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Review

Integration of progesterone receptor action with rapid signaling events in breast cancer models☆

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Abstract

Recent discoveries suggest that several protein kinases are rapidly activated in response to ligand binding to cytoplasmic steroid hormone receptors (SRs), including progesterone receptors (PRs). Thus, PRs act as ligand-activated transcription factor "sensors" for growth factor-initiated signaling pathways in hormonally regulated tissues, such as the breast. Induction of rapid signaling upon progestin binding to PR-B provides a means to ensure that receptors and co-regulators are appropriately phosphorylated as part of optimal transcription complexes. Alternatively, PR-B activated kinase cascades provide additional avenues for progestin-regulated gene expression independent of PR nuclear action. Herein, an overview of progesterone/PR and signaling cross-talk in breast cancer models is provided. Kinases are emerging as key mediators of PR action. Cross-talk between SR and membrane-initiated signaling events suggests a mechanism for coordinate regulation of gene subsets by mitogenic stimuli in hormonally responsive normal tissues, and is suspected to contribute to cancer biology.

Keywords: Progesterone receptor; Epidermal growth factor; Mitogen activated protein kinase; Cyclin D1; Breast cancer; c-Src kinase

Contents

Abbreviations: AF, activation function; AR, androgen receptor; CDK2, cyclin-dependent protein kinase 2; DBD, DNA binding domain; EGF, epidermal growth factor; ER, estrogen receptor; H, hinge; HBD, hormone binding domain; Hsp, heat shock protein; MAPK, p42/p44 mitogen activated protein kinases; MEKK, MAPK/ERK kinase kinase; MEK, MAPK/ERK kinase; MMTV, mouse mammary tumor virus; mPR, membrane progesterone receptor; PR, progesterone receptor; PRE, progesterone response element; SERM, selective estrogen receptor modulator; SH2, Src-homology two domain (interaction with phosphotyrosine residues); SH3, Src-homology three domain (interaction with proline-rich regions); SR, steroid hormone receptor; SRC, steroid receptor coactivator; STAT, signal transducer and activator of transcription; TIFs, transcription intermediary factors; TRAPs, thyroid receptor-associated proteins (known as DRIPs, vitamin D receptor-interacting proteins).

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1. Introduction

The underlying molecular mechanisms of uncontrolled cellular proliferation, survival, and maintenance of breast cancer phenotypes are poorly understood. However, it is clear that dysregulation of estrogen and/or progesterone receptor action contributes to the development and progression of a majority of breast cancers. Steroid hormones and their cognate steroid receptors (SRs) exert direct effects in the nucleus as transcription factors. In addition, SRs function at the membrane and/or in the cytosol as mediators of growth factor-initiated signaling pathways. Recent observations indicate that membrane-associated SRs rapidly activate cytoplasmic signaling pathways as an alternative route for regulating SR-induced nuclear transcriptional events. This independent avenue for coordinating gene regulation occurs by the activation of cytoplasmic kinase pathways and independently of direct SR nuclear action. Recently, progestins have been recognized as mediators of increased post-menopausal breast cancer risk when taken as part of combined hormone replacement therapy relative to estrogen alone or placebo [1]. This review will examine PR-initiated genomic and nongenomic signaling pathways in breast cancer models with the purpose of identifying key kinases involved in two branches of one integrated pathway. Integration of rapid cytoplasmic signaling events with PR nuclear actions has important implications for breast cancer progression.

2. Classical actions of PRs

PRs are activated through binding with the ovarian steroid ligand, progesterone. PRs are classically defined as ligand-activated transcription factors that regulate gene expression by binding directly or indirectly to DNA. Three PR isoforms are the product of a single gene located on chromosome 11 at q22–23 that undergoes transcription via the use of alternate promoters and internal translational start sites [2]. PR isoforms consist of the full length PR-B (116 kDa), N-terminally truncated PR-A (94 kDa), and PR-C isoforms (60 kDa). PR-positive cells usually co-express PR-A and PR-B isoforms; these receptors have different transcriptional activities within the same promoter context, but can also recognize entirely different promoters [3,4]. PR-B is required for normal mammary gland development [5], while PR-A is essential for uterine development and reproductive function [6]. PR-C is devoid of classical transcriptional activity, and instead functions as a dominant inhibitor of uterine PR-B in the fundal myometrium during labor [7]. In the absence of progesterone, PRs are complexed with several chaperone molecules including heat shock protein (hsp) 90, hsp70, hsp40, Hop and, p23; these interactions are requisite for proper protein folding and assembly of stable PR–hsp90 heterocomplexes that are competent to bind ligand [8]. Hsps also function to connect PRs to protein trafficking systems. After binding to progesterone, the receptors undergo restructuring, dimerization, and hsp dissociation. Activated receptors bind directly to specific progesterone response elements (PREs) and PRE-like sequences in the promoter regions of such target genes as c-myc [9], fatty acid synthetase [10], and MMTV [11]. Treatment with progestin also results in an upregulation of regulatory molecules without classical PREs in their proximal promoter regions, such as epidermal growth factor receptor [12,13], c-fos [14,15], and cyclin D1 [16,17]. Without canonical PREs, PR regulation of these genes can occur through indirect DNA binding mechanisms, as in the example of PR binding to specificity protein 1 to promote p21 transcription in the presence of progestin [18]. PRs may also regulate genes by tethering to activating protein 1 [19] or signal transducers and activators of transcription (STATs) [15,20].

When either directly or indirectly bound to DNA, PRs regulate the basal transcription machinery in conjunction with nuclear receptor coregulatory molecules. Coregulators modulate transcription through chromatin remodeling and recruitment of transcriptional machinery (e.g., RNA polymerase-II). Histone acetyl transferases (HATs) and histone deacetylases (HDACS) function as coactivators and corepressors, respectively. Both HATs and HDACS coordinate transcriptional activity with other regulator proteins, including the ATP-dependent chromatin remodeling complexes (SWI/SNF), arginine methyltransferases (CARM1 and PRMT1), and histone kinases (reviewed in [21]).

3. PR and signaling cross-talk in breast cancer

Normal breast development requires ERα PRs, and growth factors. Estrogen stimulates ductal elongation, and progestins induce ductal sidebranching and alveologenesis [22]. Epidermal growth factor (EGF), in addition to promoting the proliferation of terminal end-buds, augments estrogen-induced ductal outgrowth and progesterone-induced sidebranching [23]. Indeed, estrogen induces PR isoform expression only in the presence of EGF [24], suggesting the existence of important cross-talk between EGFRs and both SRs. Ligand-activated PRs and ERs are potent breast mitogens, and mammary epithelial cells that express PR also express ERα. Moreover, estrogen is usually required in order to induce the expression of PR. For these reasons, separating the effects of progesterone alone from estrogen have been difficult. Consequently, the direct role of PR isoforms in breast cancer remains poorly defined relative to the role of ERα in breast development and breast cancer.

PR and ER are expressed by a minority of non-dividing epithelial cells in the lumen of the mature mammary gland. PR- and ER-positive cells constitute only ∼7–10% of the epithelial cell population in the normal adult mammary gland. This non-proliferative condition appears to be sustained by such inhibitory molecules as TGF-beta or high levels of p27, the CDK inhibitor (reviewed in [25]). In response to communication between stromal and epithe-

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lial compartments, SR-positive epithelial cells express and secrete pro-proliferative molecules, such as Wnts or IGF-II, thereby inducing the proliferation of adjacent SR-negative epithelial cells [25,26]. Recent data indicate that SR-positive cells in the breast may support the activity of nearby stem-like progenitor cells [27]. In contrast to the normal breast, where proliferating cells are devoid of SRs, the majority of newly diagnosed breast cancers (~80%) express ER and PR. The existence of SR-positive proliferating cells in breast cancer implies that SR-positive cells undergo an early switch to autocrine stimulation and/or SR-positive lineages continue to divide. Breast cancer is not the only setting where PR-containing cells divide. In an in vivo model of the mammary gland during pregnancy, PR-B colocalizes with cyclin D1 in BrdU-stained (dividing) cells [28]. Thus, signaling pathways involved in normal mammary gland growth and development are likely reactivated during breast cancer progression.

Clinical findings indicate that PRs may play a direct role in breast cancer. Progesterone induces the estradiol-primed endometrium into a secretory phase, and thus progestins are routinely given with estrogen hormone replacement therapy (HRT) in order to protect the uterus from the proliferative effects of unopposed estrogen action, thereby reducing the risk of uterine cancer. Progestins (via PR-A) are clearly inhibitory in the uterus, but play a proliferative role (via PR-B) in the developing breast [29]. Progestin therapy increases breast cancer risk when administered with estrogen as part of combined HRT; tumors were larger and of higher grade relative to estrogen alone or placebo [1]. Experimental data in mouse models of the post-menopausal breast indicate that progestins stimulate proliferation [30]. While progestins are not carcinogens, progesterone might induce recently initiated pre-cancerous breast cell populations to inappropriately re-enter the cell cycle or stimulate dormant stem cells to undergo self-renewal. Additionally, synthetic progestins used in HRT (MPA; medroxyprogesterone acetate) interact with androgen receptors (AR), and may act as endocrine disruptors of AR signaling, which is protective in the normal breast [31]. Indeed, AR is an important mediator of breast homeostatis, and may act primarily by induction of epithelial cell apoptosis or by direct inhibition of ERα-dependent signaling. It is thus critical to distinguish among the diverse actions of synthetic progestins (which interact with numerous SRs) relative to progesterone, the natural PR ligand.

Breast tumors develop resistance to endocrine-based treatments (anti-estrogens and/or aromatase inhibitors; androgens) as they progress. However, the majority (65%) of resistant breast cancers retain high levels of SRs (ERα, AR, and PRs). In these resistant, SR-positive cancers, the rapid action of SRs at the membrane might begin to inappropriately trigger the classical transcriptional activities of SRs. In this way, PRs activated by extremely low or sub-threshold concentrations of hormone or PRs phosphorylated in the absence of hormone can activate membrane-associated signaling pathways, including c-Src kinase, EGFR, and the p42/p44 MAPK pathway. Elevation of MAPK activity and downstream signaling frequently occurs in breast cancer, providing a strong survival and proliferative stimulus to breast cancer cells. MAPK signaling downstream of EGFR or Her2 (erbB2) is also associated with resistance to endocrine therapies [32].

4. Direct PR phosphorylation in breast cancer models

Similar to other SR family members, phosphorylation–dephosphorylation events add multi-functionality to PR action (Fig. 1). Several protein kinases phosphorylate PR isoforms primarily on serine residues within the amino-termini and, to a lesser degree, on serine residues throughout the receptor [2,33]. PR contains a total of 14 known phosphorylation sites (reviewed in [34]). Serines at positions 81, 162, 190, and 400 appear to be constitutively phosphorylated in the absence of hormone [35] (Fig. 1). One to two hours after progestin treatment Serines 102, 294, and 345 are maximally phosphorylated [36]. Specific kinases have been identified that are responsible for phosphorylation of selected sites. Serines at positions 81 and 294 are phosphorylated by casein kinase II [37] and mitogen activated protein kinase (MAPK) [38,39], respectively. Progestins can also stimulate Ser294 phosphorylation independently of MAPKs by activation of an unknown kinase(s) [40]. Eight of the total 14 sites (i.e., Serines 25, 162, 190, 213, 400, 554, 676, and Thr430) are phosphorylated by cyclin A/cyclin-dependent protein kinase 2 (CDK2) complexes in vitro [35,41]. Only five of these sites (i.e., Serines 162, 190, 213, 400, 676) are proven in vivo phosphorylation sites [35,37,41].

PRs receive signals from growth factor-initiated signal transduction pathways by way of phosphorylation–

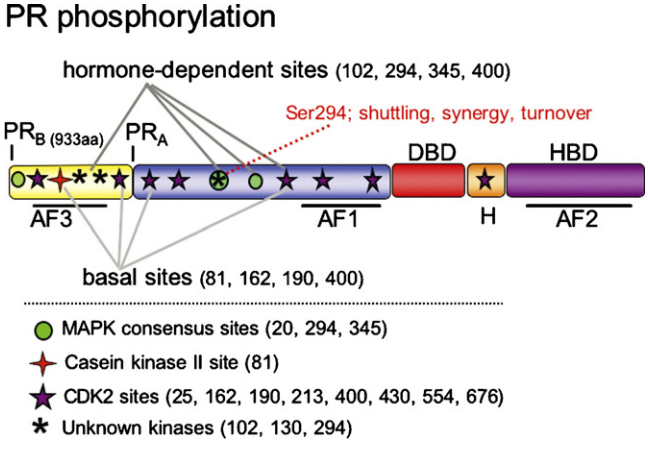

Fig. 1. Phosphorylation sites in human PR. PR phosphorylation. Thirteen serine residues and one threonine residue in human PR are shown, to represent basal (constitutive)- and hormone-induced phosphorylation sites [41] and may contribute to PR regulation by MAPK [38–40], casein kinase II [37], and CDK2 [35,41]. Individual PR phosphorylation sites may be regulated by multiple protein kinases [40] and/or in a sequential manner [88], illustrating the complexity of PR regulation by phosphorylation.

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dephosphorylation events. While the function of PR phosphorylation is incompletely understood, it might influence aspects of transcriptional regulation, such as interaction with co-regulators, as reported for ERα [42] and recently for PR [43]. PR phosphorylation is also involved in the regulation of ligand-dependent [39] and -independent [44,45] PR nuclear localization, receptor turnover, hormone sensitivity, and transcriptional activities [38,39,46,47]. As has been reported for ERα [48,49], phosphorylated PRs are hypersensitive relative to their underphosphorylated counterparts [50]. For example, following a brief (5–15 min) pre-treatment with EGF, phosphorylated nuclear PR-B receptors are transactivated by sub-physiologic progestin levels. EGF and progestins synergistically upregulate mRNA or protein levels for a number of growth regulatory genes [15], including cyclin D1 and cyclin E [12]; the regulation of cyclins by progestins is MAPK-dependent. Cyclins, in turn, regulate progression of cells through the cell cycle by interaction with cyclin-dependent protein kinases. Progestins activate CDK2 [17], and PRs are predominantly phosphorylated by CDK2 at proline-directed (S/TP) sites [35,41], perhaps allowing for the coordinate regulation of PR transcriptional activity during cell cycle progression. In support of this idea, Narayanan and co-workers [43,51] found that PR activity is highest in S-phase and lower in the G0/G1 phases of the cell cycle, but this activity is impaired during G2/M phases, concomitant with lowered PR phosphorylation. Overexpression of either cyclin A or CDK2 enhanced PR transcriptional activity; while cyclin A interacts with the N-terminus of PR, CDK2 seems to alter PR function indirectly by increasing the phosphorylation and recruitment of SRC-1 to liganded PR.

5. PR Ser294 phosphorylation in breast cancer models

PR Ser294 is rapidly phosphorylated upon exposure to ligand [36]. Ser294 is also a proline-directed or MAPK consensus site (PXXSP). Progestin-induced Ser294 phosphorylation occurs within 30–60 min independently of MAPK activation, whereas growth factor-induced Ser294 phosphorylation occurs within 3–5 min in a MAPK-dependent manner [40]. PR Ser294 is considered a significant site for PR regulation by multiple kinases [38–40,50]. Ser294 phosphorylation appears to mediate increased PR nucleocytoplasmic shuttling [40]. Rapid nuclear translocation of unliganded PR and nuclear export of liganded PR requires MAPK-dependent phosphorylation of PR Ser294 [40]. PR nuclear sequestration in response to MAPK activation might serve to protect inactive or active receptors from degradation in the cytoplasm or upon nuclear export [40]. Following ligand binding, PR undergoes rapid downregulation [52]. Phosphorylation of Ser294 greatly augments PR downregulation by making liganded PR a cytoplasmic target for ubiquitination and degradation by the 26S-proteosome pathway [38,40]. Mutant PR with alanine in place of serine at position 294 (S294A) binds ligand. In addition, mutant S294A PR, similar to wildtype PR, undergoes a characteristic upshift in gel mobility due to phosphorylation at other sites, enters the nucleus, and binds to PRE elements [38–40,50]. However, liganded S294A PR fails to exit the nucleus and undergo ubiquitination, and the receptor remains highly stable in the presence of progestins as compared to wt PR [38,40]. Interestingly, ligand-activated S294A PR is a weak transcription factor when stably expressed in breast cancer cells and fails to respond to agents that activate MAPK [39]. Conversely, generation of a Ser294 phospho-mimic receptor by replacement of Ser294 with aspartic acid (S294D) resulted in hyperactive progestin-induced transcription with increased PR turnover relative to wt PR [53]. Thus, reversible phosphorylation of PR Ser294 couples increased transcriptional activity to rapid downregulation of PR protein by the ubiquitin-proteosome pathway. Further investigation is required to determine whether the link between these events involves regulation of transcriptional events by components of the ubiquitin pathway and/or participation of nucleocytoplasmic shuttling factors or chaperones.

Recent studies support the conclusion that EGF-induced nuclear accumulation of PR is a key step in ligand-independent transcriptional activation. Labriola et al. [44] reported that exposure of T47D breast cancer cells to EGF family member, heregulin, can stimulate nuclear localization, DNA binding, and transcriptional activity of PR in the absence of hormone. Heregulin exposure also resulted in activation of MAPK and PR Ser294 phosphorylation. Qiu et al. [40] reported that PR Ser294 phosphorylation results in similar nuclear activity. However, growth factors alone failed to stimulate PR transcriptional activity or alter PR downregulation in T47D cell variants [39]. However, in the presence of ligand, MAPK activation greatly augmented both of these events [39,40]. One explanation for these apparently conflicting results is that differential expression of EGFR family members expressed on the cell surface between T47D cell line clones might lead to differences in the activation of downstream intracellular kinases, such as CDK2 (discussed below). In any case, these exciting data [40,44] suggest a continuum between PR hypersensitivity to extremely low ligand concentrations and complete ligand-independence, a phenomenon that is well-documented for AR or ERα. Regulation of PR by alternate signaling pathways, including elevated MAPK activity often exhibited by breast tumors, may contribute to disregulated gene expression and changes in cell growth and/or survival. For example, PR-B regulation of IRS-2 expression in breast cancer cells requires phosphorylation of PR Ser294 and occurs in the absence of ligand [50].

6. CDK2 regulation of PR by Ser400 phosphorylation

PR Ser400 is both basally phosphorylated and regulated by ligand in vivo, and CDK2 activity mediates Ser400 phosphorylation in vitro [35]. Progestins regulate CDK2 activity

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[17,54]. In addition to progestins, other mitogenic stimuli induce vigorous phosphorylation of PR Ser400 [45]. Therefore, Ser400 might play a role in the regulation of PR during the cell cycle. Indeed, in the presence of activated CDK2, phospho-Ser400 PR are located in the nucleus, suggesting that phosphorylation of Ser400 sequesters unliganded PRs [45]. CDK2 overexpression increased PR transcriptional activity in the absence or presence of progestin. Mutation of Ser400 to alanine (S400A) selectively blocked ligand-independent PR transcriptional activity with little effect on transcription induced in response to ligand-binding [45]. Therefore, CDK2 might positively regulate unliganded PR (i.e., heightened basal transcriptional activity) by mediating Ser400 phosphorylation, while augmenting the transcriptional activity of liganded PR, perhaps in cooperation with other CDK2 sites on PR [55] or its co-activators [43]. Interestingly, Ser400 is adjacent to a nine amino acid destruction (D)-box motif that might alter PR turnover [39]. Furthermore, activated CDK2 induces rapid PR downregulation in the presence or absence of progestins, while CDK2 inhibition blocks ligand-induced PR downregulation [45]. Although the mechanisms linking PR stability/turnover to transcriptional activity require further examination, a model is emerging in which selected SRs are regulated by protein kinases that primarily modulate receptor location or shuttling, leading to changes in transcriptional activity and/or protein turnover [45,50,56,57].

7. Extranuclear actions of PR

While the genomic effects of steroid hormone treatment are delayed by several minutes to hours (i.e., following transcription and translation), the extranuclear or nongenomic effects occur rapidly in only a few minutes. Progestin treatment of breast cancer cells causes a rapid and transient activation of MAPK signaling that is ER-dependent, but independent of PR transcriptional activity [58,59]. Migliaccio et al. [60] first reported that estradiol activates p60-Src kinase and MAPK in MCF-7 cells and that PR and ERα interact to stimulate p60-Src kinase in T47D cells [58]. Maximal activation of p60-Src kinase is observed within 2–5 min, and downstream activation of p42/p44 MAPKs occurs within 5–10 min of progestin treatment [58,59].

Human PR contains a proline-rich (PXXP) motif that mediates direct binding to the Src-homology three (SH3) domains of signaling molecules in the p60-Src kinase family in a ligand-dependent manner [59]. In vitro experiments demonstrate that purified liganded PR-A and PR-B activate the c-Src-related protein kinase, HcK; PR-B but not PR-A activates c-Src and MAPKs in vivo. PR-B with a mutated PXXP sequence prevents c-Src/PR interaction and blocks progestin-induced activation of c-Src (or HcK) and p42/p44 MAPKs. Furthermore, mutation of the PR-B DNA binding domain (DBD) abolished PR transcriptional activity without affecting progestin-induced c-Src or MAPK

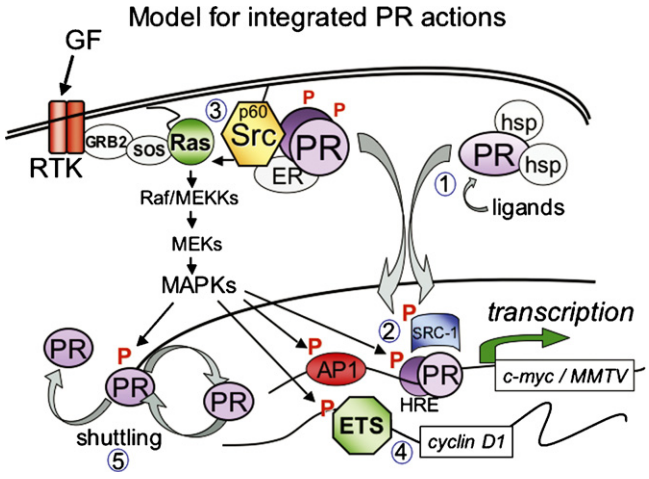

Fig. 2. Functional significance of PR phosphorylation. Phosphorylation (P) of specific sites in PRs couple multiple receptor functions, including transcriptional synergy in the presence of steroid hormones and growth factors predicted to activate MAPK and/or CDK2, and nuclear import or export (shuttling) in response to MAPK activation. Rapid ligand-dependent PR downregulation by the ubiquitin-proteasome pathway (degradation) occurs upon nuclear export. 1. Ligand binding mediates dissociation of heat shock proteins and nuclear accumulation of PR dimers. 2. Nuclear PRs mediate gene regulation via the classical pathway; phosphorylated PRs may recruit regulatory molecules that are phospho-proteins, and function in one or more inter-connected processes (transcription, localization, and turnover), perhaps linked by a common cellular machinery. 3. PRs and growth factors activate MAPKs independently via a c-Src kinase-dependent pathway, and this may result in positive regulation of PR action via "feed-back" regulation (i.e., direct phosphorylation of liganded PRs or co-activators), occurring in both the absence and presence of steroid hormone ligands and on PRE-containing or other PR-regulated gene promoters. 4. Activation of MAPKs by PRs provides for regulation of gene targets whose promoters do not contain PREs and are otherwise independent of PR-transcriptional activities but utilize PR- or SR-activated MAPKs, such as regulation of the cyclin D1 promoter by Ets factors. 5. MAPK regulation of PRs has been shown to mediate nuclear accumulation/shuttling and nuclear export that is coupled to regulation of PR transcriptional events.

kinase activation. Therefore, nongenomic MAPK activation by progestin/PR-B/c-Src complexes probably occurs by way of a c-Src-dependent mechanism involving Ras activation via phosphorylation of the c-Src substrate adaptor proteins p190 and/or Shc and followed by Grb-2 and Sos binding (Fig. 2).

Ballare et al. [61] reported that MAPK activation by progestins is blocked by anti-progestins and anti-estrogens in COS-7 cells transfected with both PR and ERα. They propose that c-Src/MAPK activation by PR is mediated indirectly by the interaction of the Src-homology two (SH2) domain of c-Src with phospho-tyrosine 537 of ERα [61]. In their model, activation of c-Src and the MAPK pathway by progestins depends upon the presence of unliganded ERα, which interacts constitutively with PR-B via two domains that flank the proline-rich sequence of PR. Deletion of either of these two ER-interacting domains in PR-B blocked c-Src/MAPK activation by progestins in the presence of ERα [61]. Mutation of PR-B's PXXP domain had no effect. In contrast, Boonyaratanakornkit et al. [59] found that ectopic PR expression

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increased basal c-Src activity in COS-7 cells in the absence of progestins and independently of added ER; co-expression of both PR-B and ERα reduced basal levels of c-Src activity. Under these conditions (i.e., low basal c-Src activity), progestin binding to PR-B clearly activated c-Src. In addition, progestins activated c-Src in PR-null MCF12A cells transduced with wt PR but not PXXP-mutant PR adenoviruses. Both groups found that ERα interacts with the SH2-domain of c-Src, but neither group tested the effects of estrogen on the ability of progesterone to activate c-Src or MAPKs [59,61].

Although discrepancies between the two models must be resolved, it is possible that overexpression of SRs in COS-7 cells leads to concentration-dependent effects resulting in the formation of different signaling complexes depending on the presence of other signaling and adaptor molecules. In support of this idea, Wong et al. [62] identified an additional ER-interacting "adaptor" protein, termed MNAR (modulator of nongenomic activity of estrogen receptor), that contains both LXXLL (nuclear receptor binding) and PXXP (SH3-domain binding) motifs. MNAR is essential for ER–Src interaction, but it is not required for progestin/PR-dependent activation of c-Src (D.P. Edwards, personal communication). In *Xenopus* oocytes, MNAR interacts with AR and appears to mediate inhibition of meiosis via Gβψ signaling; xMNAR enhanced AR transcription via c-Src kinase activation in CV1 cells [63]. Constitutive signaling via the AR/c-Src/MNAR complex occurs in androgen-independent prostate cancer cells, while transient signaling from this complex is regulated by AR ligand-binding in androgen-dependent cells [64]. A newly described protein, termed DOC-2/DAB2 (differentially expressed in ovarian cancer/disabled 2) was recently shown to antagonize AR-mediated prostate cancer cell growth by disruption of the AR/c-Src complex [65]. Taken together, these data indicate that multiple interactions contribute to direct protein kinase activation by SRs and suggest that at least some nongenomic signaling functions of amphibian PR have been conserved in mammals. Interestingly, a separate gene product encoding the putative mammalian homologue of mPR, a progesterone-binding G-protein coupled receptor first identified in spotted seatrout oocytes [66], has been described. Further studies are needed to determine if mPR plays a role in progestin-induced "rapid" signaling or if mPR interacts with classical PRs. However, studies with mPR underscore the important concept that binding proteins other than classical steroid receptors may regulate some nongenomic steroid-mediated signaling events.

8. Integration of rapid signaling and nuclear SR actions

While its role in mammalian physiology remains unclear, SR-mediated activation of cytoplasmic signaling molecules could theoretically serve to potentiate several nuclear functions of activated SRs (Fig. 2). One mechanism by which amplification of SR nuclear functions might occur is through rapid, direct phosphorylation of SRs and/or their co-regulators in response to activation of SR-induced cytoplasmic pathways that coincide with ligand binding. Clearly, such a positive feedback loop would explain the dramatic influence of activated signaling pathways on PR nuclear function. For example, several progestin-dependent functions of PR are MAPK-dependent, including upregulation of cyclins D1 and E, CDK2 activation, and S-phase entry [12,39,45,67].

Following ligand binding, most SRs stimulate a transient (3–10 min) activation of MAPKs. However, mitogenic signaling requires sustained (hours to days) MAPK activation in fibroblast cell models [68]. Recently, Faivre and Lange [69] found that in addition to rapid and transient activation of MAPK by progestin/PR-B (5–15 min), progestin-bound PR-B induced subsequent oscillations in MAPK activity that culminated in a sustained (hours to days) phase of MAPK activation that was EGFR- and c-Src-dependent. Further studies revealed the creation of an autocrine signaling loop in which PR-B triggered transcriptional upregulation of Wnt-1, leading to activation of frizzled-dependent MMPs and shedding of EGF ligands from the cell surface. This signaling cascade implicates Wnt-1-dependent transactivation of EGFR in response to progestins; PR-induced transcriptional upregulation of Wnt-1 and EGFR mRNA was sensitive to inhibition of MAPKs. Additional experiments demonstrated that progestin-induced cyclin D1 upregulation, S-phase entry, or soft-agar growth of T47D breast cancer cells was either blocked by shRNA targeted to Wnt-1 or inhibitors of MAPK, c-Src, and EGFR. Finally, progestins failed to stimulate S-phase entry in MCF-7 cells that stably express a PXXP-mutant PR-B, which is unable to bind to the SH3-domain of c-Src and activate MAPK [67]. Soft-agar growth of T47D cells stably expressing the same PR mutant (PXXP) was greatly attenuated [69]. In addition to c-Src and MAPKs, STATs are important effectors downstream of EGFR signaling. Progestins induce the tyrosine-phosphorylation and nuclear translocation of Stat5 [15] and Stat3 [20]. Proietti et al. [20] demonstrated that Stat3 phosphorylation and activation by the nongenomic actions of PR was a critical event for breast cancer cell growth; T47D cell growth and tumor growth of progestin-induced mammary adenocarcinomas in BALB/c mice was dependent on PR activation of Jak1 and Jak2, c-Src, and Stat3. Taken together, these data indicate that progesterone, via robust PR-B/c-Src signaling to MAPK, in combination with PR-dependent transcriptional events, upregulates and activates EGFR signaling to induce cell proliferation. Dysregulation of either arm of this pathway may contribute to uncontrolled proliferation of breast cancer cells.

The extranuclear actions of PRs may contribute to deregulated breast cancer cell growth [67] and/or increased breast cancer risk [1], perhaps by linking steroid hormone action to the regulation of MAPK-regulated genes (i.e., transcription factor targets of MAPK). Similarly, the extranuclear actions of liganded ERα are thought to induce a state of "adaptive hypersensitivity" during endocrine therapy in which growth factor signaling pathways are co-opted by upreg-

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ulated ERα [70]. In this model of ER-dependent MAPK activation, liganded ERα associated with the cell membrane interact with the adapter protein Shc and induce its phosphorylation, leading to recruitment of Grb-2 and Sos, followed by activation of Ras and the Raf-1/MEK/MAPK module. ERα activation of MAPK may explain why many tumors respond well to aromatase inhibitors, yet fail to respond to selective estrogen receptor modulators (SERMS) designed to inhibit ER transcriptional activity. SERMs can act as partial transcriptional agonists of phosphorylated receptors, and may not block ER-dependent MAPK activation [70]. In theory, PR-B or AR in SR-positive breast cancers could participate in MAPK-activating complexes, perhaps bypassing anti-estrogen therapies. Few groups have studied membrane-associated or cytoplasmic signaling complexes containing both ERα and PR-B or AR [71,72]. However, AR is frequently (70%) expressed in metastatic breast cancer [73], and expression of functional AR defines a subset of ER/PR-negative breast cancers [74]. These studies suggest that it will be important to target SRs that may substitute for ERα in the activation of c-Src-dependent mitogenic signaling cascades.

9. Integrated SR actions in gene expression

An important end-point of MAPK signaling is upregulation of cyclin D1. Cyclin D1 null mice exhibit deficiencies in mammary gland development, including specific defects in alveolar growth [75,76], a phenotype similar to adult female mice lacking PR-B [77]. Cyclin D1 mRNA and protein levels increase in response to estrogen, progesterone, or androgen treatment [17,78,79] and cyclin D1 is frequently elevated in breast and prostate cancers [80,81]. Interestingly, the D1a isoform of cyclin D1 acts as an androgen-induced transcriptional repressor of AR via direct binding to the AR amino-terminus [82]. However, the cyclin D1b variant promotes androgen-induced prostate cancer proliferation and is frequently overexpressed relative to D1a in prostate cancer cell lines and tumors [83].

Recent evidence suggests that SRs are often recruited to distal enhancer regions far upstream or downstream of hormone-regulated gene proximal promoters; distal HRE-containing elements function in association with pioneer-factor proteins that bind nearby to recruit and tether the distant SR complex to the proximal promoter via the creation of a chromatin loop [84,85]. Thus, SR recruitment to distant enhancer sites provides a mechanism of direct regulation of genes like cyclin D1 via the classical pathway (e.g., via SR-binding at putative distant HRE sites). As SR-driven tumors progress, membrane SRs may begin to function dominantly, leading to a switch in promoter regulation to MAPK-dependent induction via proximal promoter sites, or via post-transcriptional mechanisms that are also MAPK regulated [86]. This may explain how tumors escape the action of SR antagonists that primarily block transcriptional events, but may fail to inhibit the signaling functions of these receptors. In support of this idea, cyclin D1 expression is regulated by multiple SRs, perhaps via distant sites. However, transcriptional regulation of the cyclin D1 proximal promoter region by steroids (i.e., progestins or estrogens) is MAPK-dependent [67,87], as is progestin-induced sustained upregulation of cyclin D1 protein [69]. Thus, the activation of cytoplasmic signaling pathways by liganded-SRs not only provides enhanced SR action at specific SR-regulated genes via HRE sequences, but couples this to the regulation of additional gene products whose gene promoters clearly use SRs, but can also utilize SR-activated MAPK pathways independently of SR transcriptional activity to acheive sustained upregulation (Fig. 2).

10. Concluding remarks

Rather than acting in an obligatory or switch-like manner, phosphorylation events are generally considered to exert subtle effects on nuclear SRs, with kinase inputs primarily acting as a "rheostat" for a continuum of SR transcriptional activities. However, this conclusion is based largely on observations made with liganded receptors in the absence of controlled inhibition or activation of alternate signaling pathways. In fact, studies with human PR reviewed herein suggest that the effects of phosphorylation are quite profound in the context of multiple signaling inputs. We conclude that the phosphorylation status of a particular SR is a function of cellular kinase activities that coordinate SR responses to growth factors and steroid hormones. In the absence of alternate stimuli, independent activation of MAPKs by "extranuclear" liganded SRs may result in positive regulation of receptor action via "feedback" regulation by direct phosphorylation of SRs or their co-regulatory partners. This may theoretically occur in both the presence and absence of steroid hormone ligands and on diverse gene promoters and via distant sites in chromatin. In addition, activation of cytoplasmic kinase cascades including MAPK modules by liganded receptors provides for regulation of gene targets whose promoters can function entirely independently of SR transcriptional activities, but rely on the activity of MAPK-targeted transcription factors such as the Ets family members, Elk-1, c-myc, fos, and jun (components of AP-1). This important linkage provides for well-integrated control of a large number of genes or gene subsets coordinately regulated in response to convergence of growth factor and SR signaling. Finally, the newly discovered ability of SRs to activate kinase pathways classically defined as key regulators of cell growth underscores the concept that activation of signal transduction pathways is an integral feature of SR action. This aspect of SR function is likely to play an important role in cancer progression towards the development of resistance to endocrine therapies [70]. Targeting the relevant protein kinases (c-Src, MAPKs, and CDKs) as an integral feature of SR (PR, ER, and AR) action should provide significant improvements over the use of traditional SR blocking strategies for advanced or progressive breast cancers.

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