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Progesterone receptor transcription and non-transcription signaling mechanisms

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Abstract

The diverse effects of progesterone on female reproductive tissues are mediated by the progesterone receptor (PR), a member of the nuclear receptor family of ligand-dependent transcription factors. Thus, PR is an important therapeutic target in female reproduction and in certain endocrine dependent cancers. This paper reviews our understanding of the mechanism of action of the most widely used PR antagonist RU486. Although RU486 is a competitive steroidal antagonist that can displace the natural hormone for PR, it's potency derives from additional "active antagonism" that involves inhibiting the activity of PR hormone agonist complexes in *trans* through heterodimerization and competition for binding to progesterone response elements on target DNA, and by recruitment of corepressors that have the potential to actively repress gene transcription. An additional functional role for PR has recently been defined whereby a subpopulation of PR in the cytoplasm or cell membrane is capable of mediating rapid progesterone induced activation of certain signal transduction pathways in the absence of gene transcription. This paper also reviews recent results on the mechanism of the extra-nuclear action of PR and the potential biological roles and implications of this novel PR signaling pathway.
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1. Introduction

The diverse biological effects of progesterone (PG) are mediated by the progesterone receptor (PR), a member of the nuclear/intracellular receptor superfamily of ligand-dependent transcription factors. Thus, PG has a major role in regulating expression of specific gene networks in female reproductive tract and other target tissues [1–3]. However, not all effects of PG are genotropic. Rapid effects of PG on cell signaling pathways, independent of transcription, have been described that also contribute to selective end point biological responses [4–6].

As a member of the nuclear receptor family, PR contains three functional domains including the N-terminus, a centrally located DNA binding domain (DBD), and C-terminal ligand binding domain (LBD; Fig. 1). Three-dimensional atomic structures of isolated DBD and LBDs have revealed common motifs for these regions. By comparison, little is known about the structure of the N-terminal domain. This is the least conserved region among family members with respect to both length and amino acid sequence. The N-domain is functionally important, as it is required for full transcriptional activity of steroid hormone receptors and for many cell- and target gene-specific responses. Other functional and structural determinants have been identified within these broader three domains. In addition to binding steroid hormone, the LBD contains determinants for dimerization (DI) in the absence of DNA, binding of heat shock proteins (hsps) and for nuclear localization sequence (NLS). The DBD contains a second NLS and dimerization domain that is dependent on DNA binding. Steroid receptors contain at least two transcription activation domains (AFs), AF-1 in the N-terminal domain and highly conserved AF-2 in the C-terminal LBD. These are autonomous transferable domains required for the DNA bound receptor to transmit a transcriptional activation response and they function as specific binding sites for coactivators. AF-2 located in the LBD is hormone-dependent and becomes activated as a result of the steroid hormone inducing a conformational change that creates a hydrophobic binding pocket for members of the p160 family of steroid receptor coactivators (SRCs). The coactivators that bind to and mediate the activity of AF-1 are yet not well defined.
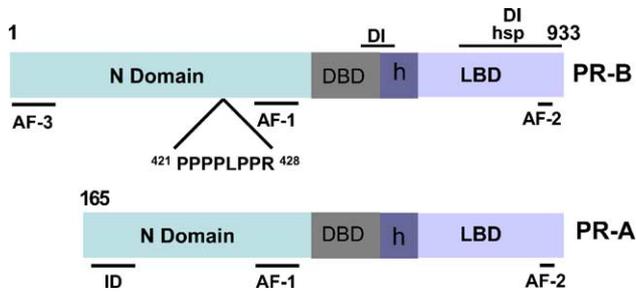
Fig. 1. Domain organization of the human PR-A and -B isoforms. N-domain, N-terminus; DBD, DNA binding domain; h, hinge; LBD, ligand binding domain. Transcription activation domains; AF-1, AF-2, and AF-3; dimerization domain, DI; inhibitor domain, ID; hsp, heat shock protein binding region; PXXPXR, class II consensus peptide ligand for Src kinase like SH3 homology domains.

The general pathway of PG inducible PR-mediated gene transcription has been well characterized. PG binding induces a conformational change(s) in PR that promote dissociation from a multi-protein chaperone complex, homodimerization and binding to specific progesterone response elements (PREs) within the promoter of target genes [7,8]. DNA bound receptors increase or decrease rates of gene transcription by influencing recruitment of RNA polymerase II to the initiation site. Through protein–protein interaction, hormone activated PR recruits coactivators that serve as essential intermediates for transmitting signals from the receptor to the transcription initiation complex. Coactivators facilitate transcription initiation through protein interactions with components of the general transcription machinery and by promoting local remodeling of chromatin at specific promoters (Fig. 2).

The human PR is expressed as two isoforms from a single gene by alternate promoter usage (Fig. 1). PR-A (94 kDa) differs from PR-B (120 kDa,) by lacking 164 amino acids (aa) at the N-terminus [9]. Although the two forms of PR have similar steroid hormone and DNA binding activities,

they have distinct transcriptional activities. PR-B in general, is a much stronger activator than PR-A. However, PR-A can be a strong activator under specific cell and target gene contexts [10,11]. The stronger activation potential of PR-B is due in part to the existence of a third activation domain (AF-3) within the first N-terminal 164 aa that is unique to PR-B (Fig. 1) [12]. Under certain cell and target promoter contexts PR-A is inactive as a transcription factor and can function as a ligand-dependent transdominant repressor of other steroid receptors including PR-B and the estrogen receptor (ER). PR-A can act in this repressor mode in response to the binding of either progestin agonists or antagonists. An inhibitory domain (ID) responsible for this transrepressor function has been mapped to the first 140 N-terminal (aa 165–305) amino acids of PR-A (Fig. 1). Since the sequence within ID is present in both PR isoforms but is only active in the context of PR-A suggests the PR-B specific N-terminal segment plays a role in suppressing the ID domain.

2. PR antagonist mechanisms

Several synthetic ligands (both steroidal and non-steroidal) for PR have been developed which compete for binding with the natural hormone and are capable of inhibiting receptor activity. Mifepristone (RU486) was the first of these PG antagonists that exhibited antiprogesterone activity in humans and has since been used in numerous clinical studies in the gynecologic and obstetrical fields. It was shown to be an effective abortifacient and postcoital contraceptive and has been used in the treatment of endometriosis, uterine myomas, and for meningiomas, which have large concentrations of PRs. This drug has also proven to be valuable for dissecting normal receptor activation mechanisms [13]. In this section, we will review our work and the work of others on the mechanism of action of RU486 and closely related compounds.
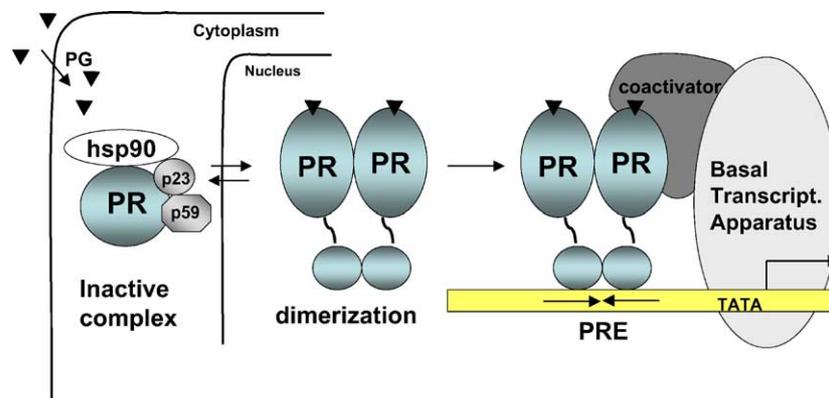

Fig. 2. PG activation of progesterone receptor. Binding of PG to the inactive receptor complex induces a conformation change which leads to immunophilin and hsp dissociation, receptor dimerization, DNA binding, and recruitment of coactivators to facilitate communication with the basal transcription apparatus. PRE, progesterone response element.

2.1. Mechanism of action of the antagonist (RU486) and related compounds

2.1.1. Binding mechanism

The simplest approach to antagonize PG is to effectively compete for binding of the physiological hormone ligand to PR with an inactive synthetic analog (steroidal or non-steroidal). RU486 is among the most widely used progestin antagonists. The main structural characteristics of RU486 that correlate with its antagonist activity are the phenyl-aminodimethyl group at the 11-β position of the steroidal skeleton (R1) and the carbon 11-side chain (R2). Although RU486 binds with high affinity to PR (in fact, it binds PR with a slightly higher affinity than PG), and effectively competes for PG binding, it does not make the same contacts in the LBD as agonist. As evidence for this, truncation of the last 42 aa at the C-terminus of PR abolishes binding to progestin agonist while retaining binding to progestin antagonist [14]. Also, a single amino acid substitution Gly → Cys at position 722 in the LBD of human PR abolishes RU486 binding while retaining high affinity binding for PG approximately equal to that of wild-type PR [15].

2.1.2. Dimerization and DNA binding by PR

The mechanism by which RU486 inactivates PR is complex and remains incompletely understood. The receptor activation steps of dissociation from hsps, dimerization and binding to PREs are not impaired. Although earlier studies suggested that RU486 might act by stabilizing the inactive PR-hsp complex, and thus prevent PR from interacting with DNA [16], the preponderance of data does not support this as a mechanism. In fact, RU486 has been observed to induce a stronger dimerization of PR and tighter association of PR with DNA than hormone agonists [17,18]. Additionally, there is no evidence that RU486 causes PR to make different contacts with PREs than PG. Based on DNA footprinting assays, PR was found to make identical base specific contacts with the HRE of mouse mammary tumor virus (MMTV) in the presence of agonist and RU486 [19,20].

We and others have shown that RU486 promotes PR binding to PREs not just in vitro but also within intact cells (in vivo). This has been shown by several experimental approaches including promoter interference assay, induction of gene activation mediated by a PR-VP16 activation domain chimeric receptor, and by the ability of the PR-RU486 complex to effectively inhibit, through competition for PREs, agonist activated GRs or a constitutively active truncated PR lacking the LBD [21,22]. Although the steroidal antagonist ZK98299 (onapristone) was initially categorized as mechanistically distinct from RU486 by preventing PR binding to DNA, this categorization was based on in vitro gel shift experiments [23]. Subsequent gel shift experiments showed that ZK98299 does induce PR binding to PREs in vitro, although the tightness of the association is lower than that induced by RU486. Additionally, ZK98299 effectively stimulated PR binding to PREs in intact cells by the approaches used to detect the influence of RU486 on PR-DNA binding in vivo. Thus, we conclude that RU486 and other structurally related steroid antagonists all work by a similar mechanism that does not involve inhibiting activation steps that lead to PR binding to specific target DNAs.

2.1.3. Altered conformation in the LBD induced by RU486 inhibits AF-2 coactivator recruitment

The mechanism for non-productive interaction of PR with DNA in the presence of RU486 is not completely worked out. However, studies with p160 coactivators and structure of LBDs bound to agonist versus antagonist have provided much insight. RU486 induces a conformation in the C-carboxyl terminal tail of PR that is distinct from that induced by hormone agonist. This alternate conformation inactivates AF-2 and does not permit interaction with SRCs [24]. There are several lines of evidence indicating that RU486 induces an alternate conformation in the C-terminus. RU486 bound PR altered the electrophoretic mobility of PR-DNA complexes when compared to agonist induced complexes in polyacrylamide native gels [9,25]. A monoclonal antibody to the C-terminus of PR (C262) recognized PR bound RU486 but not agonist bound PR, suggesting the C262 epitope is accessible in the presence of RU486 but is not available in the presence of agonist. In addition, using limited proteolytic digestion analysis, antagonist bound PR LBD gave a digestion pattern distinct from agonist bound PR LBD [26]. Modeling of the RU486 bound PR LBD crystal structure predicted that RU486 displaces helix 12 [27] and results in helix 12 disrupting the hydrophobic groove required for coactivator binding. Thus, the displacement of helix 12 induced by RU486 blocks coactivator binding to AF-2 and renders the receptor transcriptionally inactive [28].

2.1.4. Influence of RU486 on amino- and carboxyl-terminal domain interaction

Under most cellular and target promoter conditions, full transcriptional activity of steroid receptors requires functional synergy between AF-1 and AF-2 [9]. Studies with ER [29] and AR [30] suggest that this functional synergy involves a ligand-dependent intramolecular association between the N- and C-terminal domains of receptor. Using a mammalian two-hybrid interaction system, we observed a hormone-agonist dependent functional interaction between N-terminal domains (PR-A and PR-B) and the hinge LBD (hLBD) of human PR [31]. These interactions appear to involve direct protein contacts as determined by in vitro protein–protein interaction assays using purified expressed domains of PR [31]. RU486 failed to induce an interaction between the N-domains and the hLBD of PR in vitro and functionally inhibited hLBD interaction with N-domains in whole cells by mammalian two-hybrid [31]. These data indicate that RU486 fails to induce, or impairs, a physical

association between the N- and C-domains of PR. These data suggest that in addition to blocking AF-2 coactivator binding, the altered conformation in the LBD induced by RU486 may contribute to inactivation of receptor by interfering with physical association between the amino and carboxyl domains. N- and C-domain interaction may also be necessary for formation of a proper surface for additional coactivator interactions, possibly involving AF-1 coactivators, but this is still unclear at the present time.

2.2. Influence of antagonists on PR mediated transactivation through interaction with other transcription factors

The activities and mechanism of action of steroid antagonists on gene transcription have been largely defined by their ability to influence receptor function through consensus HREs. However, there is increasing evidence that a number of steroid regulated genes are not regulated by direct binding of receptor to classic HREs, but through protein–protein interaction of receptor with other sequence-specific transcription factors. Although this mode of regulation can be either positive or negative, it is more commonly a pathway for negative gene regulation by steroid receptors. As a variation of this mode of regulation are genes that contain composite response elements consisting of a less than optimal DNA binding site for the steroid receptor (often a HRE half-site) that overlaps, or is adjacent to, a binding site for another sequence-specific transcription factor. Examples of PR cross talk with other transcription factors are repression of NF-κB activity (through interaction with RelA-p65 subunit), inhibition of prolactin-induced Stat5 mediated activation of the β casein gene, repression of AP-1 (fos/jun) activity and potentiation of c/EBPβ mediated gene activation [32–35]. Because NF-κB is activated by various cytokines, cross talk with PR is thought to be involved in the immunosuppressive effects of progesterone during pregnancy. PR cross talk with AP-1, c/EBP, and Stat5 is thought to be involved in proliferative and differentiation functions of progesterone in the mammary gland and uterus, respectively. An important question is how steroid antagonists influence this mode of PR-mediated gene regulation. RU486 and ZK98299 were shown to induce PR-mediated repression of the RelA subunit of NF-κB induction of the human ICAM-1 gene in a manner similar to that of the progestin agonist R5020 [33]. We found that both RU486 and ZK98299 inhibited prolactin Stat5-mediated induction of a β-casein reporter gene, similar to that of progestin agonists [35] and PR-repression of AP-1 were reported to be induced similarly by PG agonist and RU486 [32]. Thus, it appears from these studies that PR antagonists can behave as agonists on target genes regulated through PR interaction with other transcription factors. Understanding how antagonists influence PR-mediated transcription on these indirect elements is an important consideration in profiling their biological activity in vivo.

2.3. Active antagonists

RU486 and related steroid antagonists are more potent than predicted by simple competition for PG binding and prevention of p160 coactivator recruitment by inactivation of AF-2. RU486 effectively antagonizes PG activation of PR at concentrations that are much less than substoichiometric with PG. Three mechanisms appear to contribute to this unusual potency of RU486 as an antagonist of PG. First, RU486 promotes a higher affinity interaction of PR with DNA than the agonist R5020 in vitro [36] and we and others have shown that antagonists-bound PR can effectively compete with binding of agonist-bound PR to PRE's in vivo. This provides a mechanism for PR-RU486 to inhibit PR-agonist complexes in *trans* through competition for DNA sites. A second contributing mechanism is the ability of PR bound to antagonist to heterodimerize with PR bound to R5020. We showed by coimmunoprecipitation assay in vitro [17] and a mammalian two-hybrid assay with receptor ligand specificity mutants [37], that PR bound to RU486 can heterodimerize with PR bound to an R5020. Using electrophoretic mobility shift assay (EMSA), we also observed that mixed R5020/RU486 heterodimers had a significantly reduced ability to bind to PREs [17]. Our results were similar to the results of Meyer et al. [38], who found that mixed R5020/RU486 heterodimers could not bind to PRE's using EMSA. Heterodimerization could potentially sequester a portion of cellular PR bound to agonist in an inactive form, without requiring direct binding of RU486 to PR.

A third contributing factor to the potency of RU486 to antagonize PG is the ability of PR to recruit corepressors to promoters in the presence of RU486. PR in the absence of ligand, or presence of agonist, has weak affinity for corepressors. Nuclear receptor corepressor (NCoR) was identified by yeast two-hybrid assay as a factor that interacts with RU486 bound PR but not agonist bound PR [39]. Subsequent studies revealed a direct physical association between RU486-occupied PR-B and NCoR in vitro [40] as well as interactions with NCoR and silencing mediator for retinoic acid and thyroid hormone receptor (SMRT) in mammalian cells [41]. Evidence suggests that corepressor interaction is of functional consequence, as was shown by overexpression of NCoR and SMRT which suppressed the partial agonist activity of RU486-bound PR [39]. Also, mixed agonists, which function as weak agonists or antagonists depending on the cell and promoter context [42], induced an interaction of intermediate strength with corepressors [41] in a two-hybrid assay as compared to agonists and more pure antagonists. These observations support a model in which PR has a high affinity for SMRT or NCoR only in the presence of antagonists and that the conformational change induced by agonist's increases the affinity of the receptor for coactivators, an event which is incompatible with PR-corepressor interactions [41]. Fig. 3 depicts the different mechanisms proposed to contribute to the potency of RU486 and related compounds as PG antagonists. These include the ability of
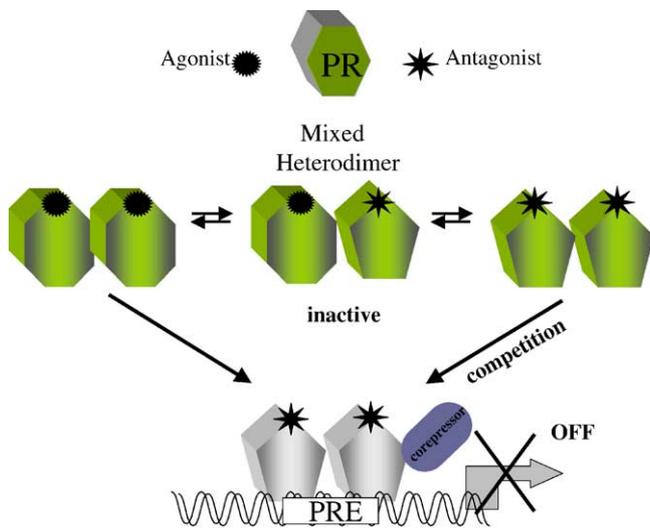
Fig. 3. Mechanism of "active" PR antagonists. PG antagonists compete with agonist for binding to PR and promote the activation steps of dimerization and binding to specific PREs of target DNA. However, antagonists induce an altered conformation in PR that is transcriptionally inactive, resulting in a non-productive interaction of receptor with DNA. Non-productive DNA-interaction is due to PR recruitment of corepressors instead of coactivators. In addition to this mechanism, the PR-antagonist complex can inhibit the PR agonist complex in *trans* by combined mechanisms of competing with the PR-agonist complex for binding to PREs and by dimerization with the PR-agonist complex to produce an inactive mixed ligand dimer (reproduced from [13]).

the PR-antagonist complex to inhibit PR complexed to PG in *trans* through heterodimerization and competition for binding to PREs, and for the PR homodimer-antagonist complex bound to PREs to recruit corepressors. This model has led to the concept that RU486 and other related steroidal compounds are "active PG antagonists."

2.4. Signal transduction cross talk potentiates partial activity of RU486

As with most steroid antagonists, RU486 is not a pure antagonist. RU486 exhibits partial agonist/antagonist activity under certain cellular conditions. The A and B isoforms of PR respond differently to antagonists; RU486-occupied PR-B can function as a partial weak agonist under certain cellular conditions whereas RU486-occupied PR-A cannot [38,43]. The partial agonist activity of RU486 is dependent on an intact AF-1 region of the receptor [38].

Our laboratory and the Horwitz group showed that the protein kinase A activator 8-bromoadenosine $3',5'$-cyclic monophosphate (8-bromo-cAMP), strongly potentiates the agonist activity of RU486 (and other related progestin antagonists) under cellular and target promoter contexts where RU486 is a complete antagonist [44,45]. However, 8-bromo-cAMP does not affect ZK98299 activity [44]. Also, RU486 in the presence of 8-bromo-cAMP is only partially effective in antagonizing R5020 action [44]. It should be noted that the activity of 8-bromo-cAMP is not restricted

to antagonists, since it will also potentiate the activity of agonist-bound PR [44,45]. The mechanism by which 8-bromo-cAMP potentiates PR activity has not yet been determined. However, it has become increasingly evident that the target of 8-bromo-cAMP activation is not receptor phosphorylation itself but receptor-interacting proteins. One such target may be the p160 coactivator SRC-1. A recent study with chicken PR (cPR) showed that 8-bromo-cAMP induced phosphorylation of two sites in SRC-1 and that this phosphorylation of SRC-1 facilitated interaction with and activation of cPR [46]. cAMP may also act by disrupting the association of PR with corepressors in the presence of RU486. It has been reported that cAMP dissociates, or inhibits, the interaction of antagonist bound PR with NCoR or SMRT in the mammalian two-hybrid assay [41]. Taken together, these results suggest that cAMP induced partial agonist activity of RU486 is due to disruption of PR corepressor interactions accompanied by facilitation of PR-coactivator interactions.

Because the partial agonist activity of RU486 is thought to be mediated primarily by AF-1, coactivators that bind AF-1 may contribute to this activity. We and others have shown that p160s and associated components of the coactivator complex (CBP and pCAF) can interact with the N-terminus of PR and mediate functional enhancement [47]. However, these associations with the N-terminal regions are much weaker than with AF-2, suggesting that AF-1 activity may be mediated by as yet undescribed proteins. SRA is a recently identified endogenous RNA transcript that functions as a selective coactivator for AF-1 of steroid receptors [48]. We recently identified a protein, Jun dimerization protein-2 (JDP-2) which functions as a PR N-terminal domain coactivator independent of AF-2 and p160s and can strongly potentiate the partial agonist activity of RU486 [49]. Although JDP-2 directly interacts with the DBD of PR, and not the N-terminus, it appears to act allosterically to recruit or stabilize other unknown coactivator interactions with AF-1 in the N-terminus.

It has become increasingly evident that the activity of steroid analogs is determined not only by the ligand and the receptor, but also by the coregulatory proteins and context of specific target gene promoters available in any given cell type. Because steroid antagonists are more effective inhibitors of AF-2 than AF-1, the relative balance between corepressors and AF-1 selective coactivators, may be more important than that of corepressors and AF-2 coactivators (Fig. 4). In theory, steroid antagonists can exhibit a broad range of tissue/cell specific agonist/antagonist activities dependent on the many possible combinatorial interactions between the conformation of receptor induced by ligand, the cellular availability of coactivators and corepressors and the composition of the accessible target gene promoters. Because of this tissue/cell selective activity of steroid antagonists, they have become more appropriately termed selective steroid receptor modulators; SERMs for ER modulators and SPRMs for PR modulators [50].
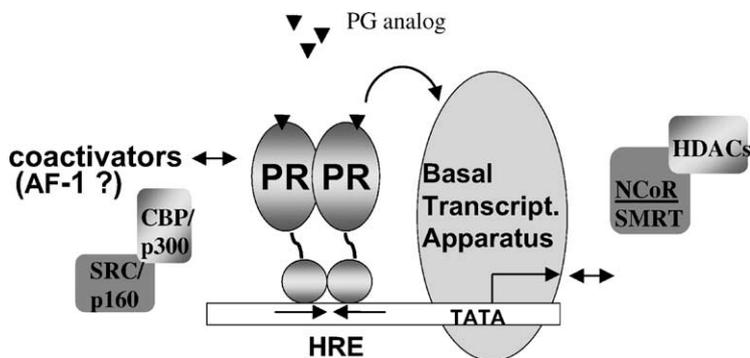
Fig. 4. Cell-specific factors that determine activity of PR in response to various ligands. PG agonist or antagonist (▶) bind PR and drives receptor to bind to promoters upstream of progesterone responsive target gene. The transcriptional activity of the receptor–ligand complex is determined by several cell specific factors. These include cellular availability of coactivators and corepressors and PR affinity for coactivators (SRC/p160 and CBP/p300) or corepressors (such as NCoR or SMRT and associated HDACs) that are determined by the specific ligand-induced conformation of the receptor. Additionally the nature of target gene promoters accessible to PR plays an important role. Shown is a consensus hormone response element (HRE; reproduced from [13]).

3. PR functions as an activator of signal transduction pathways

Not all effects of PG can be explained by the well-established roles of PR as transcription factor. There is increasing evidence that PG has rapid, membrane initiated effects independent of gene transcription to alter production of second messenger and cell signal transduction pathway. Rapid non-genomic actions of progesterone include PG-induced acceleration of *Xenopus* oocyte maturation [51,52], stimulation of acrosome reaction in sperm, modulation of neurotransmitter and neural excitability [53] and activation of Src/Ras/MAPK pathway in breast cancer cells [4]. Surprisingly, some of these rapid non-genomic effects of PG have been shown to be mediated through the same nuclear PR that regulates gene transcription [5]. Mechanisms of how nuclear PR can mediate the rapid non-genomic effects of PG are not well characterized.

3.1. PR contains an SH3 domain interaction motif that mediates rapid non-genotropic actions of progesterone on intracellular signaling pathways

Using PRA as bait, we screened a U2 osteosarcoma library by yeast two-hybrid assay for novel progestin-dependent interacting proteins. One of the proteins that interacted with PR was the Src-homology-3 (SH3) domain of the c-Cbl-associated protein (CAP). The interaction of PR with the SH3 domain of CAP was hormone dependent in the yeast two-hybrid assay and was determined by GST pull-down assays in vitro to be a direct protein interaction. A physical association between PR and select SH3 domains in mammalian cells was also observed by coimmunoprecipitation assay, either with endogenous proteins or with exogenous expressed proteins carrying tags for pull-down. PR-SH3 domain interaction in mammalian cells was progestin dependent, but was transient. Physical association was detected within a few minutes of hormone treatment

and was lost by 10 min [5]. The SH3 interaction site within PR mapped to the N-terminal domain common to PR-A and PR-B. Inspection of this region revealed a polyproline sequence motif between aa 421–428 (see Fig. 1) that conforms to a class II consensus peptide ligand for Src kinase like SH3 homology domains. SH3 domain peptide ligands are short contiguous proline sequences that form a left-handed helical conformation that recognizes the hydrophobic pocket of SH3 domains [54,55]. In the context of full length PR, we substituted three key prolines with alanines that are predicted to disrupt this motif. This mutant receptor (PR-BmPro) was expressed in baculovirus system, purified and compared with wild-type PR-B by GST-pull down assay for interaction with the SH3 domain of CAP. At the same time we extended analysis of PR-SH3 domain interaction to a range of SH3 domains containing signaling molecules including Src tyrosine kinase family members. PR-B interacts efficiently with the SH3 domain of CAP and a few other adaptor or signaling molecules, most notably with Src tyrosine kinase and related Hck. A protein chip analysis of SH3 domains from 48 different signaling/adaptor proteins indicated that PR interacts with only a subset of SH3 domains, and that most of the hits are of Src tyrosine kinase family members. Thus, PR-SH3 domain interaction is highly selective. SH3 domain interactions were abolished by the three-proline point mutations. A synthetic peptide (18 mer) containing the proline rich sequence motif (aa 421–428) plus a few flanking PR sequences effectively blocked interaction of PR with full length Src, whereas the same peptide with the three proline to alanine mutations had no effect [5]. These peptide competition results taken together with the effects of point mutations in the context of full length PR indicate that the proline rich motif is both necessary and sufficient for PR binding to SH3 domains. This ability to interact with SH3 domains of signaling molecules appears to be unique to PR. Only PR among the steroid hormone receptors contains an optimal SH3 domain interaction sequence. Other nuclear receptors tested as

purified proteins including ER, AR, GR, and TR, all failed to interact directly with SH3 domains of CAP and Src. AR does contain a polyproline sequence in the N-terminus, but it deviates from the consensus and did not in our hands make direct interaction [5].

Failure of PR-B (mPro) to interact with SH3 domains is not due to a global conformational change or instability of the mutant PR. In transfection experiments in mammalian cells, PR-B (mPro) was expressed at the same level as PR-B and exhibited the same hormone binding capacity. The mutant PR (mPro) also had the same affinity for specific PRE DNA as shown by EMSA with varying amounts of wild-type PR-B and PR-B (mPro), and it mediated a hormone-dependent induction of a PRE-controlled luciferase reporter gene to the same extent as PR-B in cell transfection assays (data not shown). These results define a previously unrecognized SH3 domain interaction motif in the N-terminus of PR that appears to be specific and separable from other functional domains of the receptor.

3.2. Functional consequence of PR interaction with the SH3 domain of Src: activation of Src/MAPK signaling pathway

Because of the central role of Src kinases in regulating a variety of cellular processes, including cell proliferation, cell cycle, differentiation, cell motility, and differentiation [54,55], we have focused our studies on the biological consequence of PR-SH3 domain interaction on Src signaling pathways. Src was also of interest because progesterone has been reported to stimulate a rapid activation of the Src/Ras-Raf/MEK-1/MAPK signaling pathway in breast cancer cells in a PR-dependent manner [4]. The rapid effect of progesterone on this signaling pathway was shown to occur independent of transcription and to contribute to the effects of progesterone on cell proliferation. The molecular

mechanism for this rapid non-genomic action of PR on Src signaling, however, was not defined.

We analyzed the functional consequence of PR-SH3 domain interaction on the activity of Src kinases, first in vitro and then in vivo. Src and closely related Hck are autoinhibited by intramolecular associations between the SH3 domain and a polyproline like helix in the linker region and between the SH2 domain and a tyrosine phosphorylation at 527 in the C-terminal tail [56,57]. Either of these intramolecular associations keeps Src and Hck in a closed inactive conformation (Fig. 5). Conversion to an open active conformation can be achieved by displacing the SH3 domain interaction with an external SH3 domain peptide ligand without dephosphorylation [58]. As a model for Src kinases we tested the ability of purified PR (bound to progestin) in vitro to activate closely related down-regulated Hck. To produce inactive enzyme, Hck was stoichiometrically phosphorylated on tyrosine 527 by CSK and then purified to homogeneity as previously described [57]. Down-regulated Hck exhibits no enzyme activity by itself. Addition of varying amounts of highly purified PR-B caused a dose-dependent activation of Hck. No significant difference was observed between PR-A and PR-B. Mutant PR (mPro), which is unable to bind SH3 domains in vitro, also failed to activate Hck at any concentrations tested, indicating that PR activates Src kinases through an SH3 domain displacement mechanism (Fig. 5). The apparent Kact for PR, which is the concentration that gives half maximal activation of Hck, was estimated from these curves as 28 nM. This places PR in the category of a potent activator of Src kinases. Kacts for other known Src activators, through SH3 domain displacement, have been reported to be in the 100–200 nM range [56–58].

We next examined the consequence of PR-SH3 interaction on Src kinase activity and signaling pathways in mammalian cells (in vivo) in reconstituted transfection systems. In Cos7 cells (PR negative) cotransfected with PR-B and Src, progestin stimulated a rapid activation of Src kinase without
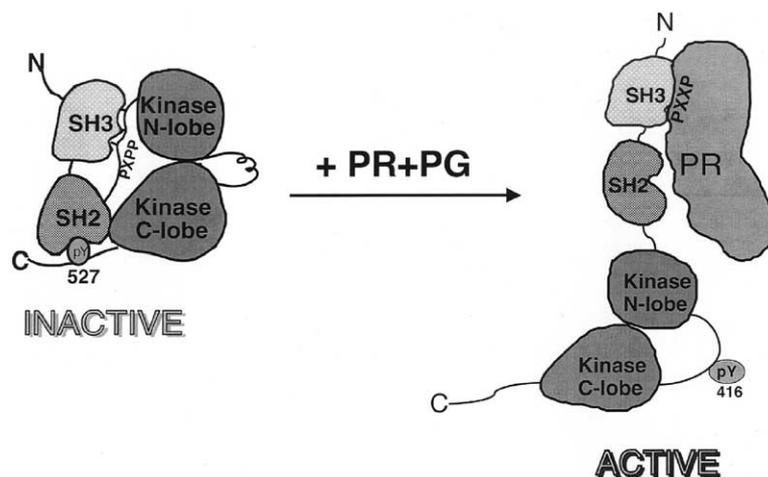

Fig. 5. Proposed mechanisms for PR activation of Src kinases. PR interacts directly with Src. The PXXP motif in the N-terminal domain of PR interacts with the SH3 domain of Src and converts Src from an inactive "closed conformation" to an active "open conformation" by an SH3 displacement mechanism.
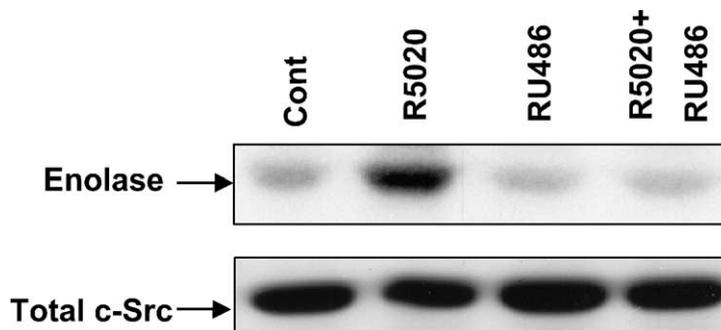
Fig. 6. Rapid progesterone activation of Src kinase signaling pathway is inhibited by PR antagonist, RU486. Cos7 cells were transfected with PR-B and Src kinase activity was measured before and after treatment of cells for 2 min with R5020, RU486, or R5020 + RU486 (all at 10 nM). Src kinase activity (upper panels) in cell lysate was assayed by ^{32}P incoporation into the substrate enolase and total Src protein levels (lower panel) were detected by immunoblot with Src specific antibody (327 antibody; reproduced from [5]).

any change in total Src protein. Src activity was measured by an immunoprecipitation kinase assay, using incorporation of ^{32}P ATP into enolase as a substrate. Activation induced by R5020 was rapid and transient with optimal induction occurring at 2–5 min, and decay of the response by 10 min. This effect is dependent on PR. In cells that lack PR, no progestin activation of Src was observed. In addition, the PR antagonist RU486 itself failed to stimulate Src kinase activity but effectively inhibited induction by the agonist R5020 (Fig. 6). As compared with wild PR-B that supports R5020 stimulation of Src kinase activity, no progestin activation was observed in cells expressing PR-B (mPro). Thus, the point mutations that abolished PR-SH3 interaction and Src kinase activation in vitro also abolished progestin activation of Src in vivo.

Progestin induced activation of Src is not dependent on PRs ability to function as a transcription factor. PR bearing a point mutation in the DBD that prevents binding to DNA, or in AF-2 that cripples transcription activity, are fully capable of mediating rapid progestin stimulation of Src kinase activity. A nuclear localization (NLS) mutant form of PR that localizes to the cytoplasm in the presence and absence of hormone is also fully capable of mediating rapid progestin activation of Src (not shown). To determine the consequence of PR-SH3 domain interactions on down stream effectors in the Src/Ras/Raf/MAPK signal pathway, we analyzed the influence of progestins on MAPK activation in MCF-7 breast cancer cells. In cells expressing PR-B, R5020 stimulated a rapid and transient activation of both p42/44 ERK 1 and ERK-2. Stimulation was 25% of the magnitude of EGF. In contrast, R5020 had no effect on MAPK activity in cells expressing mutant PR-B (mPro) or no PR. Thus, the integrity of the SH3 recognition motif in PR is essential not only for Src activation in cells but also for down stream MAPK activation. These data taken together indicate that rapid activation of Src through PR-SH3 domain interaction is an extra-nuclear function of receptor, completely separable from its function as a nuclear transcription factor.

To begin to explore whether PR activation of Src (or other signaling pathways) through SH3 domain interactions is of physiological relevance, we examined the role of this property of PR in mediating the effects of progestins on cell cycle arrest. In breast cancer cells, progestins stimulate a transient proliferation through one round of the cell cycle followed by cell cycle arrest. Prolonged treatment of progestins results in a significant decrease in the percentage of cells in S-phase and a concomitant increase in percent of cells in G1 [59]. In breast epithelial cells expressing mPro, progestin effects on cell cycle kinetics were substantially diminished as compared to cells expressing wild-type PR-B [5]. These data suggest that this non-genomic action of PR on cytoplasmic signaling pathways contributes to the overall effects of progesterone on cell cycle progress and proliferation.

4. Summary

Progesterone antagonists repress the biological actions of progesterone by "actively" inhibiting PR activation. PR bound to an antagonist can inhibit PR bound to an agonist in *trans* through heterodimerization and competition for DNA binding. Once bound to target gene promoters, the PR antagonist complex can recruit corepressors and actively repress gene transcription. Cell specific factors are also important determinants of the activity of PR. The agonist/antagonist activity of compounds like RU486 is thought to be a reflection of the balance between expression and availability of coactivators and corepressors and the context of specific target promoters available in any given cell type. The currently available PG antagonists have provided insights into the mechanism of action of PR and suggest that it may be possible to develop tissue/cell specific modulators of PR with minimal cross-reaction with GR and other steroid hormone receptors.

We propose that PR is a dual function protein capable of activating signaling pathways in the cytoplasm or at the cell membrane, and of functioning in the nucleus in its well-established role as a transcription factor (Fig. 7). Identification of mutations that separate these functions of PR provides the opportunity to further analyze the physiological
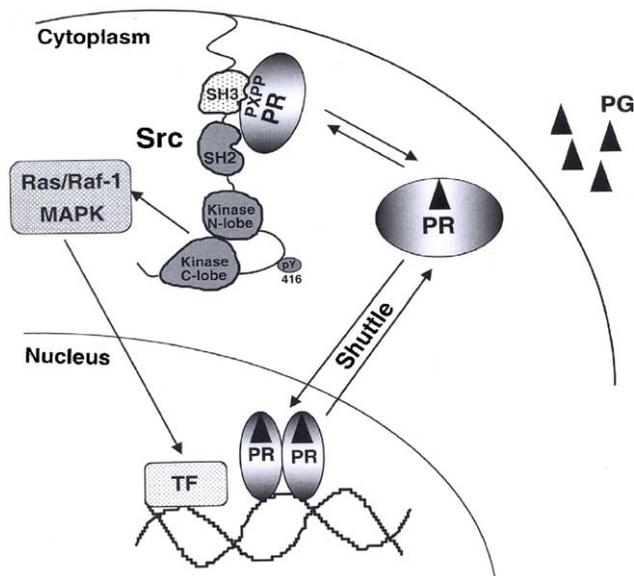
Fig. 7. Model for PR as a dual function protein. PR is proposed to function in the cytoplasm as an activator of Src signal transduction pathways and in the nucleus as a transcription factor (reproduced from [5]).

role of these two distinct PR signaling pathways in vivo. How PR, which is predominantly a nuclear protein, interacts with cytoplasmic or cell membrane signaling molecules in vivo is an important unanswered question. PR as well as other steroid receptors is known to actively shuttle between the cytoplasm and nucleus by active import and export mechanisms. This dynamic localization implies that PR is capable of encountering signaling molecules in the cytoplasm and of having extra-nuclear functions. The rapid extra-nuclear action of PR on cell signaling pathways may provide an alternative mechanism for PG to activate genes without direct receptor interaction in the nucleus. MAPKs are capable of activating nuclear transcription factors unrelated to nuclear receptors, raising the possibility that PR-SH3 domain signaling pathways may be a way for progesterone to regulate a different set of genes than the classical nuclear targets regulated by direct interaction with PR.

Acknowledgements

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