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REVIEW

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Ligand-independent activation of steroid hormone receptors

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Abstract In addition to the conventional hormone-dependent regulation of the activity of steroid/thyroid receptor family members, many studies have shown that there is substantial cross-talk between signal transduction pathways and steroid receptors. In a number of cases the modulation of kinase/phosphatase activity in cells leads to activation of steroid receptors in the absence of hormone. This novel mechanism may not be ubiquitous as the glucocorticoid receptor appears to be refractory to activation in the absence of hormone. However, estrogen receptors, progesterone receptors, androgen receptors, retinoic acid receptors, retinoid X receptors, and vitamin D receptors all exhibit ligand-independent activation under appropriate conditions. Whether a steroid receptor responds to a signal by inducing transcription of a target gene in the absence of hormone depends upon the cell type, promoter, and activator. The mechanism(s) by which ligand-independent activation is induced is currently a subject of great interest. Because the signals that activate receptors induce protein phosphorylation, altered phosphorylation of the receptors, and/or proteins that associate with the receptors are likely to be key to ligand-independent activation. In the case of the estrogen receptor there is good evidence that altered receptor phosphorylation plays a role in ligand-independent activation. Other likely targets are proteins in the heat shock protein complexes, corepressors, and/or coactivators of steroid receptors.

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Key words Ligand-independent · Estrogen receptor · Progesterone receptor · Androgen receptor · Phosphorylation

Abbreviations *AR* Androgen receptor · *CAT* Chloramphenicol acetyltransferase · *CHO* Chinese hamster ovary · *CT* Cholera toxin · *EGF* Epidermal growth factor · *ER* Estrogen receptor · *ERE* Estrogen response element · *GR* Glucocorticoid receptor · *IBMX* 3-Isobutyl-1-methylxanthine · *IGF* Insulin-like growth factor · *PK* Protein kinase · *PR* Progesterone receptor · *PRE* Progesterone response element · *PSA* Prostate-specific antigen · *RAR* Retinoic acid receptor · *RXR* Retinoid X receptor · *tk* Thymidine kinase · *TOT* *trans*-Hydroxytamoxifen · *vit* Vitellogenin

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Introduction

The steroid receptors belong to a large family of ligand-activated transcription factors that include the classical

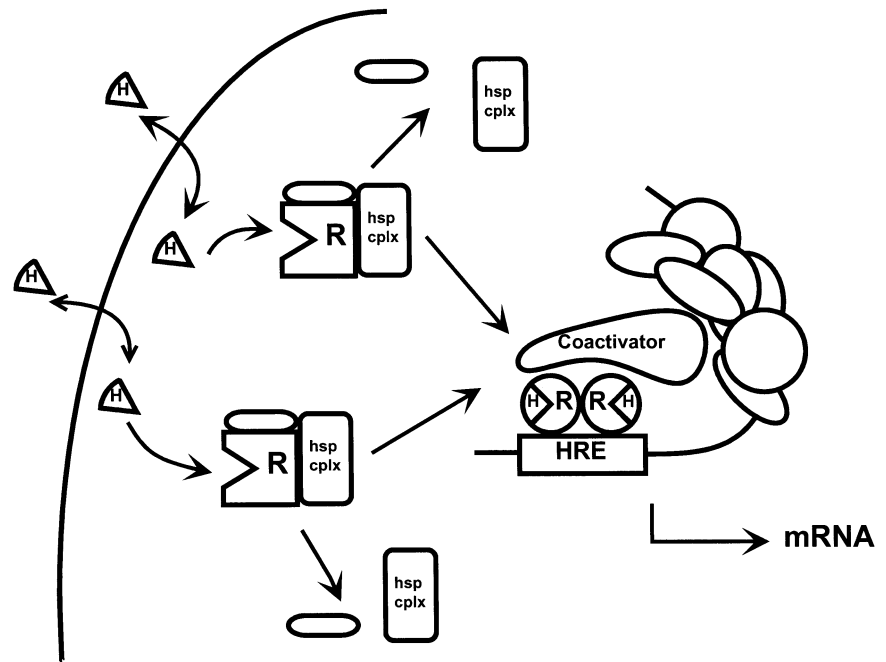

Fig. 1 Activation of steroid hormone receptors. In the absence of hormone, receptor monomers (*R*, receptor monomer) are associated with a preformed heat shock protein complex (*hsp cplx*) as well as other proteins (*oval* associated with the receptor). Hormone (*H*) diffuses into the cell, binds to the receptor resulting in dissociation of associated proteins, dimerization of the receptor and binding to target DNA sequences (*HRE*). Other proteins including coactivators and transcription factors bind to the DNA and to the receptor producing a transcriptionally active complex

steroid receptors as well as the thyroid, retinoid, and vitamin D receptors [1, 2]. Also included are many proteins that have been termed orphan receptors because their ligands and/or functions have yet to be identified [2]. All of the family members contain carboxyl terminal ligand-binding domains that are also important for receptor dimerization [3] (for a review of steroid receptor structure see [4]). Also common to all family members are DNA-binding domains containing two Zn finger motifs that are located amino terminal of the hormone-binding domain. The amino termini of the receptors are extremely variable with respect to both length and sequence; this region is important for transcriptional activation and/or repression (reviewed in [5]).

The receptors can be separated into two classes based on their association with other proteins in the absence of hormone. Figure 1 shows the classic ligand-dependent activation of the steroid/thyroid hormone receptor family members that interact with heat shock proteins. Included in this group are the estrogen (ER), progesterone (PR), androgen (AR), glucocorticoid (GR), and mineralocorticoid receptors. In the absence of hormone each receptor monomer is associated with a protein complex that contains hsp90 as well as a number of other proteins [6, 7]. This receptor complex is incapable of binding to DNA and is either cytoplasmic or loosely bound in the nucleus. The steroid diffuses into the cell, binds to the ligand-binding domain of the receptor inducing a conformational change that favors dissociation of the protein complex [7] and tight binding to DNA. The receptors bind as homodimers to specific steroid response elements that consist of inverted palindromes separated by three nucleotides [8]; they then interact with basal transcription factors [9], coactivators [10], and other transcription factors to induce and/or repress transcription of the target gene.

Receptors such as the thyroid hormone, retinoic acid, and vitamin D receptors that do not interact with the heat shock proteins bind to DNA in the absence of ligand repressing the transcription of the target gene [4] (Fig. 2). These receptors typically form heterodimers with retinoid X receptors [11–13]; however, some orphan receptors can act as monomers. Recent studies indicate that in the absence of ligand, these receptors interact with repressor proteins [14, 15] such as NCoR, blocking the activity of the dimer. Ligand-binding induces dissociation of the corepressor, allowing interaction with other transcription factors and coactivators which results in induction of the target gene.

Most if not all of the members of the nuclear receptor family are phosphoproteins (reviewed in [16]). Consequently the fact that the functions of these proteins are regulated by phosphorylation and by signal transduction pathways is not surprising. However, the finding that some of the steroid receptor family members can be activated by modulation of signal transduction pathways in the absence of hormone is contrary to the models depicted in Fig. 1 and 2 showing that the ligand plays a central, irreplaceable role in receptor function. Although dual regulation of target genes by signal transduction pathways and by steroid receptor had been noted, it was originally assumed that these were separate pathways leading to induction of the same target gene.

With the cloning of the steroid receptors it became possible to determine whether a pathway is receptor dependent or receptor independent by transfecting the receptor into cells lacking receptor and measuring the resulting activity. Denner et al. [17] first found that the chicken PR can be activated in the absence of hormone in CV1 cells cotransfected with an expression plasmid for cPR and a reporter, progesterone response element

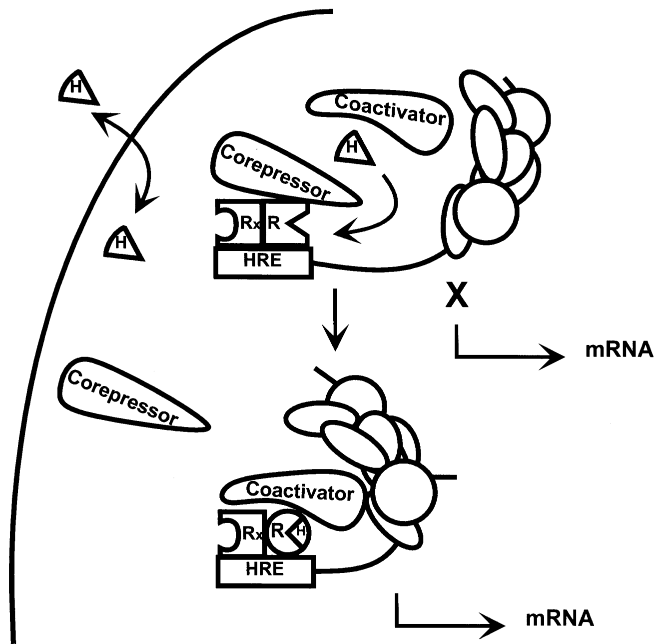

Fig. 2 Activation of steroid/thyroid hormone receptor family members that do not bind heat shock proteins. In the absence of hormone (*H*), these receptors (*R*, receptors) bind to DNA typically as a heterodimer with RXR (*Rx*); corepressors interact with this complex blocking the action of the receptor. Binding of hormone induces a conformational change that causes dissociation of the corepressor allowing interaction with coactivators and other transcription factors and inducing synthesis of RNA

(PRE) thymidine kinase (tk) chloramphenicol acetyltransferase (CAT). Treatment of the transfected cells with either 8-Br cAMP, which activates protein kinase (PK) A, or with okadaic acid, an inhibitor of phosphatases 1 and 2_A, induced transcription of the target gene in the absence of hormone. The activity was strictly dependent on the PR expression plasmid but was due to ligand-independent activation of the receptor. This surprising finding led to studies with other receptors. Power et al. [18, 19] subsequently found that several steroid receptors including the COUP orphan receptor can be activated through activation of D_1 type dopamine receptors in transfected cells, demonstrating that signals emanating from a membrane receptor can activate steroid receptors. Of particular interest was the finding of Ignar-Trowbridge et al. [20, 21] that ER can be activated by epidermal growth factor (EGF) treatment both in vivo and in cell culture.

These initial studies led to a great deal of interest in alternate pathways for activation of steroid receptor family members. Whether a receptor can be activated in the absence of hormone appears to depend upon a number of factors including the receptor type, the cell and promoter context, and the signaling pathway. To date much remains to be determined about the relative importance of this pathway and about the mechanism(s) of ligand-independent activation. These may depend both on the receptor and the signaling pathway. Factors that have been found to activate steroid receptors in the absence of hormone all share the common characteristic of altering the activity of kinases or phosphatases, suggesting that phosphorylation of the receptors or of associated proteins plays a critical role in ligand-independent activation. Other questions to be answered include whether the activation alters the balance between receptors in heat shock complexes and those bound to DNA, or whether these pathways cause the activity per receptor molecule bound to DNA to increase greatly; in some cases both aspects of receptor function may be altered. Because of potential differences among receptors studies of the various receptors are discussed below.

Progesterone receptors

With the exception of the rabbit progesterone receptor, avian and mammalian PRs studied to date are expressed as two forms termed A and B [22–24]. Both arise from the same gene either through transcription of different mRNAs [25] or through alternate initiation of translation at two sites within the same mRNA [26]. The two forms share common hormone-binding and DNA-binding domains, but the shorter A form lacks the 128 (chicken) [26] to 164 (human) [25] amino terminal amino acids of the corresponding B form. In the case of the chicken progesterone receptor both forms are active, although their promoter preference differs [27]. In contrast, there is evidence that the A form of the human receptor acts predominantly as a repressor [28], and that the B form is the activating form under most conditions [29, 30].

Chicken progesterone receptor

The initial observation of ligand-independent activation of a steroid receptor was made using the A form of cPR_A. Denner et al. [17] found that activation of PKA by treatment of transfected CV1 cells with 8-Br cAMP results in activation of cPR_A. Treatment of these cells with either a cell-permeable fragment of the specific PKA inhibitor PKI or a less specific inhibitor, H8, caused a reduction in the ligand-independent activation as well as the hormone-dependent activation of the progesterone receptor. These data intimate that PKA plays a role in both hormone-dependent and ligand-independent activation of the receptor. Subsequent studies by Power et al. [19] revealed that cPR_A can be activated by treatment of transfected cells with dopamine. Although the demonstration of ligand-independent activation of cPR has for the most part focused on cPR_A, both Denner et al. [17] and Power et al. [19] report that cPR_B is activated in a similar fashion. Zhang et al. [31] found that the EGF and inhibitors of phosphatases (calyculin A and vanadate) also activate cPR_A. This response can be elicited in HeLa cells as well as in CV1 cells. To determine whether the response is unique to the PRE-tk-CAT reporter which contains a portion of the tk promoter region as well as two PREs, a

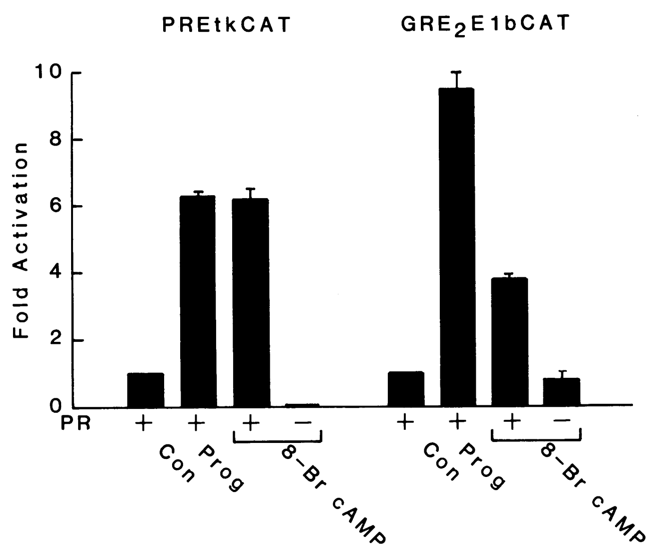

Fig. 3 Effect of 8-Br cAMP on PR_A-mediated transcription in CV1 Cells. CV1 cells were cotransfected with 1 μg PR_A plasmid and 5 μg PRE-tk-CAT (*left*) or glucocorticoid response element 2 E1b-CAT (*right panel*) as previously described [17]. Cells lacking receptor (*PR*) were transfected with 5 μg reporter and 1 μg of the parent vector (p91023b) lacking the PR_A insert. Twenty-four hours after the transfection cells were untreated (*con*), treated with 10^{-7} M progesterone (*Prog*), or treated with 1 mM 8-Br cAMP (*8-Br cAMP*). Cells were harvested, and CAT activities were determined as described [31]. (Reproduced with permission from [31])

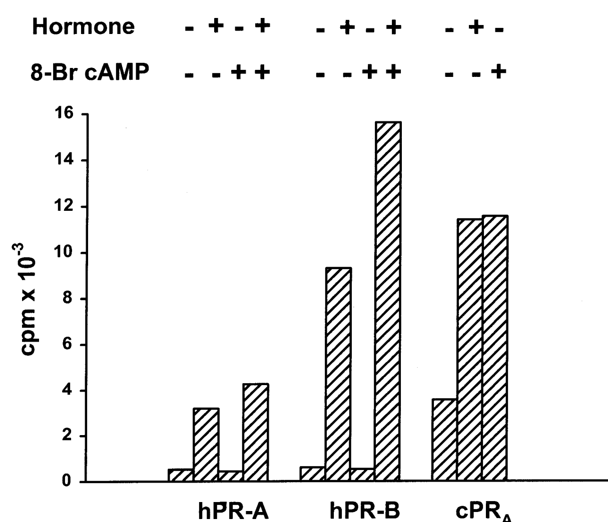

Fig. 4 Neither hPR-A nor hPR-B are activated by 8-Br cAMP treatment under conditions that activate cPR_A. CV1 cells were transfected with either 1 μg cPR_A, 0.5 μg hPR-A, or 0.5 μg hPR-B expression plasmid and 5 μg PRE-tk-CAT as previously described [17]. Twenty-four hours after transfection 10^{-8} M R5020 was added to hormone-treated hPR-A and hPR-B and 10^{-8} M progesterone to hormone-treated cPR_A. 8-Br cAMP-treated samples received 1 mM 8-Br cAMP. Cells were harvested after an additional 24 h and assayed for CAT activity as described previously [31]

simpler promoter containing two PREs linked to the TATA box of the E1b gene and the coding sequence of CAT was used. As shown in Fig. 3, this promoter was also activated in a receptor-dependent, but ligand-independent manner [31]. The relative activation compared to hormone-dependent activation was less than with the more complex promoter, suggesting that interaction with other DNA-binding factors plays a role in determining the magnitude of ligand-independent activation.

Rat progesterone receptor

Turgeon and Waring [32] have shown 8-Br cAMP or gonadotropin-releasing hormone dependent activation of rat PR in primary rat pituitary cells using a transfected reporter. These responses were blocked by the progesterone antagonist mifepristone (RU486), demonstrating that the activation is progesterone receptor dependent.

Evidence that PR can be activated in vivo comes from studies of rat sexual behavior. Mani et al. [33] found that ovariectomized estrogen-primed rats that receive injections of dopamine agonists of the D_1 type directly into the third ventricle display a high rate of lordosis, similar to that observed with progesterone. That this response occurred through activation of the progesterone receptor was demonstrated by the ability of progesterone antagonists [RU486 or onapristone (ZK98299)] to block the response to dopamine agonists. Subsequently Mani et al. [34] showed that preadministration of antisense, but not sense, oligonucleotides coding for PR blocks the response to dopamine, further confirming that the dopamine response is PR mediated.

Human progesterone receptor

In contrast to the chicken and rat PR, several groups have failed to detect ligand-independent activation of the human PR. Beck et al. [35] found that the activity of endogenous PR in T47D breast cancer cells stably transfected with an MMTV-CAT reporter is stimulated by 8-Br cAMP or tetradecanoyl 12-phorbol 12-acetate (a PKC activator) in the presence of the progestin agonist promegestone (R5020), but that 8-Br cAMP alone does not activate PR. Although these studies were performed under somewhat different conditions than the cPR studies, we have directly compared the activity of cPR_A and the A and B forms of hPR and shown that neither hPR-B nor hPR-A are activated under the conditions that activate cPR_A (Fig. 4).

Subsequent studies by Beck et al. [36] revealed that treatment with the combination of the antagonist RU486 and 8-Br cAMP activates hPR. This antagonist/agonist switch is dependent upon use of a progesterone antagonist such as RU486 that promotes DNA binding as judged by in vitro DNA-binding assays whereas ZK98299 in combination with 8-Br cAMP is inactive. Sartorius et al. [37] reported similar results and subsequently demonstrated that only the B form of the PR is

activated by RU486 and 8-Br cAMP [38]. The reasons for these species-specific differences in PR responses have yet to be determined. Nonetheless, there have been a few reports of ligand-independent activation of hPR under other conditions. Kazmi et al. [39] reported that they observed ligand-independent activation of hPR in COS-1 cells cotransfected with a PR expression vector and the reporter PRE-tk-CAT. However, the activation relative to optimal hormone-dependent activation was not assessed due to the use of suboptimal (0.1 nM) levels of R5020. Philpott and Shahid [40] have also reported ligand-independent activation of hPR-B by dopamine in CV1 cells transfected using lipofectin to introduce exogenous DNA.

Mechanism of ligand-independent activation of cPR

The finding that stimulation of different kinase pathways activates cPR raises the question of whether there are multiple mechanisms leading to ligand-independent activation of the progesterone receptor or whether the signal transduction pathways converge leading to a single mode of activation. That at least two pathways must be operating was demonstrated by Power et al. [19] who showed that substitution of a Thr for Ser-628 the hormone-binding domain of cPR_A produced a receptor that is activated in response to hormone and to treatment with okadaic acid, but not to treatment with dopamine. The means by which this occurs remains to be determined. Bai et al. [41] showed that mutation of two of the phosphorylation sites in cPR [42, 43] reduced the ligand-independent activation in response to either 8-Br cAMP or to a dopamine agonist, but the mutations did not completely eliminate the activation. Moreover, they found that treatment with 8-Br cAMP does not change the phosphorylation of cPR_A [41] suggesting that altered phosphorylation of a protein that interacts with cPR may be required for ligand-independent activation. With the recent finding that cAMP response element binding protein binding protein [44–46] and other coactivators such as SRC-1 [10], RIP140/160 [47], and TIF1 [48] interact with steroid receptors and mediate their activities, one or more of these proteins may be the target of the signaling pathways and likely play important roles in mediating transcription from complex promoters that contain both cAMP response elements and steroid receptor response elements. However, to date the evidence suggests that simple overexpression of cAMP response element binding protein binding protein [46] or SRC-1 [10] does not induce ligand-independent activation of steroid receptors.

Estrogen receptors

There is abundant evidence for ligand-independent activation of mammalian ER in transiently transfected cells, in cells containing endogenous receptors and endogenous target genes, as well as in vivo.

Transient transfection studies

That the wild-type hER can be activated by dopamine in transfected cells and that this activation is inhibited by ICI-164384 was shown by Smith et al. [49]. However, cotreatment with the partial estrogen antagonist *trans*-hydroxytamoxifen (TOT) and dopamine resulted in activity greater than that of dopamine alone. Interestingly, the Val-400 mutant of ER did not respond to dopamine in the absence of ligand but was synergistically activated in the presence of TOT and dopamine. ICI-164384 in combination with dopamine does not activate the Val-400 mutant.

Ignar-Trowbridge et al. [20] demonstrated that mouse ER cotransfected with a reporter plasmid into Ishakawa cells can be activated by EGF or transforming growth factor α, and that this activation is inhibited by ICI-164384 [21]. Activation of endogenous ER in BG-1 human ovarian cancer cells cotransfected with a vitellogenin (vit) estrogen response element (ERE) CAT reporter plasmid was detected in response to either EGF or to estrogen and was blocked in each case by administration of ICI-164384.

Other growth factors can also activate the ER. Newton et al. [50] have shown that either insulin or insulin-like growth factor (IGF) 1 can activate the endogenous ER in GH_3 pituitary cells transiently transfected with a reporter plasmid. IGF-1 and IGF-2 can activate ER in the neuroblastoma cell line SK-ER3 stably transfected with an ER expression vector. Moreover the growth factors induce growth arrest and differentiation of this cell line similarly to estrogen whereas the parental line is unaffected. Ignar-Trowbridge et al. [51, 52] have also shown that IGF-1 can activate ER in ovarian adenocarcinoma cells as can activators of PKA and PKC. Moreover, inhibitors of PKC do not block growth factor dependent activation indicating that there are multiple pathways to ligand-independent activation of the receptor [51].

Pietras et al. [53] have shown that treatment of MCF-7 cells with heregulin transcriptionally activates the endogenous ER in cells transfected with an ERE-CAT reporter in the absence of hormone. This treatment also causes phosphorylation of tyrosine in the ER. Heregulin should also activate the mitogen-activated protein kinase pathway leading to phosphorylation of Ser-118 which plays an important role in the activation of the ER [54].

Whether the ligand-independent activation is due to the tyrosine phosphorylation is unknown. The role of phosphorylation of Tyr-537 in ER function is unclear. Although there have been reports that phosphorylation of this site is necessary for hormone-binding [55] or DNA-binding [56], others have shown that mutation of this site produces active, hormone-binding receptor and, depending upon the amino acid substitution, produces a receptor that shows a high degree of constitutive activity [57].

Regulation of endogenous genes by endogenous receptors

Aronica and Katzenellenbogen [58] have found that the levels of PR (an estrogen-regulated gene) in primary rat uterine cells can be stimulated by cAMP or by IGF-1 as well as by estrogen, and that stimulation by any of these compounds can be inhibited by antiestrogens; this indicates that the actions of cAMP and IGF-1 are mediated by the ER.

Ligand-independent activation of estrogen receptor in vivo

Ignar-Trowbridge et al. [20] showed that administration of EGF to ovariectomized mice induces markers of estrogen action including uterine DNA synthesis and phosphatidylinositol lipid turnover; these responses were reduced by the ER antagonist, ICI-164384, consistent with the activation of the ER by EGF. Furthermore, treatment with EGF caused nuclear retention of the ER and an increase in the proportion of receptor with slower mobility on sodium dodecyl sulfate gels that is characteristic of altered phosphorylation of ER. Consistent with the requirement for ER for many EGF-mediated responses in mouse uterus was the finding of Curtis et al. [59] that EGF fails to induce DNA synthesis and PR mRNA in the ER knockout mouse as it does in the wild type. That the EGF receptor is functional in the ER knockout mouse mice was demonstrated by EGF receptor autophosphorylation and induction of c-*fos* in response to EGF treatment.

Mechanism for ligand-independent activation of estrogen receptor

Fujimoto and Katzenellenbogen [60] found that the effects of TOT on the activity of ER in cells that have elevated levels of cAMP or are transfected with an expression vector for the PKA catalytic subunit depend on the promoter used to detect the ER activity. Whereas activation was observed with several promoters, transcription of an ERE tk reporter was unaffected by increased levels of cAMP in combination with TOT. Consistent with the observations of Smith et al. [49], ICI-164384 does not stimulate ER-dependent transcription in the presence of elevated levels of cAMP.

That ligand-independent activation of ER is a function of the cell type and reporter used was clearly shown by Ince et al. [61]. Whereas the wild-type ER transfected into 3T3 cells exhibited ligand-independent activation in response to treatment with cholera toxin (CT) and 3-isobutyl-1-methylxanthine (IBMX) (causing an increase in cAMP levels) when measured using an ERE-vit-CAT reporter, no activation was seen when measured using an $(ERE)_2$-tk-CAT promoter. In contrast, a small amount of ligand-independent activation was detected in Chinese hamster ovary (CHO) cells when the $(ERE)_2$-tk-CAT plasmid was used, but not when the ERE-vit-CAT was used. In some cases the combination of estradiol and increased cAMP levels greatly increased transcription; in others the effect was minimal. Of particular interest was the finding that some ER mutants that exhibit little activity in the presence of estrogen become transcriptionally active in response to elevated levels of cAMP [61]. An ER containing glutamine at position 540 instead of leucine (L540Q) binds estrogen normally, but exhibits almost no hormone-dependent activation in CHO cells when receptor activity is measured using an $(ERE)_2$-TATA-CAT reporter. Although IBMX/CT alone, under these conditions, does not activate the mutant, addition of IBMX/CT in combination with estradiol produces a transcriptionally active receptor. Interestingly, both TOT and ICI-164384 enhance the activity of this mutant in the presence of IBMX/CT. Under identical conditions ICI-164384 has little effect on the activation of the wild-type ER. In other cell/promoter contexts the combination of IBMX/CT and estradiol activate the L540Q mutant but TOT and ICI-164384 do not synergize with IBMX/CT. In 3T3 cells IBMX/CT is sufficient to activate both the wild type and L540Q receptors when assayed using the ERE-vit-CAT reporter, and ICI-164384 blocks activation of both receptors. Collectively these experiments demonstrate that the ability of a receptor to produce a transcriptionally productive complex depends on the proteins with which the receptor must interact to induce transcription of a target gene. The ability to interact with other proteins to induce transcription may depend on the conformation of the ligand-binding domain, the complement of proteins expressed in a particular cell type, other proteins that bind to the same promoter, and the effects of signal transduction pathways on the levels and interactions of these proteins.

Ligand-independent activation of ER is accompanied by altered phosphorylation of ER. Aronica and Katzenellenbogen have shown that treatment of rat uterine cells with estrogen, 8-Br cAMP, or IGF-1 increase the phosphorylation of endogenous rat ER [62]. Subsequent studies of hER expressed in COS cells have revealed that activation of PKA pathways caused increased phosphorylation of the carboxyl terminal portion of ER although the specific phosphorylation sites have not been identified [63].

Several lines of evidence suggest that EGF-mediated activation of ER differs from the dopamine- and cAMP-mediated pathways. First, the Val-400 mutant can be activated by treatment with EGF [64] but not by dopamine [49]. Second, studies of EGF-mediated induction have implicated the amino terminus of the ER as being required for this activation [51, 64]. Chimeras between GR, which does not exhibit ligand-independent activation, and ER show that an ER-GR chimera containing the ER amino terminus and the GR ligand-binding domain can be activated by EGF whereas the reciprocal chimera cannot [64]. Moreover, a mutant lacking AF-2 activity is still capable of responding to EGF [64], sug-

gesting that it is the AF-1 transactivation domain in the amino terminus rather than the carboxyl terminal AF-2 transactivation domain that is required for EGF-mediated activation. Several groups [54, 64, 65] have shown that ER can be phosphorylated on Ser-118 by mitogen-activated protein kinase, a kinase that can be activated through a cascade initiated by binding of EGF to its receptor. Mutation of this serine to an alanine abolishes EGF-mediated activation [64]. Substitution of a glutamic acid, which can mimic a phosphorylated amino acid, permits EGF-mediated activation but does not produce a constitutively activated receptor [64]. These data suggest that phosphorylation of Ser-118 is necessary, but insufficient, to induce EGF-mediated transcriptional activation. Whether the additional required activity for EGF-dependent ER activation is phosphorylation of another site in ER or alteration of other factors remains to be determined.

In contrast to the EGF response, insulin activation of ER appears to act through the carboxyl terminal of the ER in neuroblastoma cells as demonstrated by Patrone et al. [66]. This pathway involves ras but not phosphatidylinositol 3′ kinase.

Taken together, these studies provide clear evidence for multiple pathways leading to activation of the ER. In contrast to the studies to date of PR, ligand-independent activation of ER causes changes in phosphorylation of ER, and these changes are dependent upon the activating signal.

Androgen receptors

Ligand-independent activation of AR has not been studied as extensively as that of ER and PR, and reports have been mixed.

Transient transfections

Culig et al. [67] have shown that androgen receptor, transiently expressed in DU-145 prostate cancer cells that lack endogenous androgen receptor, can be activated by treatment of the cells with growth factors. They found that IGF-1 is most efficient in activating AR, and that activation can be detected using both a simple reporter consisting of two androgen response elements linked to a TATA box and the coding sequence for CAT or a more complex promoter consisting of a portion of the 5′ flanking region of the prostate-specific antigen (PSA) gene. By contrast, keratinocyte growth factor activates AR when assayed with the simple promoter, but not the PSA reporter. In both cases growth factor dependent activation is blocked by the pure antiandrogen casodex. Although weak activation was detected with EGF and the simple promoter, neither basic fibroblast growth factor nor IGF-2 activated the androgen receptor under these conditions. Culig et al. [68] have recently shown that luteinizing hormone releasing hormone or dibutyryl cAMP can weakly activate AR transiently transfected into DU-145 cells, but that these compounds act synergistically with androgens to stimulate transcription.

Nazareth and Weigel [69] found that hAR can be activated in CV1 cells cotransfected with an AR expression plasmid and a reporter consisting of two glucocorticoid/androgen response elements and the TATA box of the E1b gene linked to the coding region for CAT by treating the cells with forskolin to stimulate adenylyl cyclase activity, increasing the levels of cAMP. This activation is blocked by the androgen antagonists flutamide and casodex and requires a functional DNA-binding domain in the AR. Interestingly, inhibition of the PKA pathway blocks forskolin-dependent activation of AR and reduces androgen-dependent activation without reducing levels of AR expression or reducing the activation of a control, constitutively active reporter. This provides evidence for involvement of this signaling pathway in ligand-dependent activation of the AR. Forskolin-dependent activation is also detected in PC3 prostate cancer cells that lack endogenous AR when these cells are transfected with an AR expression vector and a reporter consisting of a portion of the 5′ flanking region of the probasin gene linked to the coding region for CAT. Because probasin is an androgen-regulated, prostate-specific gene [70], this complex promoter should be representative of a natural target gene.

Other investigators have not detected ligand-independent activation of the AR. Ikonen et al. [71] found that the activity of rat AR transiently cotransfected into CV1 cells is enhanced by treatment with 8-Br cAMP, okadaic acid, or the PKC activator phorbol myristate acetate in combination with testosterone; none of the compounds activated the receptor in the absence of testosterone. Although there are several differences between these experiments and those of Nazareth et al. [69] (rat AR vs. human AR, different reporters, different transfection procedures, and different means of elevating cAMP levels), the reasons for the observed differences have yet to be resolved. Reinekainen et al. [72] found that growth factors modulate the activity of rat AR transfected into CV1 cells but are incapable of activating AR in the absence of androgen. Finally, de Ruiter et al. [73] have reported synergistic activation of stably transfected hAR expressed in CHO cells with phorbol myristate acetate in combination with androgen but not with phorbol myristate acetate alone regardless of the promoter used. In this cell line 8-Br cAMP does not enhance the androgen-dependent activity of the receptor nor does it activate the receptor alone.

Activation of endogenous target genes by endogenous androgen receptors

To detect activation of an endogenous AR, Culig et al. [67] assessed the effect of growth factors on the secretion of PSA from LNCaP prostate cancer cells that contain an endogenous AR with a mutation in the ligand-

binding domain. Only IGF-1 induced secretion of PSA and, as in the transfected cells, casodex blocks the activity. Treatment of LNCaP cells with luteinizing hormone releasing hormone does not affect PSA secretion [68], whereas treatment with dibutyryl cAMP in combination with the synthetic androgen methyltrienolone (R1881), enhances secretion over that observed with R1881 alone.

Mechanism for ligand-independent activation of androgen receptor

To elucidate the mechanism of ligand-independent activation of AR, Nazareth and Weigel [69] examined the effects of activators and inhibitors of transcriptional activation on the nuclear localization and DNA binding of AR. Using a subcellular fractionation assay, they found that treatment with forskolin slightly enhances the nuclear localization of AR, and that cotreatment with androgen antagonists blocks the increased nuclear localization. Similarly, treatment of cells with forskolin slightly enhances the DNA-binding activity of the AR, and this also is blocked by cotreatment with antagonists.

Although AR phosphorylation studies have not been carried out under conditions that cause ligand-independent activation, there is some evidence that phosphorylation may be altered. Jenster et al. [74] have shown that the AR appears as a doublet when analyzed by sodium dodecyl sulfate gel electrophoresis, and that the proportion of the slower mobility form increases in response to hormone treatment due to enhanced phosphorylation. Weigel and Nazareth [69] reported that treatment with forskolin increases the proportion of receptor with increased mobility, suggesting that the phosphorylation pattern differs from that of control or hormone-treated receptors.

Collectively these initial studies of the AR indicate that it can be activated in the absence of hormone under specific conditions, but that ligand-independent activation depends strongly on the cell type, promoter, and activators utilized.

Glucocorticoid receptors

A number of studies have shown that GR is refractory to ligand-independent activation. Power et al. first showed that GR is not activated by dopamine under conditions that activate cPR and ER [19]. Subsequent studies have shown that the hormone-dependent activity of GR can be stimulated by activators of PKA [75, 76] and PKC [76], but neither pathway activates the receptor alone. This enhanced activation of GR does not appear to involve altered phosphorylation of GR [77]. Nordeen et al. [78] showed that, similar to hPR, the combination of the progesterone/glucocorticoid receptor antagonist RU486 with 8-Br cAMP induces an antagonist/agonist switch in GR. Because of the cell type, promoter, and activator dependence of ligand-independent activation of other steroid receptors, it is possible that the conditions required to activate GR simply have not been identified.

Other steroid/thyroid hormone receptor family members

The receptor family members that do not form stable hsp complexes include the retinoic acid receptors (RAR) α, β, γ, retinoid X receptors (RXR) α, β, γ, vitamin D receptor, and thyroid hormone receptors. In addition, there are numerous orphan receptors whose ligands have yet to be identified.

Huggenvik et al. [79] demonstrated that RARα can be activated in the absence of retinoic acid by cotransfection of RARα, the catalytic subunit of PKA, and an RAR-dependent reporter into CV1 or HeLa cells. Matkovits and Christakos [80] subsequently found that RARα, RARβ, RXRα, RXRβ, and RXRγ, but not RARγ, can be activated in the absence of their cognate ligands when transfected into CV1 cells that were subsequently treated with the phosphatase inhibitor, okadaic acid. However, almost no ligand-independent activation was observed when the cells were treated with dopamine. These same investigators demonstrated that vitamin D receptor, transfected into CV1 cells, can be activated by okadaic acid or by dopamine [80].

Very little is known about the activation of the orphan receptors. However, a COUP-PR chimera containing the DNA-binding domain of PR is activated by dopamine [18]. Other orphans or receptor variants may be regulated by phosphorylation instead of by ligand-binding. A likely candidate is Nur77, whose expression, phosphorylation, and activity is regulated by signal transduction pathways; the phosphorylation state of Nur77 depends upon whether the factor is induced by growth factors or by membrane depolarization [81, 82]. These data demonstrate that signal transduction pathways also modulate this subset of the steroid/thyroid hormone receptor family.

Summary

Several of the steroid receptor family members exhibit ligand-independent activity in vivo as well as in cell culture under specific conditions. That the activation is frequently cell type, promoter, and activator-specific suggests that ligand-independent activation depends more upon additional factors than does hormone activation. A model of these two pathways is shown in Fig. 5. In the conventional hormone-dependent pathway the binding of hormone causes conformational changes in the receptor resulting in dissociation of the heat shock protein complexes and potentially of other inhibitory molecules. The receptor dimerizes, binds to a hormone response element, interacts with basal transcription factors as well as coactivators, and induces transcription of the target gene. During the activation process phosphorylation of the re-

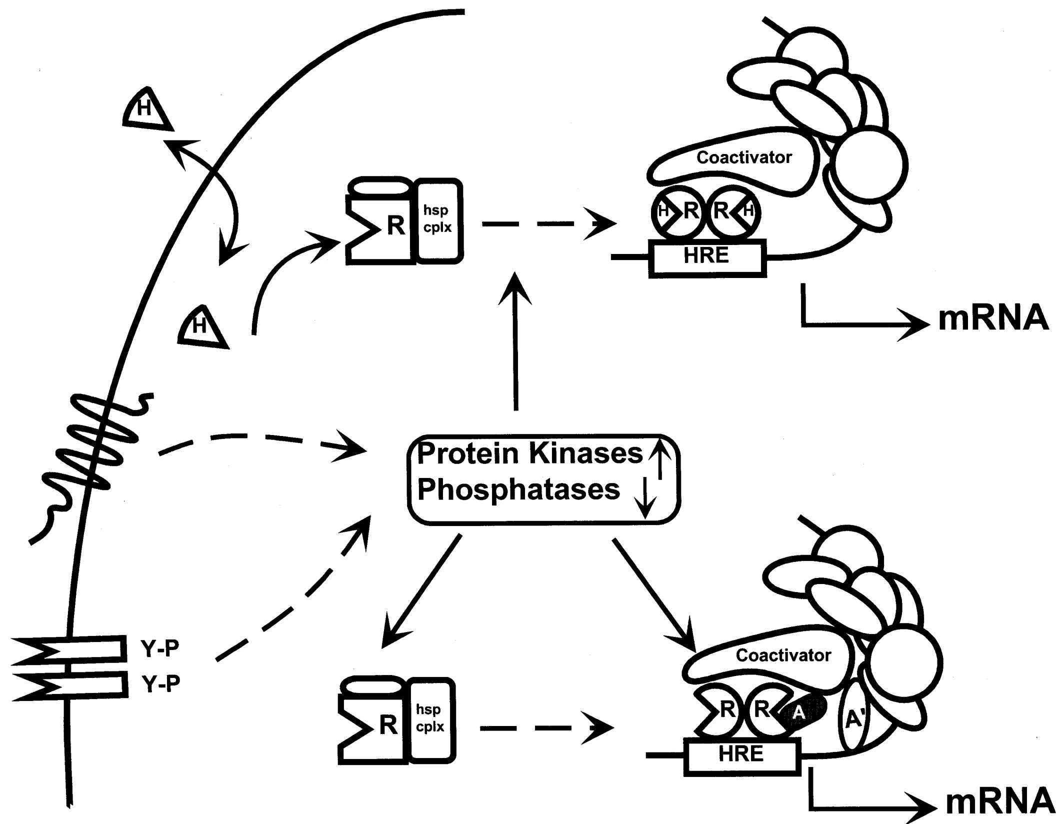

Fig. 5 A model for ligand-independent activation of steroid receptors. *Above*, the conventional hormone-dependent activation of steroid receptors described in Fig. 1. The hormone-dependent activity is modulated by the cellular content of kinases and phosphatases. *Below*, a proposed model for ligand-independent activation. Signals emanating from membrane receptors through signal transduction cascades alter the activities of kinases and/or phosphatases in the cells, resulting in altered phosphorylation of receptors and/or interacting proteins and activation of the receptor. This pathway may require additional factors that interact directly with the receptor (A) or with specific sites on the DNA (A′). *R*, Receptor monomer; *H*, hormone; *HRE*, hormone response element; *hsp cplx*, hsp complex that associates with receptor

ceptor is typically increased [42, 63, 83], and these phosphorylations play a role in producing optimally active hormone-bound receptors [63, 84, 85]. In the case of ligand-independent activation a signal emanating from the membrane activates the receptor through altered phosphorylation of the receptor itself (ER) and/or proteins that interact with the receptors producing transcriptionally active complexes. That ligand-independent activation is not universally observed with the same reporters and cell types as is the hormone-dependent pathway suggests that there are additional cell specific factors that may interact with the receptor directly (A) or through binding sites on the DNA (A′) to promote or inhibit ligand-independent activation.

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