REVIEW ARTICLE

Steroid hormone receptors and their regulation by phosphorylation

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The steroid/thyroid hormone receptor superfamily of ligand-activated transcription factors encompasses not only the receptors for steroids, thyroid hormone, retinoids and vitamin D, but also a large number of proteins whose functions and/or ligands are unknown and which are thus termed orphan receptors. Recent studies have highlighted the importance of phosphorylation in receptor function. Although most of the phosphorylation sites are serine and threonine residues, a few of the family members are also phosphorylated on tyrosine. Those steroid receptor family members that are bound to heat-shock proteins in the absence of ligand typically are basally phosphorylated and exhibit increases in phosphorylation upon ligand binding. Most of these sites contain Ser-Pro motifs, and there is evidence that cyclin-dependent kinases and MAP kinases (mitogen-activated

protein kinases) phosphorylate subsets of these sites. In contrast, phosphorylation sites identified thus far in members of the family that bind to DNA in the absence of hormone typically do not contain Ser-Pro motifs and are frequently casein kinase II or protein kinase A sites. Phosphorylation has been implicated in DNA binding, transcriptional activation and stability of the receptors. The finding that some of the steroid receptor family members can be activated in the absence of ligand by growth factors or neurotransmitters that modulate kinase and/or phosphatase pathways underscores the role of phosphorylation in receptor function. Hence this family of transcription factors integrates signals from ligands as well as from signal transduction pathways, resulting in alterations in mRNA and protein expression that are unique to the complex signals received.

INTRODUCTION

The steroid/thyroid hormone receptors are members of a very large family of nuclear ligand-activated transcription factors that includes the steroid receptors [those for progesterone (PRs), androgen (ARs), oestrogen (ERs), glucocorticoids (GRs) and mineralocorticoids] and receptors for thyroid hormone, retinoids and vitamin D, as well as an even larger group of proteins termed orphan receptors whose ligands and/or function are as yet unknown [1,2]. These receptors play key roles both as transcriptional activators and as repressors in all aspects of biological function, including regulation of development, metabolism and reproduction.

Functionally, the receptor family can be divided into two groups based on their interactions or lack of interactions with heat-shock proteins, as well as on their tendency to form homodimers or heterodimers. Figure 1 shows a simplified model of the mechanisms of action of the two sides of the family. In the absence of hormone, steroid receptors are associated with preformed complexes of proteins which include hsp90 (heat-shock protein of 90 kDa) and hsp70 [3]. These complexes may be cytoplasmic or loosely associated with the nucleus. Studies from several laboratories suggest that, in the absence of ligand, the receptors shuttle between the cytoplasm and nucleus, with the relative amount of cytoplasmic receptor dependent upon the receptor type [4,5]. Thus in the absence of hormone the GR [6] and perhaps AR [7] appear to be predominantly cytoplasmic, whereas the PR and ER are predominantly nuclear [4,5,8]. Ligand binding leads to dissociation of the heat-shock proteins, receptor dimerization and binding to a specific steroid response

element whose consensus sequence is an inverted repeat separated by three nucleotides. GRs, PRs, ARs and mineralocorticoid receptors all bind to the same consensus sequence (AGAACA_{nnn}TGTTCT), whereas ERs bind to the sequence AGGTC_{nnn}TGACCT [9]. Although these consensus sequences have been identified, there are clearly other sequences to which the receptors bind alone or in combination with other proteins [10–14]. The receptor dimer then interacts with basal transcription factors [15], other DNA-binding proteins [16,17] and co-activators, resulting in transcription of the target gene.

In contrast, as shown in the lower portion of Figure 1, the thyroid hormone, retinoid, vitamin D and orphan receptors that have been studied all appear to be tightly bound to the nucleus at specific sites in the DNA in the absence of ligand. These proteins typically form heterodimers with retinoid X receptors (RXRs) [18–20] rather than homodimers, and these receptor forms recognize sequences that match the ER half site with variations in nucleotide spacing and the direction of the repeated elements (for a review of DNA binding specificity, see [13]). Ligand and DNA binding may also cause dissociation of repressor molecules [21,22], allowing association with general transcription factors, other DNA-binding proteins and/or co-activators, resulting in transcription of the target gene.

While much remains to be learned about this family of proteins, a great deal is known about steroid receptor structure and function; various aspects have been the subjects of previous reviews (for a comprehensive review of receptor structure and function, see [23]). Of special interest are the very recent studies that reveal the complexity of the regulation of receptor function and cross-talk with other transcription factors and signalling

Figure 1 Activation of steroid/thyroid hormone receptors

The upper portion of the Figure depicts activation of the steroid receptor members of the family. In the absence of hormone, each receptor (R) monomer is associated with a heat-shock protein complex (hsp). These complexes may be cytoplasmic or loosely bound in the nucleus. Binding of steroid (S) causes a conformational change resulting in dissociation of the heat-shock complex and allowing dimerization of the receptor and DNA binding to a hormone response element (HRE) to produce a transcriptionally productive complex. The lower part of the Figure describes the action of the non-steroid receptors (R'). In the absence of ligand these proteins are bound to their hormone response elements, typically as heterodimers with RXRs (RX) or as homodimers, or in a few cases as monomers, and repress transcription of the target gene. Binding of hormone (H) changes the conformation so that the receptor can form an active transcription complex.

pathways. These regulatory mechanisms, which include phosphorylation, interaction with other steroid receptor family members, interactions with other transcription factors and interactions with co-activators and co-repressors, are the subject of this review.

STEROID RECEPTOR STRUCTURE AND FUNCTION

The steroid/thyroid receptor family is characterized by three regions of homology; these consist of a highly conserved DNA-binding domain that contains two Zn²⁺-containing motifs, and two less highly conserved regions in the C-terminal region of the protein. The region N-terminal of the DNA-binding domain

exhibits the greatest variability with respect to both length and sequence similarity [24].

Shown in Figure 2 are the locations of the functional domains of a typical receptor. Although all receptors contain DNA- and hormone-binding domains, the remaining functions are a composite from studies of various receptors, and some members may lack specific functional regions. Deletion studies suggested that the portion of the receptors required to maintain ligand binding was surprisingly large (about 25 kDa) for a steroid ligand [25]. Affinity labelling studies of GRs and PRs [26,27] confirmed that the steroid interacts with amino acids that are more than 100 residues apart in the primary structure. The hormone-binding domain also contains regions important for dimerization [28]. X-

Figure 2 Location of functional regions in a typical steroid receptor

A/B represents the region N-terminal to the DNA-binding domain; C contains the DNA-binding domain; D contains the hinge region; E contains the ligand-binding domain; F is a region originally found in ER that can be deleted without eliminating hormone binding.

ray crystallographic structures of the DNA-binding domains of the GR [29] and the ER [30] revealed that this region is important both for DNA binding and for receptor dimerization.

Both the N-terminal and C-terminal regions of steroid receptors contain sequences important for trans-activation (reviewed in Sartorius et al. [31]). These were initially termed TAF-1 and TAF-2, and more recently have been renamed AF1 and AF2. The human PR B form is unique in that it contains a third activation function at the extreme N-terminus, termed AF3 [31].

Other receptor functions include a nuclear localization signal, which is in the hinge region between the DNA- and hormone-binding domains [32]. This region and the C-terminal region are important for binding of heat-shock proteins [33].

Binding of hormone initiates a series of events that result in activation or repression of target genes. Several lines of evidence demonstrate that hormone binding changes the conformation of the receptor. Studies from Gorski's laboratory initially showed that ligand binding changed the hydrophobicity of the ER, implying a major conformational change [34]. Allan et al. [35], using *in vitro* translated ³⁵S-labelled PRs, showed that binding of hormone caused the ligand-binding domain to become protease-resistant. Using an antibody that reacts with the C-terminal 14 amino acids of the PR, Weigel et al. [36] demonstrated that this region is accessible in the unliganded receptor, but is not recognized once the agonist, R5020, is bound. Finally, a comparison of the three-dimensional structure of the ligand-binding domain of the retinoic acid receptor (RAR) bound to ligand with the RXR in the absence of ligand suggests that a helical region moves to cover the ligand upon binding [37,38].

That ligand binding significantly alters the structure of receptors was also shown by functional studies that include the work of Smith [39], who has shown that binding of progesterone blocks reassociation of the PR with heat-shock proteins, and by the observation that, for some steroid receptors, ligand binding either is required for DNA binding [40] or enhances DNA binding [41].

The mechanism of action of antagonists has been of interest both mechanistically and clinically for many years. Antagonists are either steroidal or non-steroidal compounds that compete with agonists for binding and prevent activation of receptors. Although it was initially assumed that the binding sites for agonists and antagonists are the same, there is now evidence that they can be different, although overlapping. Vegeto et al. [42] showed that a human PR mutant lacking the C-terminal 42 amino acids no longer bound R5020, but still bound the

antagonist RU486. Moreover, RU486 acted as an agonist for this mutant. A comparison of partial protease patterns of agonist-bound versus antagonist-bound PRs revealed that a smaller limit fragment lacking the C-terminal amino acids was produced when antagonist was bound [35]. The antibody C262, which does not interact with agonist-bound receptor, does interact with antagonist-bound receptor, suggesting that different conformations are induced [36]. Many of these antagonists induce dissociation from heat-shock proteins and binding to DNA; in some cases these compounds act as partial agonists. It is thought that the conformation of the antagonist-bound receptor blocks productive interaction with one or more proteins needed to induce transcription. As described below, likely candidates for this function are the recently discovered co-activators. A second class of antagonists (e.g. ZK98299, a PR antagonist, and flutamide, an AR antagonist) fail to promote DNA binding efficiently [40,43]. One of the ER antagonists, ICI 164,384, seems to function in part by reducing the stability of the ER [44]. Thus there appear to be multiple mechanisms by which antagonists can inhibit receptor action. All antagonists identified to date are synthetic compounds. An important question yet to be answered is whether there are naturally occurring antagonists for these proteins with physiologically important functions.

REGULATION OF RECEPTOR FUNCTION BY MEANS OTHER THAN PHOSPHORYLATION

Multiple forms of steroid receptor family members as a means of regulating the response to a steroid

In most avian and mammalian species, the PR is expressed as two major DNA-binding forms, PR-A and PR-B [45–47]. The exception is the rabbit receptor, which is expressed only as the larger B form [48]. Both forms are expressed from the same gene, either from different mRNAs [49] or by alternative initiation of translation [50]. The human PR-B contains an additional 164 amino acids at the N-terminus [49], and this region contains an additional trans-activation domain [31]. Both forms are biologically active, although their relative ability to induce target genes differs [51,52]. A third form, the C receptor, which contains the C-terminal hormone-binding domain and a portion of the DNA-binding domain, was detected more recently [53].

A and B forms have recently been described for the AR [54]. In contrast with the PR, the A form of the AR is expressed at substantially lower levels than the B form [54], and its contribution to androgen action is currently being evaluated.

The GR also is expressed as two forms, α and β , that are produced by alternative splicing [55]. The α form is the classical GR and is activated by glucocorticoids. The β form differs at the C-terminus, does not bind glucocorticoids and can repress the activity of the GR α form [55]. The relative expression and importance of the β form has yet to be determined.

In addition to the variants described above, numerous splice variants, particularly of the ER, have been described [56]. For the most part, these consist of receptors lacking one of the exons found in the full-length ER. They have primarily been detected at the mRNA level, and their expression and role *in vivo* is an active subject of investigation.

Receptor variants and heterodimerization play key roles in the regulation of the receptors that do not interact with heat-shock proteins. There are multiple forms of RARs and RXRs that arise from distinct genes [57–59], as well as splice variants of these genes. There are also multiple forms of the thyroid hormone receptor [60–62].

Although some of the receptor family members that do not interact with heat-shock proteins are capable of forming homodimers, many of these proteins function as heterodimers with RXRs. Although RXRs can form homodimers, they apparently play a larger role as heterodimer partners. Thus the response of a receptor such as the vitamin D receptor will depend in part upon its RXR partner. The ligand response of the heterodimers is dependent both upon the heterodimer pair and upon the DNA response element. For example, the retinoid X ligand, 9-*cis*-retinoic acid, can inhibit the response to the ligand of the heterodimer partner, have no effect on its response, or stimulate the response [63,64]. The cellular complement of receptors, the ligands present and the DNA response elements will dictate the response of individual cells to these receptors.

Regulation of receptor function by physical or functional interactions with other proteins

Numerous studies utilizing artificial as well as natural promoters have demonstrated that the activity of a steroid receptor will in part be determined by proteins that bind to nearby or even overlapping DNA-binding sites. In the simplest case of two adjacent steroid hormone response elements, both the DNA binding of receptor dimers [65] and the resulting transcription of a target gene are greatly enhanced compared with a single steroid response element. In the complex mouse-mammary-tumour-virus (MMTV) promoter, which contains a number of near-consensus sequences as well as half sites, mutation of any of these full or half sites reduces the transcriptional activity [13]. Using a series of artificial promoters containing a single steroid response element and single sites for other transcription factors such as NF1 or SP-1, Schule et al. [16] showed that both GRs and PRs functionally synergize with these factors to enhance transcription. Other types of interactions are typified by the pfl promoter, which contains a negative glucocorticoid response element; binding of the GR can inhibit or stimulate transcription of this gene depending upon whether a Fos-Jun or Jun-Jun complex occupies the adjacent AP-1 site [66,67].

Steroid receptors interact with other transcription factors such as AP-1 [68,69] and RelA [70] independent of DNA binding, and also reciprocally affect each other's function. The less active form of the PR, PR-A, can repress the activity of PR-B [71,72]. Moreover, McDonnell's group has shown that PR-A also can repress the activity of GRs, mineralocorticoid receptors and even ERs [71,73]. Kraus et al. [74] have also reported inhibition of the ER by the PR. Because PRs and ERs bind to different response

elements, this inhibition must be through a mechanism other than competition for a DNA-binding site.

Two important classes of interacting proteins, co-activators and co-repressors, have recently been described. Co-activators bind to the receptor itself, and presumably serve as bridges to the general transcription factors. Those that interact with the C-terminal portion of the receptors [75–77] have been characterized as interacting with receptors only in the presence of agonist. Co-repressors have been identified that interact with some of the non-heat-shock-binding members of the family [21,78]. The proteins that have been characterized interact with the receptor in its hinge region and typically are released upon binding of ligand.

Clearly, regulation of steroid receptor function is a complex phenomenon involving many factors and many steps. Any or all of these are targets for regulation by phosphorylation, as outlined below.

PHOSPHORYLATION OF THE STEROID/THYROID RECEPTOR SUPERFAMILY

Although the steroid/thyroid hormone receptors are a family of ligand-activated receptors, they are also phosphoproteins and their functions are regulated by phosphorylation. Studies of other transcription factors have shown that phosphorylation can play roles in nuclear translocation, DNA binding (either positive or negative), interactions with other proteins and trans-activation (for a review, see [79]). Transcription factors are frequently multiply phosphorylated and, as in the case of c-Jun, may contain phosphorylations that enhance activity as well as others that reduce activity [80]. That the phosphorylation and dephosphorylation reactions are accomplished by multiple enzymes indicates that the cell can alter the activity of the protein in response to different signalling pathways. Although steroid receptor phosphorylation has not been as well characterized as that of some of the other transcription factors, recent studies have revealed that regulation of receptor activity by phosphorylation is complex and is involved in multiple aspects of receptor action. The finding that some of the steroid receptors can be activated by stimulation of kinase pathways in the absence of hormone (reviewed below), and that these pathways can alter responses to antagonists, confirms the importance of phosphorylation in the activity of steroid receptors.

Analysis of receptor phosphorylation

That the steroid receptors are phosphoproteins has been known for many years [81,82]. Phosphopeptide mapping studies of chicken [83], human [84,85] and rabbit [86,87] PRs showed that the proteins are multiply phosphorylated and that, in the case of the chicken [83] and human [84,88] PRs, novel sites are phosphorylated in response to hormone treatment. For the chicken [83] and human [84,88] PRs, this enhanced phosphorylation is accompanied by a change in mobility on SDS/PAGE gels. The ER [89–91] and GR [92] also exhibit enhanced phosphorylation in response to hormone treatment. Studies of the AR have yielded conflicting results. Analysis of endogenous AR in LNCaP cells showed enhanced phosphorylation of the receptor in response to androgen [93], whereas AR expressed in COS cells showed no net increase in phosphorylation per molecule, but rather a general enhancement of the stability of the receptor [7]. Sites have been identified by direct characterization of phosphorylation sites in endogenous receptors, as well as by

recombinant DNA technology. In some cases the results have been consistent; in others they are conflicting.

Phosphorylation of PRs

Using ^{32}P -labelled oviduct tissue minces as a source of chicken PR (cPR), Denner et al. [83] first identified three phosphorylation sites by isolating and sequencing phosphotryptic peptides. A fourth site was identified subsequently by Poletti and Weigel [94]. Figure 3 shows the location of the four phosphorylation sites in the cPR. All four are in the region common to the PR-A and PR-B receptors and contain the consensus sequence Ser-Pro within the phosphorylation sites. Subsequent studies of other steroid receptors (see below) revealed that the majority of the phosphorylation sites in these receptors contain Ser-Pro sequences, suggesting that the proline-directed kinases, which include cyclin-dependent kinases [95], mitogen-activated protein (MAP) kinases and stress-activated protein kinases [96,97], play roles in receptor phosphorylation. As shown in Figure 3, three of the sites are in the N-terminus, which has been identified as a region important for transcriptional activation, and the fourth is in the region between the DNA-binding and hormone-binding domains (the hinge region). Two of the N-terminal sites, Ser²¹¹ and Ser²⁶⁰, are phosphorylated in the absence of hormone and their phosphorylation is increased in response to hormone treatment [83], whereas the other sites, Ser⁵³⁰ and Ser³⁶⁷, are detected after hormone treatment [83,94]. Of particular interest is the hinge-region site, Ser⁵³⁰. All of the steroid receptors contain either a Ser-Pro or a Thr-Pro at essentially the same position [83]. This site is also phosphorylated in the AR [98], and an analogous site has been reported in the mouse ER [99].

Phosphorylation of the cPR expressed in yeast (*Saccharomyces cerevisiae*) is indistinguishable from phosphorylation of the endogenous chicken oviduct receptor [100]. More recent studies have shown that the cPR expressed in CV1 cells is also identical with the endogenous receptor [101], so that, in this case, expression in different systems results in comparable phosphorylation patterns.

The roles of some of these sites have been examined using site-directed mutagenesis to replace the target serines with alanine. Substitution of an alanine for Ser²¹¹ blocks the hormone-dependent change in mobility on SDS/PAGE gels [102], demonstrating that phosphorylation of this site is required for the mobility shift. A comparison of the activity of the Ala²¹¹ mutant with wild-type receptor in cells transiently co-transfected with a receptor expression vector and a reporter showed that the activity of the mutant receptor was reduced in a cell- and promoter-specific manner [102]. The most significant reduction was observed in HeLa cells, where the mutant exhibited 25% of wild-type activity when a simple promoter consisting of two progesterone/glucocorticoid response elements and a TATA box from the E1b gene was linked to the coding region for chloramphenicol acetyltransferase. In contrast, receptor activity was reduced only slightly when the mutant was tested in CV1 cells using a complex promoter containing a portion of the thymidine kinase promoter as well as two progesterone response elements. This cell- and promoter-specific variation in activity has been observed for other steroid receptors (see below), suggesting that these sites are involved in interactions with proteins whose expression may be cell-type-specific. A model of this mechanism is presented in Figure 4.

A very different phenotype was detected when Ser⁵³⁰ in the hinge region was replaced with alanine. Although the activities of the wild-type and mutant receptors were essentially identical at saturating concentrations of hormone, the activity of the mutant

was several-fold less than wild-type at 0.1 nM progesterone [103]. That this decrease in activity was not a result of a change in hormone-binding affinity was shown by hormone binding assays both *in vitro* and in whole cells. This led to a model suggesting that this phosphorylation maintains the receptor in an activated state such that dissociation of hormone does not promote rebinding of the receptor to heat-shock protein complexes.

Phosphorylation of the human PR has not been characterized as thoroughly. Using peptide mapping of ^{32}P -labelled receptor isolated from T47D breast cancer cells, Sheridan et al. [84] initially showed that the phosphorylation of the human PR was substantially more complicated than that of the cPR, and that the patterns for PR-A and PR-B differed. Subsequent studies using a DNA-binding mutant suggested that some of the hormone-dependent phosphorylation of PR may also be DNA-dependent [104]. Using T47D breast cancer cells labelled with ^{32}P as a source of PR, Zhang et al. [85] identified three phosphorylation sites found only in the B form of the receptor. All reside within the N-terminal region unique to this form. Additional peptide mapping studies revealed that there are a number of basal phosphorylation sites whose phosphorylation is enhanced within a few minutes of hormone administration, and three sites that are phosphorylated slowly in response to hormone [88]. One of these is a PR-B-specific site. All five of the sites that have been identified are N-terminal to the DNA-binding domain; four contain the consensus sequence Ser-Pro, whereas the other site contains a casein kinase II consensus sequence [85]. *In vitro* phosphorylation studies suggest that one site is a casein kinase II site [85] and that three basal sites are substrates for cyclin A-CDK2 (cyclin-dependent kinase 2) complexes [105]. Based on the peptide mapping studies, as many as five additional sites are as yet unidentified.

Beck et al. [106] compared the effects of agonists and antagonists on PR phosphorylation and found that treatment with RU486 caused phosphorylation of the same hormone-dependent sites as did that with the agonist R5020. However, treatment with the pure antagonist ZK98299 failed to induce the hormone-dependent phosphorylations [106]. Hence treatment with some antagonists does not promote normal phosphorylation.

Other than a preliminary report that mutation of two potential phosphorylation sites (which have not yet been confirmed as authentic phosphorylation sites) results in reduced transcriptional activation [107], no studies of human PR phosphorylation-site mutants have been published. Consequently the roles of the many sites in the human PR remain to be determined.

Phosphorylation of GRs

Bodwell et al. [108] have identified seven phosphorylation sites in the mouse GR; all of the sites are in the region N-terminal to the DNA-binding domain, and many contain Ser-Pro sequences. One of the sites is a threonine and other sites may be phosphorylated either by casein kinase II or by other unidentified kinases. Hormone treatment increases phosphorylation at all sites, but the phosphorylation of one site, Ser²²⁰, is preferentially increased [109]. Hu et al. [110] have shown that GRs overexpressed in Chinese hamster ovary cells undergo cell-cycle-dependent changes in phosphorylation, with basal phosphorylation highest in G2/M and hormone-dependent phosphorylation most evident in S phase. Hsu and De Franco [111] found that the relative phosphorylation of GR phosphorylation sites in cells synchronized in G2 differed from the phosphorylation in unsynchronized cells; these receptors are transcriptionally inactive, but are still capable of acting as repressors.

Figure 3 Location of phosphorylation sites in the cPR

The structure of the cPR and the location of the four phosphorylation sites within the receptor are shown. As indicated by the internal start for PR-A, all four sites are found in the region common to PR-A and PR-B. The presence and size of the 'P' denotes the state and extent of phosphorylation in the absence and presence of progesterone.

Mason and Housley [112] were the first to characterize the activity of steroid-receptor phosphorylation-site mutants. In these studies, the activity of mouse GRs having alanine replacing one or more of the phosphorylation sites in the receptor was measured in COS cells using MMTVCAT (a portion of the MMTV long tandem repeat-linked coding sequence for chloramphenicol acetyltransferase) as a reporter. Under these con-

ditions, none of the single mutants showed a significant alteration in activity, but a receptor lacking the five sites conserved in mouse, rat and human GRs showed a small (22%), but statistically significant, reduction in activity. More recent studies by Jewell et al. [113] have suggested that phosphorylation may affect GR stability and thus alter receptor activity.

Phosphorylation of ERs

In contrast with the studies of PRs and GRs, where individual laboratories have identified the phosphorylation sites, several laboratories have contributed to the identification of phosphorylation sites in the ER. At present, these studies have led to conflicting identifications. Auricchio's group first reported that the ER contains phosphotyrosine [114] and subsequently identified the site as Tyr⁵³⁷ in the ligand-binding domain [115]. Arnold et al. [116] have also identified Tyr⁵³⁷ as a phosphorylation site in the human ER. Other groups have not detected phosphotyrosine, but have detected serine phosphorylation [91,117].

Several groups have identified serine phosphorylation sites. Three separate groups studying phosphorylation of ERs transiently expressed in COS cells identified Ser¹¹⁸, a site containing the consensus sequence Ser-Pro, as the major hormone-dependent phosphorylation site [90,91,118]. Mutation eliminates the change in mobility detected on SDS gels as a result of hormone treatment *in vivo* [118]. The corresponding site, Ser¹²², has been identified in the mouse ER [99]. However, although Arnold et al. [119] identified Ser¹¹⁸ as a phosphorylation site in the endogenous ER from MCF-7 cells, they reported that Ser¹⁶⁷, a casein kinase II site, was the major hormone-inducible site [120]. Lahooti et al. [99] have found that the corresponding mouse site, Ser¹⁷¹, may also be phosphorylated, and reported two additional sites, Ser¹⁵⁶ and Ser¹⁵⁸, which are located in sites that contain neither Ser-Pro

Figure 4 A potential role for Ser²¹¹ and other receptor phosphorylation sites that exhibit cell- and/or promoter-specific changes in activity

As shown in the upper portion of the Figure, the phosphorylation induces an interaction with a protein that forms a bridge between the general transcription factors and the receptor.

sequences nor a casein kinase II consensus sequence. LeGoff et al. [91] have also identified either Ser¹⁰⁴ or Ser¹⁰⁶ (both Ser-Pro motifs) as phosphorylation sites in human ERs expressed in COS cells, and found no evidence for phosphorylation of Ser¹⁶⁷ or Ser²⁹⁴, which corresponds to the hinge site found in the mouse ER. Although the differences between the phosphorylation patterns of human ERs expressed in COS and MCF-7 cells [91,120] may be a result of cell-specific phosphorylation, the phosphorylation of the recombinant baculovirus-expressed ER mirrors that of the MCF-7 cell ER [120]. Additional sites remain to be identified in the ER expressed in COS cells [91], as well as in the MCF-7 ER [120]. These discrepancies may be resolved once the identification of the sites is completed.

The activities of several of these sites have been examined by site-directed mutagenesis. Ali et al. [90] reported that mutation of Ser¹¹⁸ to Ala reduced the activity of the ER to as little as 25 % of wild type, but that the decrease was cell- and promoter-specific and in some cases was much smaller. LeGoff et al. [91] found that mutation of Ser¹¹⁸ alone only marginally affected activity, but mutation of the three serines at positions 104, 106 and 118 reduced activity to about 50 %. Lahooti et al. [99] reported that mutation of Ser¹²² or Ser²⁹⁸ had little effect on the transcriptional activity of the wild-type mouse ER, but mutation of Ser¹²² in the presence of a mutated Af2 region substantially decreased the transcriptional activity.

In addition to studies showing that phosphorylation regulates transcriptional activation, others suggest that DNA binding is affected. Denton et al. [89] found that treatment of ERs with phosphatase decreased DNA binding. Arnold et al. [116] have subsequently reported that phosphorylation of Tyr⁵³⁷ is important for dimerization and for DNA binding, and have also shown that phosphorylation of Ser¹⁶⁷ enhances DNA binding [119]. Taken together, these studies suggest that at least DNA binding and transcriptional activation of the ER are modulated by phosphorylation.

Kato et al. [121] and Arnold et al. [119] have both shown that Ser¹¹⁸ can be phosphorylated *in vitro* by MAP kinase. Kato et al. [121] have further shown that activation of MAP kinases enhances ER phosphorylation *in vivo* and that this phosphorylation is on Ser¹¹⁸. Expression of ras, an intermediate in the MAP kinase pathway, stimulates the activity of wild-type ERs, but not of a Ser¹¹⁸ mutant. Joel et al. [118] reported that phorbol ester treatment also stimulated phosphorylation of Ser¹¹⁸. Taken together with the *in vitro* evidence that Ser¹⁶⁷ is a casein kinase II site [120] and the finding that Tyr⁵³⁷ can be phosphorylated by Src kinase *in vitro* [116], it is evident that the phosphorylation of the ER is regulated by multiple kinases.

Phosphorylation of ARs

The AR is phosphorylated on multiple sites, but little is known about its phosphorylation. The receptor is detected as a doublet of 110 kDa and 112 kDa on SDS/polyacrylamide gels, and it is the 112 kDa species that is phosphorylated [122]. ARs expressed in COS cells exhibit hormone-independent phosphorylation [7], whereas a specific hormone-induced peptide was detected in ARs from LNCaP cells [122]. Zhou et al. [98] have identified three phosphorylation sites in ARs expressed in COS cells. All three, Ser⁸¹, Ser⁹⁴ and Ser⁶⁵⁰, are in Ser-Pro motifs; these studies indicate that additional sites remain to be identified. Mutation of Ser⁶⁵⁰ to Ala reduces the activity of the receptor by approx. 30 %.

Other receptor family members

The other receptors that have been examined are also phosphoryl-

ated, but their phosphorylation patterns are different from those of the steroid receptors. The sites that have been identified to date are not Ser-Pro sites. Ser²⁰⁵ (Ser²⁰⁸ depending upon the numbering system) is the major hormone-dependent phosphorylation site in the vitamin D receptor [123,124]. This site is a casein kinase II site. Interestingly, when this site is mutated to glycine, the activity of the receptor is reduced slightly [125], but when it is mutated to alanine the activity is not affected [123,125]. Analysis of the alanine substitution mutant revealed that alternative phosphorylation occurred at a nearby site [123]. Co-expression of the vitamin D receptor and casein kinase II stimulated the activity of the wild-type receptor and of the alanine mutant, but not of the glycine mutant [125].

Analysis of thyroid hormone receptor phosphorylation is incomplete. Thyroid hormone receptors are phosphorylated [62,126–129] and phosphorylation enhances DNA binding [128,129]. On the other hand, DNA binding of the TR2 variant, which does not bind ligand, is negatively regulated by phosphorylation [62].

The RARs are all phosphoproteins, but a number of findings suggest that the phosphorylation patterns of the different receptors, and even of splice variants, differ. Interestingly, RAR β 1 and RAR β 3 show enhanced phosphorylation in response to ligand, but RAR γ and RAR β 2 do not [130]. RAR β , in contrast with the others, contains a phosphotyrosine [130]. Ser³⁶⁹ in RAR α 1 is phosphorylated in COS-1 cells in response to increased protein kinase A activity, and *in vitro* by purified protein kinase A, but is not phosphorylated in the absence of protein kinase A stimulation [131]. Additional phosphopeptides were also detected in the COS-1-expressed receptor. Finally, Lefebvre et al. [132] have shown that dephosphorylation decreases the DNA binding of both RAR α and RXR α .

Little is known about the phosphorylation of orphan receptors. However, the phosphorylation of Nur77(NGFIB) (nerve growth factor-inducible protein-B) is dependent upon the activator utilized; activation by nerve growth factor produces a phosphorylation pattern distinct from that found as a result of membrane depolarization [133,134]. Davis et al. [135] have reported that one of the sites phosphorylated *in vivo*, Ser³⁵⁴, is phosphorylated *in vitro* by pp90^{rk}; this site is in a region important for DNA binding and may affect the DNA binding of the protein.

STEROID RECEPTORS AND SIGNAL TRANSDUCTION PATHWAYS

The steroid receptors have classically been viewed as ligand-activated transcription factors. With the surprising discovery that modulation of kinase activity in cells can cause activation of some of the steroid receptor family members in the absence of hormone [136,137], it became apparent that there are alternative means of activating some of these receptors. Since then, many examples of cross-talk between steroid receptor pathways and other signal transduction pathways have been described.

Denner et al. [136] first reported that cPR-A, co-transfected with a reporter gene into CV1 cells, can be transcriptionally activated by treatment of the cells with 8-bromo-cAMP (an activator of protein kinase A) or with okadaic acid (an inhibitor of phosphatases 1 and 2A). Subsequent studies showed that activation of membrane-receptor-mediated signal transduction pathways by dopamine [138] or by epidermal growth factor (EGF) [139] also activated the cPR.

There is also indirect evidence that some of the endogenous mammalian PRs can be activated in the absence of progesterone, both in cells and in animals. Turgeon and Waring [140] found

that a gonadotropin-releasing-hormone pathway can activate the PR in rat pituitary cells. Mani et al. [141] have shown that dopamine-induced lordosis in ovariectomized rats can be blocked by progesterone antagonists, implying that dopamine activates the PR. Despite these studies, an analysis of the human PR revealed that, although 8-bromo-cAMP stimulated the hormone-dependent activation of the PR in T47D breast cancer cells, the PR was not activated in a ligand-independent manner [142]. However, the combination of the antagonist RU486 with 8-bromo-cAMP caused receptor activation and the antagonist to act as an agonist [40,143]. In contrast, the antagonist ZK98299, which does not promote DNA binding as effectively as RU486, remained inactive in combination with 8-bromo-cAMP. This antagonist/agonist switch appears to be rather specific, in that activation of protein kinase C with phorbol esters does not induce the switch, although it does stimulate agonist-dependent transcription. Subsequent studies by Sartorius et al. [144] showed that only the B form of the receptor responds to 8-bromo-cAMP and RU486.

That the ER can be activated by signal transduction pathways in the absence of ligand was first suggested by the study of Ignar-Trowbridge et al. [137], showing that EGF treatment of ovariectomized mice resulted in nuclear translocation and altered phosphorylation of the ER, as judged by a change in mobility of the receptor on SDS/polyacrylamide gels. Moreover, EGF-stimulated uterine DNA synthesis was reduced by treatment with the anti-oestrogen ICI 164,384. Subsequent studies in transiently transfected Ishikawa endometrial adenocarcinoma cells confirmed that EGF can activate the ER and that the N-terminal portion of the receptor was more important for this activation than the C-terminus [145]. Moreover, Auricchio et al. [146] found that treatment of MCF-7 cells with vanadate, a phosphotyrosine phosphatase inhibitor, stimulated tyrosine phosphorylation of the ER and enhanced cell growth. The stimulation of growth was blocked by ER antagonists, suggesting that vanadate acts through the ER.

Other signalling pathways also activate and/or change the phosphorylation of the ER. Rat uterine ERs can be activated in the absence of oestrogen by cAMP and by insulin-like growth factor-1, and these treatments enhance the phosphorylation of the receptor [147].

A number of studies have examined the ligand-independent activation of the human ER. This receptor can be activated by dopamine [148] and by EGF [147]. Other studies have shown that the ability to exhibit ligand-independent activation and the level of the activation is to some extent dependent on the promoter and cell context, so that in some cases little or no ligand-independent activation is detected, but there is substantial stimulation of hormone-dependent activity. In addition, these signalling pathways substantially modify the response to agonists and antagonists [149,150]. Treatment of MCF-7 breast cancer cells with activators of protein kinase A in the presence of antagonist induces transcriptional activity; the magnitude of the response is promoter-dependent [150]. Of particular interest is the finding that some mutants that cannot be activated by oestrogen alone are activated by treatment with 8-bromo-cAMP in combination with either agonists or antagonists [151].

Despite a number of attempts to demonstrate ligand-independent activation of the GR, it appears that this receptor requires a ligand for activation. Similar to the human PR, the receptor activity is stimulated by agents that activate protein kinase A and protein kinase C pathways [152]. This activation is not accompanied by changes in GR phosphorylation [152]. The GR also exhibits an antagonist-to-agonist switch in response to 8-bromo-cAMP, but not to phorbol esters [153].

Attempts to induce ligand-independent activation of the AR have led to mixed results. Some investigators have failed to detect ligand-independent activation, but have seen stimulation of hormone-dependent activity [154,155]. However, Culig et al. [156] have reported that the AR can be activated by growth factors, and that the magnitude of the response is dependent upon the growth factor as well as the promoter.

Taken together, these studies suggest that some of the steroid receptor family members are activated in the absence of ligand, but their ability to induce transcription in response to these signals will be highly dependent upon the cellular context and on the target genes.

SUMMARY

The studies summarized above demonstrate that the level of receptor protein and of its cognate hormone is only one factor in determining the final biological response. The relative abundance of the receptor relative to other receptor forms, as well as to that of other transcription factors and co-activators or co-repressors, will affect the resulting activity. In addition, the sequence, location and number of receptor-binding sites and adjacent protein-binding sites will affect the level of induction of a specific target gene within the cellular milieu. These factors presumably control the cell- and/or tissue-specific responses to hormones, and all of these interactions are potential targets for regulation by receptor phosphorylation.

Many of the phosphorylation sites in the receptors have been identified, and initial analyses indicate that at least DNA binding and transcriptional activation are modified substantially by phosphorylation. The regulation of receptors by phosphorylation is complex, and individual phosphorylations can act either to enhance or to inhibit the activity of the receptor. As with most sites in transcription factors, none of the sites in the receptors that bind ligand has been found to function as an on/off switch. That the receptors are multiply phosphorylated by several different kinases suggests that subsets of sites may subserve specific roles that are dependent upon the physiological state of the cell. Moreover, it is probable that the current methods used to assay receptor function fail to measure fully the roles of the individual phosphorylation sites. The activity of receptor mutants is typically measured in transient transfection assays, with both an artificial promoter and a receptor expression plasmid being introduced into cells that do not normally express the receptor. Since most transfection procedures are inefficient, receptors are typically overexpressed in the successfully transfected cells in order to measure their activity. This creates two potential problems. First, the higher level of receptor may partially mask changes in affinity for DNA or for other proteins. Secondly, the important interacting proteins may be in limiting quantities in these cells. That this is a problem is evident from studies of receptors that form heterodimers with the RXRs. Although most cells contain endogenous RXRs and some activity is obtained when a receptor such as the vitamin D receptor is expressed, this activity is typically much higher if additional exogenous RXR is provided.

A second limitation in evaluating fully the role of phosphorylation is the use of artificial promoters transiently introduced into cells. Under these conditions, both the context of the natural gene and the structure imposed by chromatin are lacking. As techniques for measuring steroid receptor function in a more natural context are developed, the importance of the individual phosphorylation sites will be more clearly defined.

The finding that some of the steroid receptors can be activated

Figure 5 Steroid receptor activation

The upper portion depicts the classical steroid (S)-dependent activation pathway. The lower portion depicts signals emanating from receptors such as the dopamine receptor or growth factor receptors that alter the kinase/phosphatase balance of the cell such that the receptors are activated in the absence of ligand. L represents a ligand binding to a membrane receptor that acts through a cAMP pathway. Y-P indicates the active form of a growth-factor receptor. R, receptor; hsp, heat-shock protein complex.

in the absence of hormone has forced a re-evaluation of steroid receptor action and the interaction of steroid receptors with other signal transduction pathways. A model depicting the two pathways of steroid receptor activation is presented in Figure 5. In the upper portion is the classical hormone-dependent pathway showing that binding of ligand to receptor in the heat-shock protein complex initiates a series of steps that results in an active liganded receptor homodimer binding to DNA and inducing transcription. The lower portion shows signals generated by membrane-bound receptors such as those for dopamine or growth factors following a signal cascade that results in activation of the receptor in the absence of ligand. Although there is evidence in the case of the ER that the phosphorylation of the receptor is altered during this process, the role of this phosphorylation in the activation process remains to be determined. In addition to the receptor, the heat-shock protein complex associated with the unliganded receptor, as well as proteins that interact with receptor to produce the active transcription complex, are likely targets for these signalling pathways. Major questions yet to be answered include the mechanism by which receptors become active in the absence of ligand, and the relative importance of this pathway *in vivo*. Nonetheless, these studies highlight the importance of the interactions between signal transduction cascades and steroid hormone receptors in producing a biological response.

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