signaling pathways that induce hormone-independent action or cause antagonist/agonist switches also potentiate the

corresponding hormone-dependent activity and have been discussed above.

Human progesterone receptor

Human PR requires Cdk2 (cyclin-dependent kinase 2) for hormone-dependent activation of at least some of its target genes [Narayanan et al., 2005a]. Inhibition of Cdk activity with the cyclin-dependent kinase inhibitor roscovitine blocks hormone-dependent induction of a PR-responsive reporter or of the endogenous metallothionein gene; reducing expression of Cdk2 also strongly inhibits PR activity. The Cdk2 partner, cyclin A₂, is a PR coactivator that depends on binding Cdk for its potentiation of PR activity. Although PR can be phosphorylated by cyclin A₂/Cdk2 *in vitro* [Knotts et al., 2001], the potentiation of PR activity is independent of PR phosphorylation [Narayanan et al., 2005a]. Rather, the target is likely to be the p160 coactivator, SRC-1. In ChIP (chromatin immunoprecipitation assays), roscovitine treatment has no effect on the binding of either PR or cyclin A to an MMTV (mouse mammary tumor-like virus) promoter, but it prevents the hormone-dependent recruitment of SRC-1 [Narayanan et al., 2005a]. Consistent with the idea that cyclin A/Cdk2 is important for PR function, PR-dependent activation of the MMTV promoter is cell cycle-dependent [Narayanan et al., 2005b]. PR activity is highest in S phase, where cyclin A is most highly expressed, and lower in early G₁ or in G₂/M phase. In cells with low levels of the cyclin-dependent kinase inhibitor, p27, overexpression of activated Cdk2 induces hormone-independent activation of PR and this hormone-independent activation is blocked by mutation of Ser⁴⁰⁰, a PR phosphorylation site [Pierson-Mullany and Lange, 2004].

Other kinases and phosphatases also modulate PR action, although their targets have not been elucidated. Treatment with EGF, resulting in activation of p42/p44 MAPK, enhances hormone-dependent activity [Daniel et al., 2007]. In contrast, activation of p38 MAPK by MKK6 inhibits PR activity [Proia et al., 2006]. Phosphatases also potentiate PR activity. The PP1 and PP2A inhibitor, okadaic acid, stimulates PR activity [Beck et al., 1992]. In contrast, overexpression of the phosphatase, PPM1D (p53-induced serine/threonine phosphatase, protein phosphatase 1D magnesium-dependent, delta isoform), enhances PR activity; reducing expression of endogenous PPM1D in MCF7 breast cancer cells inhibits PR activity [Proia et al., 2006].

ER α and ER β

Estrogen receptor activity is also modulated by cyclin A/Cdk2, but the activity is dependent upon the ability of the kinase to phosphorylate ER α [Rogatsky et al., 1999]. Signaling pathways that induce hormone-independent activation also potentiate hormone-dependent actions. There are additional reports of kinases regulating ER activity in specific cell types. In contrast to its effects on PR, activation of p38 MAPK enhances the activity of ER α and increases its nuclear localization in endometrial cells [Lee and Bai, 2002]. However, p38 MAPK has been reported to play a role in ERBB2/ERBB3-dependent

inhibition of ER β (estrogen receptor β) activity [St-Laurent et al., 2005]. GSK3 β (glycogen synthase 3 β) enhances ER α activity in neuronal cells [Mendez and Garcia-Segura, 2006]. Phosphatases also play a role in regulating activity. Overexpression of PP5 (protein phosphatase 5) reduces Ser¹¹⁸ phosphorylation in ER α and transcriptional activation. Moreover, reducing expression of PP5 enhances the transcriptional activity of ER α [Ikeda et al., 2004].

Glucocorticoid receptor

Studies of GR show that cyclin-dependent kinases, Cdk1 and Cdk2, can phosphorylate GR *in vitro* and that GR expressed in yeast lacking the corresponding kinase activity is less active than in a wild type strain [Krstic et al., 1997]. In contrast, a number of kinases inhibit GR activity. JNK (Jun N terminal kinase) inhibits rat GR activity through direct phosphorylation of GR [Rogatsky et al., 1998a]. Similarly, GSK3 inhibits rat GR through direct phosphorylation [Rogatsky et al., 1998b]; it has no effect on human GR, which lacks the corresponding phosphorylation site. Although p38 MAPK also inhibits GR activity, its effects are independent of the previously identified phosphorylation sites in GR [Szatmary et al., 2004]. Similar to ER α , PP5 is a negative regulator of GR activity [Zuo et al., 1999].

Androgen receptor

AR activity is also regulated by cyclin-dependent kinases. Similar to PR, overexpression of cyclin A enhances AR activity [Narayanan et al., 2005a]. Roscovitine reduces AR activity and expression [Chen et al., 2006]. Although roscovitine inhibits many cyclin-dependent kinases, studies with more specific inhibitors suggest that Cdk1 is the kinase required for optimal AR protein expression. Consistent with a different cyclin-dependent kinase requirement compared to PR, the cell cycle dependence of AR activity exhibits a different pattern, with a decrease in activity at the G1/S boundary [Martinez and Danielsen, 2002].

Cell signaling pathways regulate both the transcriptional activity and the stability of AR protein. Although inhibition of HER2 decreases AR protein levels and activity at low levels of hormone, the downstream kinase(s) responsible for regulating AR activity and expression has not been identified. HER2 activates Akt, but constitutively active Akt cannot compensate for inhibition of HER2 [Mellinghoff et al., 2004]. The role of Akt in AR action is controversial. Some investigators have found that Akt enhances AR activity, whereas others have reported that it inhibits AR activity [Lin et al., 2003; Lin et al., 2001; Taneja et al., 2005]. Thus, actions of Akt are likely to be context-dependent. Akt inhibits the activity of GSK-3, a kinase that has been reported to inhibit AR activity [Salas et al., 2004]. Thus, in cells with high levels of GSK-3 activity, activation of Akt through inhibition of GSK-3 may increase AR activity. Mitogen-activated protein kinases (MAPK) also modulate AR activity. MEK1, an upstream activator of p42/p44 MAPK, increases AR activity [Abreu-Martin et al., 1999]. In contrast, inhibiting either JNK or p38 MAPK using siRNA for their upstream

activators, MKK4 and MKK6, increases the expression of prostate specific antigen (PSA), an androgen-regulated gene in LNCaP prostate cancer cells [Gioeli et al., 2006].

Steroid receptor coregulators

In addition to directly modifying the receptors, there is increasing evidence that receptor activities are regulated by changes in the phosphorylation state of coactivators and corepressors. For example, the p160 coactivators are extensively phosphorylated and are targets of multiple signaling pathways. Sites in SRC-1 are important in potentiating hormone-independent activities of chicken PR and human AR [Rowan et al., 2000; Ueda et al., 2002]. Phosphorylation of SRC-1 also plays a role in the interaction between human PR and SRC-1 [Narayanan et al., 2005a]. The related p160 coactivator, TIF2 (transcription intermediary factor 2)/GRIP1 (glucocorticoid receptor interacting protein 1)/SRC-2, is phosphorylated by p42/p44 MAPK, and this phosphorylation plays a role in potentiating AR activity [Gregory et al., 2004]. Phosphorylation of GRIP1 by p38 MAPK enhances its potentiation of ER α activity [Frigo et al., 2006]. The third p160 coactivator, AIB1 (amplified in breast cancer 1)/SRC-3, plays a broader role in modulating the activities of multiple transcription factors. It is highly phosphorylated and different transcription factors, including steroid receptors, require phosphorylation of subsets of the phosphorylation sites for optimal potentiation of activity [Wu et al., 2002; Wu et al., 2004]. In some cases, the receptors themselves can alter phosphorylation of SRC-3. Estradiol treatment of ER α -containing cells increases the phosphorylation of SRC-3 at specific sites [Zheng et al., 2005].

Phosphorylation of corepressors also influences their ability to repress transcription. Phosphorylation of the corepressor, SMRT (silencing mediator of retinoid and thyroid hormone receptors), by MEK1 (MEK kinase 1), causes nuclear export, relieving repression [Jonas and Privalsky, 2004]. MEK1 also causes dissociation of N-CoR (nuclear receptor corepressor) complexes from androgen and estrogen receptors, enhancing transcriptional activity [Zhu et al., 2006]. Other coregulators are also phosphoproteins and altered cell signaling is likely to influence association and activities of these proteins as well.

Kinases as coactivators

The responsiveness of steroid receptor function to a variety of cell signaling pathways suggests that there may be means to preferentially enhance or inhibit the phosphorylation of components of steroid receptor pathways in addition to the changes induced by global changes in kinase/phosphatase activities. The simplest mechanism would be direct interactions of receptors or coactivators with kinases or phosphatases, enhancing proximity to other potential substrates in the pathway. Indeed, one of the first candidate coactivators identified, TIF1 α , is a protein kinase whose phosphorylation is enhanced upon interaction with activated nuclear receptors [Fraser et al., 1998]. A novel nuclear protein

kinase (ANPK) was identified as an AR-interacting protein that enhanced AR activity, but did not phosphorylate AR, leading to the conclusion that it acts through phosphorylation of AR coregulators or modification of chromatin proteins [Moilanen et al., 1998]. Other, better characterized kinases have also been found to be associated with nuclear receptors. AR interacts with Cdk9, a component of P-TEFb, a kinase that phosphorylates the CTD (carboxyl terminal domain) of Pol II, facilitating elongation of transcripts [Lee et al., 2001]. PR recruits cyclin A/Cdk2 to the promoter of target genes. Inhibition of its activity blocks transcription of an MMTV promoter and prevents recruitment of SRC-1, but not recruitment of another coactivator complex containing TRAP220 (thyroid receptor associated protein 220) [Narayanan et al., 2005a].

Receptors also interact with phosphatases, thereby limiting activity. AR interacts with small carboxyl terminal domain phosphatase 2, which reduces the transcriptional activity of AR [Thompson et al., 2006]. PP5 binds to ER α and ER β ; reducing PP5 expression enhances induction of a number of ER α target genes including c-myc and pS2 [Ikeda et al., 2004]. Other phosphatases and kinases are brought to receptor complexes by proteins that interact with receptors. For example, I κ K (I κ B kinase) associates with SRC-3, a substrate for the kinase; the kinase regulates the activity of the coactivator [Wu et al., 2002]. Kinase inhibitor studies show that Ser¹¹⁸ is at least in part phosphorylated by I κ K α [Weitsman et al., 2006]. Thus, binding of SRC-3 to ER α may facilitate the hormone-dependent phosphorylation of Ser¹¹⁸. The phosphatase, PP5, interacts with GR through hsp90 complexes and reduces GR phosphorylation. Reducing expression of PP5 has target gene-specific effects on GR activity [Wang et al., 2007] ranging from no detectable effect to substantial inhibition of hormone-induced transcription.

Receptor phosphorylation

Identification of phosphorylation sites

Early attempts to identify phosphorylation sites in steroid receptors relied on radiolabeling followed by direct protein sequencing or comparisons of the radiolabeling patterns of wild type and mutant receptors [Bodwell et al., 1991; Denner et al., 1990a; Le Goff et al., 1994; Zhang et al., 1997; Zhang et al., 1995; Zhou et al., 1995]. More recent approaches to the identification of sites have included mass spectrometry [Gioeli et al., 2002; Knotts et al., 2001].

All of the steroid receptors contain multiple phosphorylation sites. The receptors are partially phosphorylated in the absence of hormone and are more highly phosphorylated after hormone treatment. Some of the sites exhibit enhanced phosphorylation in response to hormone, while phosphorylation of others is almost exclusively hormone-dependent. Most of the sites are serines or threonines in the amino terminal regions of the receptors, although there is a positionally-conserved Ser-Pro or Thr-Pro in the hinge regions of the steroid

receptors. This site has been shown to be phosphorylated in chicken [Denner et al., 1990a] and human PR [Knotts et al., 2001], mouse ER α [Lahooti et al., 1995], and human AR [Zhou et al., 1995]. Whether it is also phosphorylated in other receptors has not yet been determined. Most of these sites are located in Ser/Thr-Pro motifs, indicating that the receptors are direct targets of proline-directed kinases including the cyclin-dependent kinases and the MAPK family, which is consistent with studies showing that these kinases modulate receptor activity. Sites identified as authentic *in vivo* sites in the human steroid receptors are shown in Figure 2.

Some of the phosphorylation sites in steroid receptors are conserved across species, whereas others are unique to specific species. There is a GSK3 β phosphorylation site in rat GR that is absent in human GR [Rogatsky et al., 1998b]. Chicken PR contains four Ser-Pro phosphorylation sites [Denner et al., 1990a; Poletti and Weigel, 1993] and all are in the regions common to the PR-B and PR-A isoforms. In contrast, human PR is highly phosphorylated. There are a number of phosphorylation sites common to the PR-A and PR-B isoforms, but PR-B contains at least four additional phosphorylation sites in its unique amino-terminus [Knotts et al., 2001]. Although Ser²⁹⁴ is in the primary sequence of both PR-A and PR-B, studies with a phosphorylation site-specific antibody reveal that Ser²⁹⁴ is phosphorylated only in the PR-B isoform [Clemm et al., 2000]. The two isoforms have different biological activities and this difference may reflect unique conformations in the amino-termini of the two isoforms; alternatively, a protein may bind to the PR-A isoform, thereby occluding the site.

In addition to the Ser/Thr-Pro sites, sites in other consensus sequences have been identified. Additional receptor sites include Ser¹⁶⁷ in ER α , a casein kinase II consensus site [Arnold et al., 1994] that is also phosphorylated by Rsk [Joel et al., 1998], as well as by Akt [Shah and Rowan, 2005], and a site in PR (Ser⁸¹), which is in a consensus CKII site and can be phosphorylated by CKII *in vitro* [Zhang et al., 1994]. Additional sites are detected when specific cell signaling pathways are activated. For example, activation of p38 MAPK causes phosphorylation of Thr³¹¹ in ER α [Lee and Bai, 2002] and activation of Protein Kinase A (PKA) induces phosphorylation of Ser³⁰⁵ in ER α [Al-Dhaheri and Rowan, 2007]. In cells with high Akt activity, AR Ser²¹³ is phosphorylated in response to DHT (dihydrotestosterone); this phosphorylation is inhibited by the PI-3K inhibitor, LY294002, suggesting that the phosphorylation is Akt-dependent [Taneja et al., 2005].

In addition to the Ser/Thr phosphorylation sites in steroid receptors, there is limited evidence to support tyrosine phosphorylation. In ER α , phosphorylation of Tyr⁵³⁷ in the hormone binding domain has been described, although others have not detected phosphorylation of this site, suggesting that the stoichiometry of phosphorylation may be low and/or it is only phosphorylated under specific conditions [Al-Dhaheri and Rowan, 2006; Arnold et al., 1995]. Two recent studies have shown that Src can

phosphorylate AR on Tyr⁵³⁴ in the amino-terminus of AR and there is some evidence that other tyrosines can be phosphorylated to a lesser extent [Guo et al., 2006; Kraus et al., 2006]. Consistent with the failure to detect tyrosine phosphorylation of AR previously, an analysis of AR in LNCaP cells revealed that tyrosine phosphorylation in response to hormone was a rapid and transient event. Using a phosphorylation site-specific antibody, Guo et al. have shown that the site is phosphorylated in AR in prostate tumors [Guo et al., 2006].

Applications of phosphorylation site-specific antibodies

Because each receptor is multiply phosphorylated, identifying the kinases that phosphorylate the individual sites *in vivo* has been challenging. Site-specific antibodies have been made for a number of the sites and some are now commercially available. Studies with these antibodies reveal that some sites are targets for multiple kinases, allowing the receptors to respond to a variety of stimuli. For example, Ser¹¹⁸ in ER α is phosphorylated by p42/p44 MAPK as a result of EGF treatment, but cyclin H/Cdk7 phosphorylates the site in response to hormone treatment [Chen et al., 2000; Chen et al., 2002]. Similarly, p42/p44 MAPK phosphorylates Ser²⁹⁴ in PR-B in response to EGF, but hormone-dependent phosphorylation of the site occurs despite blocking p42/p44 MAPK activation [Narayanan et al., 2005b].

Site-specific antibodies have also been useful in resolving whether candidate sites are authentic *in vivo* sites. Initial studies in LNCaP prostate cancer cells failed to identify Ser²¹³, a consensus Akt phosphorylation site in AR, as an authentic *in vivo* phosphorylation site [Gioeli et al., 2002]; however, Ser²¹³ is phosphorylated by Akt *in vitro* [Lin et al., 2001]. Subsequent studies with a site-specific antibody revealed that Ser²¹³ is phosphorylated in wild type AR, but in a cell type-specific manner [Taneja et al., 2005]. Interestingly, Ser²¹³ is phosphorylated in AR in LAPC-4 prostate cancer cells. The LNCaP AR contains an amino acid mutation in the ligand binding domain (T877A). Surprisingly, when wild type and mutant ARs were expressed by transient transfection, the wild type receptor displayed much more Ser²¹³ phosphorylation than did the mutant [Taneja et al., 2005]. Thus, the AR in LNCaP cells likely is minimally phosphorylated on this site, consistent with a failure to detect phosphorylation by direct analyses of phosphopeptides.

Phosphorylation site-specific antibodies to phosphorylated Ser¹¹⁸ and Ser¹⁶⁷ in ER α have been used to examine breast cancer specimens. Some studies show a correlation between phosphorylation of Ser¹¹⁸ and a favorable response to Tamoxifen [Murphy et al., 2004], as well as a correlation between Ser¹⁶⁷ phosphorylation and response to endocrine therapy [Yamashita et al., 2005]. The studies to date are limited and not all studies have found such correlations (reviewed in [Murphy et al., 2006]). Phosphorylation of Ser¹¹⁸ also correlated with PR expression, suggesting that the phosphorylation is an indication that the ER α is active in those samples and that an ER α antagonist is likely to be beneficial.

Phosphorylation and receptor function

Transcriptional activation

Initially, studies of the role of phosphorylation in receptor function were limited to measuring transcriptional activity of transiently transfected reporters. More recent studies have revealed that receptor phosphorylation contributes to a variety of functions including receptor stability, nuclear localization, transcriptional activity, interaction with coregulators, and other activities such as splicing. In some cases, the phosphorylation may determine whether a subsequent posttranslational modification will occur. As better assays are developed, it is probable that additional functions will be identified.

Using transient transfection assays to measure the activities of wild type and mutant receptors, Bai and Weigel found that an amino-terminal site in chicken PR (Ser²¹¹) was required for optimal transcriptional activity and that the hinge site was required for response to low levels of hormone [Bai et al., 1994; Bai and Weigel, 1996]. Analyses of human PR phosphorylation site mutants revealed modest reductions in transcriptional activity [Takimoto et al., 1996]. In contrast, an Ala²⁹⁴ PR-B mutant stably transfected into a PR-negative breast cancer cell line was much less active than the corresponding line containing a stably transfected wild type receptor, suggesting that cell context and/or stable rather than transient transfection alters responsiveness [Daniel et al., 2007; Qiu and Lange, 2003]. Transient transfection assays of the activities of ER α and AR phosphorylation mutants also revealed some decreases in activity [Le Goff et al., 1994; Zhou et al., 1995].

Additional roles for receptor phosphorylation

Analyses of individual receptor functions have revealed more specific roles for phosphorylation. Mutation of the phosphorylation sites in GR increases receptor stability [Webster et al., 1997]. A GR-interacting protein, TSG101, preferentially interacts with hypophosphorylated GR and protects it from degradation [Ismaili et al., 2005]. Substitution of an alanine for Ser²⁹⁴ in human PR increases receptor stability. PR is degraded by the proteasome pathway and elimination of the phosphorylation site decreases ubiquitination of PR, suggesting that the phosphorylation may serve as a signal for ubiquitination [Lange et al., 2000]. In ER β , phosphorylation of Ser¹⁶ enhances ER degradation [Cheng et al., 2000]. This serine is also a site for O-GlcNAc modification [Cheng et al., 2000; Cheng and Hart, 2001]. The competing modifications likely regulate the stability of ER β .

Phosphorylation can enhance or inhibit protein/protein interactions. For example, phosphorylations in the amino terminus of ER β induced by p42/p44 MAPK enhance SRC-1 association and induce ligand-independent activation [Tremblay et al., 1999]. In contrast, activation of Akt reduces interaction between ER β and CBP. Mutation of Ser²⁵⁵ in ER β restores CBP binding and blocks inhibition of ER β activity by this signaling pathway [Sanchez et al., 2007].

Phosphorylation also plays a role in regulating nuclear localization of receptors. Phosphorylation of Thr³¹¹ in ER α increases nuclear localization [Lee and Bai, 2002]. In contrast, stress kinase-induced phosphorylation of Ser⁶⁵⁰ in AR enhances cytoplasmic localization [Gioeli et al., 2006]. Phosphorylation of Ser²⁹⁴ is required for EGF-induced nuclear localization of human PR-B, but not for hormone-dependent nuclear localization [Qiu et al., 2003]. Phosphatase activity is also required for GR relocation to the nucleus, although the target of the phosphatase is unknown [Dean et al., 2001; Galigniana et al., 1999]. Thus, phosphorylation plays a role in subcellular distribution of most of the steroid receptors.

Phosphorylation and ER α function

The role of phosphorylation in the regulation of receptor functions has been most extensively characterized for ER α and these studies highlight the wide range of kinases that can phosphorylate a receptor and the diverse roles for phosphorylation in regulating receptor function. Eight phosphorylation sites in ER α (serines 104, 106, 118, 167, 236, and 305, Thr311 and Tyr537), distributed throughout the receptor (see Figure 2), have been identified and antibodies have been prepared to study their phosphorylation [Al-Dhaheri and Rowan, 2006]. Phosphorylation of serines 104, 106, and 118 has been implicated in optimal interaction with several coactivators [Dutertre and Smith, 2003; Likhite et al., 2006].

Phosphorylation of Ser¹¹⁸ appears to be particularly important for physical and/or functional interactions with a variety of coregulators. It has been implicated in the interaction with (SF)3a p120, a splicing factor, and the potentiation of splicing [Masuhiro et al., 2005]. On the other hand, phosphorylation of this site enhances interaction with the estrogen receptor repressor SPBP (stromelysin-1 platelet-derived growth factor-responsive element-binding protein) [Gburcik et al., 2005]. Ser¹⁶⁷ phosphorylation plays a role in optimal DNA binding *in vitro* and in binding to endogenous promoters *in vivo* [Castano et al., 1997; Likhite et al., 2006; Shah and Rowan, 2005]. Ser²³⁶ phosphorylation has been implicated in inhibition of hormone-independent dimerization and DNA binding, but this inhibition is overcome by the addition of estradiol [Chen et al., 1999]. Phosphorylation of Ser³⁰⁵ increases transcriptional activity and has been implicated in preventing acetylation of Lys³⁰³ [Cui et al., 2004].

Interestingly, Lys³⁰³ is often mutated to Arg in breast cancer and this mutation has been associated with hypersensitivity to estrogen [Fuqua et al., 2000]. As discussed above, phosphorylation of Thr³¹¹ enhances nuclear translocation [Lee and Bai, 2002]. The role of phosphorylation of Tyr⁵³⁷ has been controversial [Yudt et al., 1999]. Mutation of the site alters estradiol binding kinetics and some amino acid substitutions promote hormone-independent activation [Weis et al., 1996]. However, these changes may not be a reflection of the phosphorylation states. *In vitro* phosphorylation with Src, which phosphorylates Tyr⁵³⁷, as well as at least one other tyrosine, enhances affinity for estradiol [Likhite et al.,

2006]. As more sophisticated means of measuring receptor functions are developed, it is likely that additional roles for site-specific receptor phosphorylation will be identified.

Activation of cell signaling pathways by steroid hormones

There is abundant evidence that steroids can activate a variety of cell signaling pathways that influence transcription and enzyme activities independent of the genomic activities of the receptors [Watson and Lange, 2005] and, as discussed above, these pathways also modulate receptor function. These actions are mediated by steroid receptor family members, as well as by other proteins that respond to steroids. A detailed discussion of this aspect of steroid action is beyond the scope of this review. However, these pathways offer a potential means of autoregulation of the transcriptional activity of the receptors. For example, AR and ER interact with Src and MNAR (modulator of nongenomic actions of the estrogen receptor) [Haas et al., 2005; Wong et al., 2002]. Hormone binding results in activation of Src and the downstream activation of p42/p44 MAPK, kinases that regulate receptor function through phosphorylation of coactivators and, in some cases, phosphorylation of the receptors themselves. PR directly interacts with Src family tyrosine kinases through a proline-rich motif in the NTD of PR, which also activates a kinase cascade [Boonyaratanakornkit et al., 2001]. PR also induces long term activation of p42/p44 MAPK through induction of Wnt-1 and the resulting activation of the EGF receptor [Favre and Lange, 2007].

Summary

Cell signaling pathways that regulate phosphorylation of steroid receptors and their coactivators are critical factors in determining the activities of steroid receptors under different physiological conditions. While many of the phosphorylation sites in the steroid receptors and some of the sites in the coregulators have been identified, others are still unknown. In particular, there are likely to be sites that are phosphorylated only in response to a specific signaling pathway; these phosphorylations may be transient, but important for specific biological responses. A number of additional candidate sites have been identified by *in vitro* phosphorylation studies. Some of these sites likely are authentic targets under some circumstances, while others may be artifacts of the conditions (high concentrations of purified receptor and kinase with no competing substrates) and are not authentic *in vivo* phosphorylation sites. Moreover, because phosphorylation studies using high specific activity γ [³²P] ATP can yield a substantial signal despite a low percent or stoichiometry for the phosphorylation, some signals may be due to phosphorylation of a small amount of denatured receptor. The sites shown in Figure 2 are restricted to those that have been shown to be phosphorylated in receptors expressed in mammalian or insect cells. As additional antibodies are generated for

candidate sites, it is likely that others will be established as genuine sites.

Some sites are targets of multiple kinases, thereby permitting integration of signals from multiple pathways. Studies under special conditions (for example, serum-free medium or activation of a kinase pathway through a stress signal) can identify a pathway or kinase capable of phosphorylating a specific site, but these experiments should be interpreted with caution. Under other circumstances, another kinase may be the dominant regulator of the site. Although roles for some phosphorylation sites have been identified, many others have not been characterized. Roles for specific sites may also be context-dependent. This is suggested by the role of Ser¹¹⁸ not only in recruiting proteins that contribute to ER α activation, but also in the recruitment of a repressor of ER α activity. It is likely that there are phosphorylation “codes” dependent upon the signaling pathways activated under a specific physiological condition that determine the function of the receptor. These alterations will determine the phosphorylation of specific subsets of receptor phosphorylation sites, as well as the level of phosphorylation of coregulators.

While some functions may depend upon a single phosphorylation, others will have more complex requirements. For example, EGF-dependent activation of ER α requires Ser¹¹⁸ phosphorylation [Bunone et al., 1996]; although substitution of a negatively-charged glutamic acid restores responsiveness to EGF, the Glu¹¹⁸ mutant is not constitutively active. Therefore, at least one additional EGF-induced modification of receptor or other proteins is required. In the case of AR, mutation of Ser⁶⁵⁰ in the hinge region of AR has been reported to reduce AR transcriptional activity by about 30% [Zhou et al., 1995], but this site is also the target of JNK phosphorylation [Gioeli et al., 2006] and in the context of activated JNK, phosphorylation of this site reduces AR activity. As better assays for individual receptor functions are devised and more phosphorylation site-specific antibodies for receptors and coregulators are developed, it is likely that many additional roles for phosphorylation in receptor function will be identified.

Studies of the regulation of endogenous target genes by phosphorylation site mutants are likely to reveal target- and perhaps tissue-specific requirements for phosphorylations. In contrast to simple transiently transfected reporters with multiple hormone response elements, natural targets require receptor interactions with a variety of other transcription factors and coregulators to appropriately modify chromatin and induce transcription. The contributions of individual phosphorylation sites to overall biological function can be tested for conserved sites by generating mice with individual amino acid substitutions and examining their phenotypes. There is evidence that many, but not all, phosphorylation sites are conserved. Thus, differential phosphorylation is also a potential regulator of species-specific actions of receptors.

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