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MAP kinases couple multiple functions of human progesterone receptors: degradation, transcriptional synergy, and nuclear association☆

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Abstract

Breast cancers often have increased mitogen-activated protein kinase (MAPK) activity; this pathway influences breast cancer cell growth in part by targeting steroid hormone receptors. Bidirectional cross-talk between these two pathways is well documented; progestins increase the expression of type I growth factor receptors that couple to MAPK activation, and in turn, activation of p42 and p44 MAPKs increases ligand-dependent progesterone receptor (PR) transcriptional activity, and parodoxically, augments PR downregulation. Breast cancers that have become steroid hormone resistant often remain highly sensitive to growth factors. We believe that the mechanism of steroid hormone resistance is biochemically linked to the acquisition of growth factor responsiveness. Using in vitro models, we have established numerous regulatory links between signal transduction pathways elicited by peptide growth factors and PR. Of note is the role of phosphorylation of human PRs by MAPKs. Phosphorylation of PR on a key serine residue (Ser294) by MAPKs couples multiple receptor functions, including ligand-dependent PR downregulation by the ubiquitin–proteasome pathway, transcriptional synergy between progestins and growth factors, and nuclear localization of PR proteins. Linkage of these events suggests a mechanism for steroid hormone receptor "hypersensitivity" induced by growth factors. The uncoupling of these events during breast cancer progression is predicted to profoundly influence hormone responsiveness, as PR with altered stability may be driven primarily by upregulated growth factors.

Keywords: Progesterone receptors; Phosphorylation; Mitogen-activated protein kinase; Ubiquitin; Breast cancer

1. Bidirectional regulation of epidermal growth factor receptor (EGFR) and progesterone receptor (PR) signaling in breast cancer

The ovarian steroid hormones, estradiol and progesterone, are involved in breast cancer development, but at the time of diagnosis only one-third of tumors are steroid hormone dependent. As tumors progress, they are more likely to become resistant to endocrine therapies, yet often retain their nuclear steroid receptors. In fact, receptor loss or mutation accounts for only 10–20% of clinically observed steroid hormone-resistant tumors [1]. EGFR expression is significantly associated with loss of steroid hormone sensitivity regardless of receptor status [2]. Thus, it has been postulated that in the vast majority of resistant tumors, control over growth is assumed by locally acting autocrine or paracrine peptide growth factors; invasive cancers with the worst prognoses are those that are growth factor receptor positive and steroid hormone resistant [3].

EGF may also play a role in steroid hormone responsive breast cancers. Several studies have documented a transcriptional and/or proliferative synergy between EGF and progesterone or estrogen [4,5], and that progesterone upregulates EGFR expression on the cell surface [5–8]. We found that progesterone, acting through its nuclear steroid receptor, greatly potentiates the activities of several downstream signaling pathways that are initiated by growth factor receptors, including mitogen-activated protein kinases (MAPKs) [9,10]. Progestin-mediated biochemical changes in signaling molecules may contribute to a switch in the proliferative responsiveness of breast cancer cells from steroid hormone-dependent pathways to those activated by peptide growth factors [9,11]. Progesterone appears to act as a "priming factor" for the actions of secondary agents. In addition to increasing high affinity EGFR numbers, progesterone affects the phosphorylation state of both EGFR and c-erbB2 receptors [5]. Sarup et al. [8] reported that both the synthetic progestin, R5020, and progesterone increased EGFR

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number. Interestingly, during EGF-stimulated T47D cell growth, PR numbers but not affinity decreased, indicating bidirectional effects of each agent on the others receptor [8]. Upregulation of EGFR by progesterone in conjunction with downregulation of PR by EGF evokes a regulatory loop that may contribute to the loss of hormone responsiveness and simultaneous assumption of growth factor-mediated proliferation observed clinically during breast tumor progression. Growth factors act on PR signaling by: (1) enhancing PR transcriptional activity; and (2) driving the degradative loss of PR. In advanced breast cancers that are hormone resistant, yet still contain functional estrogen receptor (ER) and/or PR, abnormally stabilized but highly active steroid hormone receptors may be uncoupled from control of receptor downregulation; such stable receptors may drive gene transcription, but may respond only weakly to steroid receptor antagonists.

2. Steroid receptors are downstream targets of MAPK pathways

The mechanisms by which growth factors stimulate cell proliferation are complex (Fig. 1). Growth factor binding induces dimerization of tyrosine kinase growth factor receptors and autophosphorylation of tyrosine residues that are located in the cytoplasmic domains of the receptor dimer. This in turn stimulates binding to the receptor of specific regulatory proteins via their SH2 and SH3 domains, which then activate key signaling molecules, such as Ras, triggering downstream MAPK cascades (reviewed in [12]). For example, EGFR stimulation by either EGF or transforming growth factor-α leads to Ras activation. Ras (several family members exist) simultaneously activates cytoplasmic Ser/Thr protein kinases from both the Raf and MEK kinase (MEKK) families [13]. Raf and MEKK can independently phosphorylate and activate MAPK kinases (MEKs), leading to phosphorylation and activation of MAPKs. Expression of constitutively activated MEKK1 can lead to selective activation of other MAPK family members, including Jun kinase (JNK) [14] and p38 MAPK [15], most likely via activation of specific upstream MEK isoforms (Fig. 1). MEKK1, but not Raf, is a potent activator of the c-*myc* oncogene [16], one of the most common gene activations in breast cancer [17]. JNK, also known as stress-activated protein kinase (SAPK), and p38, are activated in response to stress stimuli such as UV irradiation and heat-shock [18]. These kinases are also activated by growth factors, including EGF.

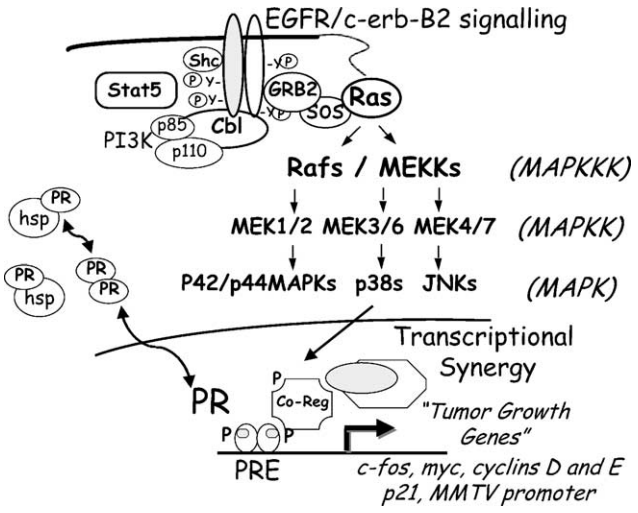

Fig. 1. Cross-talk between growth factors and progesterone receptors. Growth factor activation of EGF and c-erbB2 receptors leads to successive phosphorylation and activation of kinases in the MAPK cascade. MAPKs translocate into the nucleus and phosphorylate transcription factors, including steroid hormone receptors and/or their co-activators. Phosphorylation events lead to synergistic regulation of genes important for tumor cell growth.

One end point of MAPK cascade activation is the modulation of the phosphorylation state, and thus the activities of nuclear transcription factors that regulate early response genes, such as c-*jun*, c-*fos*, and c-*myc*. They in turn regulate the genes necessary for cell division. In breast cancer cells, this same set of genes is under steroidal control. For example, both c-*myc* and cyclin D1 are induced in human breast cancer cell lines stimulated to proliferate by estrogen and progesterone [19,20], while growth inhibition by antiestrogens or antiprogestins is accompanied by >80% decrease in c-*myc* mRNA [21]. Immortalized "normal" mammary epithelial cells transfected with c-*myc* are able to grow in soft agar following treatment with several growth factors, including EGF [22]. Additionally, both ER and PR are substrates for MAPKs [23,24]. Regulation of steroid hormone receptors by growth factor-induced signaling pathways may explain transcriptional synergy in response to EGF and progestins on several gene promoters (Fig. 1), including c-*myc* [15], cyclins D and E [25], c-*fos* and p21 [10], and MMTV [26].

3. Phosphorylation of steroid hormone receptors is an important regulatory mechanism

At least 14 serine residues on human PR are known to be phosphorylated in vivo, either basally, or as a result of hormonal stimuli and/or following protein kinase activation [27–31]. The significance of phosphorylation of these sites with regard to PR function remains largely undefined. Phosphorylation of human steroid hormone receptors is generally believed to positively or negatively modify their transcriptional activity rather than act as an "on–off" switch. Phosphorylation–dephosphorylation events may instead predominantly serve to "fine-tune" aspects of receptor regulation, integrating signals from other pathways by altering subcellular localization or trafficking of receptor complexes, protein–protein interactions, or degradation of receptor proteins [29,30]. However, the traditional view that phosphorylation of human steroid hormone receptors, including PR, is largely a means of accomplishing relatively subtle alterations in receptor regulation is changing, as novel phosphorylation

sites continue to be identified and characterized [27]. We were the first to define a role for phosphorylation of PR on Ser294 by MAPK in ligand-dependent receptor downregulation by the ubiquitin–proteasome pathway [24].

Similar to human PR, ER is also heavily phosphorylated. However, in contrast to PR, phosphorylation of ER-α is a well-known mechanism for the modulation of its transcriptional activity. Phosphorylation of Ser118, located in AF1, is mediated by MAPK in vitro and in cells treated with growth factors in vivo, and enhances the transcriptional activity of ER elicited by either estrogen or tamoxifen [23]. Lee et al. [32] recently reported that MEKK1 increased the agonist activity of ER-α induced by either estradiol or 4-hydroxytamoxifen in endometrial and ovarian cancer cells. Interestingly, this effect was mediated through activation of JNK and p38 MAPK, but not p42/p44 MAPKs. Although independent of known phosphorylation sites on ER-α, p38 MAPK efficiently phosphorylated the receptor in immunocomplex kinase assays in vitro [32]. Thus, in the presence of estrogen, ER undergoes a state of "hyperactivation" following growth factor stimulation of multiple MAPK pathways. Our results suggest that this regulatory paradigm also holds true for PR signaling [15].

4. PR is phosphorylated on Ser294 in response to progestins and MAPK activation

Using in vitro models, we have investigated the regulation of human PR by growth factor-initiated signaling pathways. Advanced breast cancers often contain constitutively active p42 and p44 MAPKs [33,34]. To mimic conditions of persistent MAPK activation, we overexpressed the constitutively active C-terminal kinase domain of MEKK1 [35] in HeLa cells together with the B isoform of human PR and a PRE-driven luciferase reporter construct (Fig. 2A). MEKK1 is a strong activator of MEK1 kinase activity in transient expression systems [13,15,35]. MEKK1 expression resulted in robust activation of p42 and p44 MAPKs (inset) and phosphorylation of PR on Ser294 in HeLa cells (Fig. 2C); JNK was not appreciably activated in these cells (not shown). MEKK1-mediated activation of MAPKs greatly increased PR transcriptional activity in the presence of the synthetic progestin, R5020 (Fig. 2A). MEKK1 expression typically resulted in a five- to eight-fold increase in ligand-dependent PR transcriptional activity compared to that in vector controls and also induced a slight increase in basal transcriptional activity; these results reproduced in T47D human breast cancer cells [15]. We (and others) have also reported transcriptional synergy between progestins and EGF on several non-PRE containing promoters [9,10,25]. Phosphorylation of PR Ser294 occurred in the presence of MEKK1 and correlated with increased PR transcriptional activity in response to R5020 (Fig. 2C). However, MEKK1 promoted nearly complete PR protein downregulation under the same conditions (Fig. 2B). Thus, an inverse relationship exists

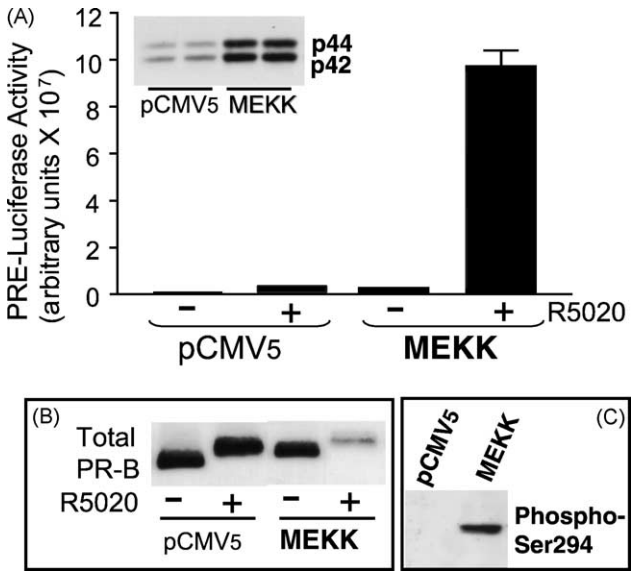

Fig. 2. Synergistic activation of PR by progestins and MAPKs. Triplicate cultures of HeLa cells were transiently transfected with PR-B, a PRE-luciferase reporter construct and either pCMV5 control vector or MEKK1. Cells were treated without or with R5020 (10 nM) for 24 h and luciferase activity in cell lysates was determined. Inset: MAPK (p42 and p44) activity was measured in cell lysates from cells expressing either vector control plasmid (pCMV5) or MEKK1. Total PR (B) and PR phosphorylated on Ser294 (C) in HeLa cells expressing either control vector or MEKK1 were visualized using monoclonal antibodies as described [15]. Adapted from Shen et al. [15].

between PR transcriptional activity and PR protein levels [15]. The MEK1/2 inhibitors, PD98059 and UO126, inhibited MAPK activation, phosphorylation of PR Ser294, and transcriptional synergy in response to R5020, indicating that these events are MAPK dependent [15]. These changes were reproduced using endogenous gene products known to be regulated by progestins and growth factors; during a state of heightened PR transcriptional activity, MAPK activation augments PR downregulation in the presence of progestins [15] (see Fig. 7A).

5. Transcription and protein stability are functionally linked

An inverse relationship exists between protein stability and the transcriptional activity of several ubiquitinated steroid hormone receptors, including ER-α [36], thyroid hormone receptor [37], and PR [15]. The classic view of proteasome-mediated protein downregulation predicts that ubiquitination serves to tag regulatory proteins for destruction by the multisubunit proteasome complex in order to rapidly attenuate the signal (i.e. transcriptional activity in the case of transcription factors). However, several recent studies suggest additional exciting functional roles for ubiquitination and/or proteasome subunits. Salghetti et al. [38] first noted overlap between the activation domains and destruction elements or "degrons" of several unstable

transcription factors, including E2F-1, fos, jun, and p53. Furthermore, a close correlation exists between the ability of an acidic activation domain to both activate transcription and signal proteolysis. Phosphorylation is a prerequisite for degron function of the yeast cyclins, Cln2 [39] and Cln3 [40]. These elements (i.e. degrons) activated transcription when fused to a DNA-binding domain [41]. Salghetti et al. [41] speculate that the negative charge conferred by phosphorylation of degrons mimics acidic activation functions. Thus, short-lived transcription factors may be destroyed because of their ability to activate transcription well; perhaps these processes, both triggered by a single phosphorylation event, are coupled through common cellular machinery.

6. PRs are substrates for the ubiquitin–proteasome pathway

Inspection of the PR primary sequence near Ser294 (in both A and B isoforms) revealed a nine-amino acid consensus motif known as a "destruction box" (Fig. 3A). This sequence (the D-box) was originally defined in A- and B-type cyclin molecules and is required for their rapid cell cycle-dependent degradation by the ubiquitin–proteasome pathway [42,43]. Enzymes called ubiquitin-ligases (E3 enzymes) bind to their substrates via the D-box and covalently link 76-amino acid ubiquitin molecules (or poly-ubiquitin chains) to distant (usually downstream) lysine residues (reviewed in [44,45]). In PR, the MAPK consensus site at Ser294 is nested within a D-box motif (Fig. 3A). We confirmed that PR are substrates for the ubiquitin–proteasome pathway by treatment of several breast cancer cell lines with lactacystin [24], a highly specific inhibitor of the 26S proteasome [46]. Ubiquitinated species of protein substrates for this pathway are relatively short-lived, but can be effectively "trapped" by addition of lactacystin (Fig. 3B). Thus, high migrating, ubiquitinated PR intermediates accumulate following a brief exposure to R5020 in lactacystin-treated cells (lane 4). We further showed that ligand-dependent PR protein downregulation was entirely blocked by lactacystin following long-term exposure to R5020 [24,47]. These data suggest that PR degradation by the ubiquitin–proteasome pathway is the primary means by which PRs are rapidly downregulated following ligand binding.

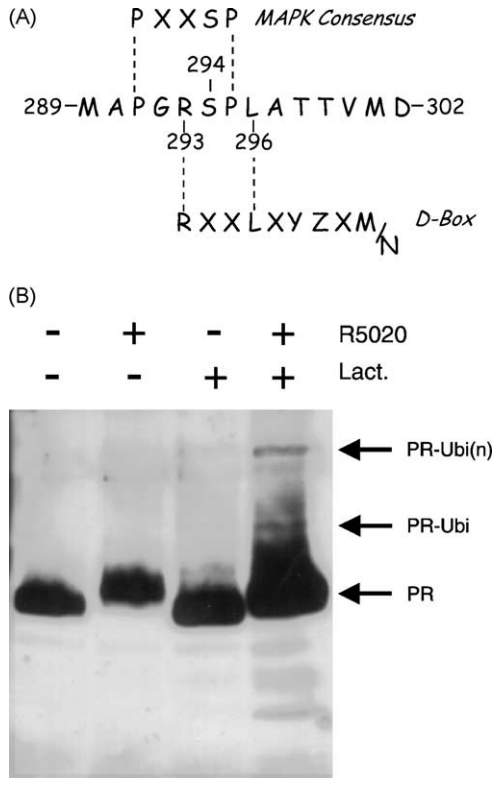

Fig. 3. PRs are substrates for the ubiquitin pathway. (A) PR contains a D-box motif. Ser294 is within a consensus MAPK phosphorylation site and lies between required residues (R293 and L296) in a consensus destruction box motif. (B) PR are ubiquitinated. T47D-YB cells were pretreated for 4 h without or with lactacystin, followed by R5020. PR were immunoprecipitated and visualized by immunoblotting with PR-specific antibodies. High molecular weight ubiquitinated forms of PR in immunoprecipitates are indicated by arrows.

We then tested the role of MAPKs in targeting PR to the ubiquitin–proteasome pathway. T47D-YB (stably expressing only the PR-B isoform) breast cancer cells were serum-starved to effectively "quiet" the MAPK pathway and then pretreated with MEK inhibitor prior to progestin treatment (Fig. 4). Similar to the results obtained with lactacystin [24], the MEK inhibitor blocked ligand-dependent PR downregulation, suggesting that this process is also MAPK dependent. The specificity of PR Ser294 phosphorylation as the "tag" for ubiquitination and ultimate PR destruction was confirmed directly by mutation of PR Ser294 to alanine. Serines at positions 344 and 345 of PR (also a MAPK consensus site) were also replaced by alanine as a control (S344/345A PR-B). Each PR Ser to Ala mutant was expressed stably in PR-null T47D cells and a time course of PR downregulation in response to R5020 was completed (Fig. 5A and B). Mutation of PR Ser294 to alanine (S294A) completely blocked the ability of the progestin to downregulate PR; S294A PR remained highly stable 24 h after R5020 treatment [15]. Similar mutations at Ser344/345 were without effect [24]. Co-immunoprecipitation experiments using HA-tagged ubiquitin and flag-tagged PR-B demonstrated that in contrast to wt PR, S294A PR fail to be ubiquitinated (Fig. 5C) and therefore are not targeted to the 26S proteasome [24]. These results demonstrate a clear role for phosphorylation of PR on Ser294 in ligand-dependent PR downregulation and suggest that MAPKs may directly regulate this site in vivo, although we have not ruled out a role for additional protein kinases in the regulation of this site [47].

7. PR stability and transcriptional activity are inversely related

In addition to altering protein turnover, mutation of PR Ser294 resulted in a functionally impaired receptor with

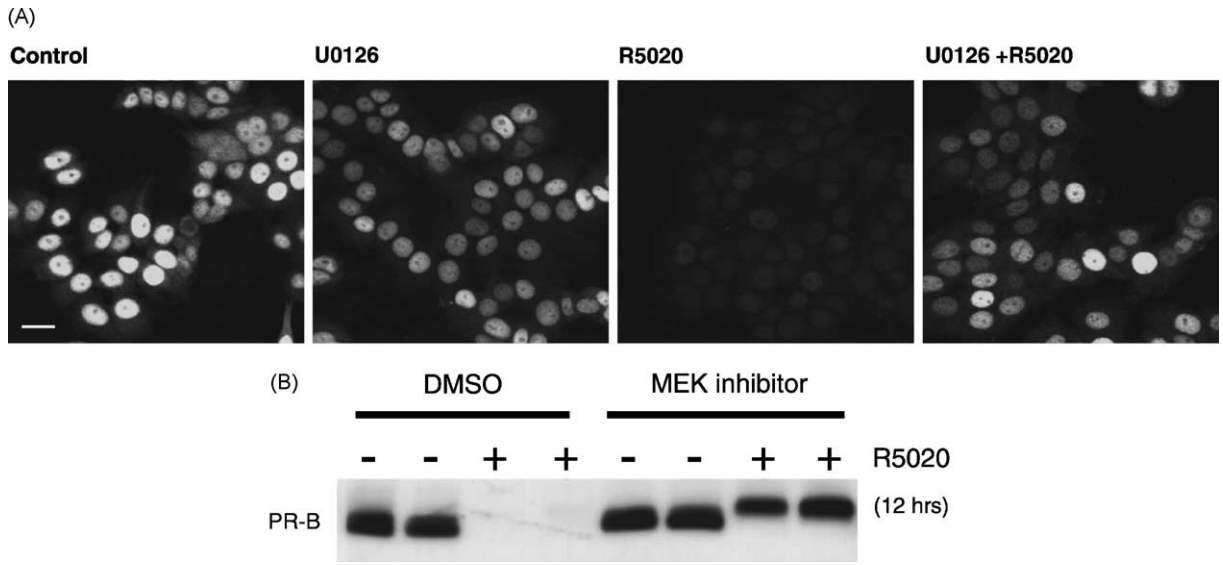

Fig. 4. Blockade of PR-B downregulation by the MEK1/2 inhibitor. (A) T47D-YB cells were cultured on cover-slips, placed in serum-free medium, and pretreated with DMSO vehicle (control) or U0126 (10–20 μM) for 30 min prior to treatment with either EtOH vehicle (control) or R5020 (10 nM) for 18 h. Immunohistochemistry was performed on fixed cells using PR-specific monoclonal antibodies. Scale bar: 20 μm. (B) T47D-YB were treated as in (A), except that R5020 was added for 12 h prior to cell lysis, and PR were visualized by Western blotting with PR-specific monoclonal antibodies as described [24,47]. Adapted from Lange et al. [24] and Qiu et al. [47].

weak transcriptional activity [15]. However, it is important to note that we initially examined the ability of transiently expressed wt and S294A PR to stimulate transcription from a PRE-luciferase reporter construct in several cell lines, including T47D, COS, and HeLa. Concentration curves performed in transcription assays following transient transfection of PR constructs revealed no significant differences in progestin-stimulated transcriptional activity between wt and S294A PR (Fig. 6A). Indeed, Takimoto et al. [48] have shown only modest effects of mutation of phosphorylated residues on the transcriptional activity of steroid hormone receptors using transient systems. Since our early studies [24] indicated that only stably expressed PR downregulate with reproducible kinetics that approximate that of endogenous PR in T47D and other breast cancer cells (Fig. 5A), we re-examined the transcriptional activity of S294A PR in stable expression systems [24]. Several experiments revealed a tight association between the rate of PR turnover and PR transcriptional activity, and that these aspects of PR function are inversely related. For example, we showed previously that wt PR are "stabilized" (i.e. resist ligand-induced downregulation) by several means, including following inhibition of the 26S proteasome with lactacystin, inhibition of p42/p44 MAPKs with MEK inhibitors (PD98059 or U0126; Fig. 4), inhibition of nuclear export (PR are degraded in the cytoplasm), and by mutation of PR Ser294 to Ala [24,47]. Each of these manipulations produces highly stable PR molecules that are paradoxically non-functional in that their transcriptional activity is weak or nil [15,47]. Interestingly, when expressed in COS cells, PR fail to downregulate in the presence of ligand. In contrast to other cell line models, PR expression actually greatly increases upon progestin treatment and transcriptional responses are actually blunted (i.e. instead of increased) in response to MAPK activation in COS cells (again recapitulating an inverse relationship).

Thus, to avoid the use of transient transfection systems, we have recently begun to examine the expression of endogenous gene products in cells stably expressing either wt or S294A PR-B. For example, expression of the c-*myc* oncogene is regulated by progestins in breast epithelial cells [49,50]. We have confirmed this result, and further demonstrated a synergistic effect in the presence of progestin and EGF [15] (Fig. 6B). Notably, PR (wt) protein is downregulated by the time increased c-myc protein is visible; PR protein loss is augmented in the presence of EGF and progestin, at which time c-myc protein expression is highest (Fig. 6B). Conversely, S294A PR fail to downregulate in all conditions (their expression may increase in response to EGF alone) and c-myc expression is very weak in comparison to cells stably expressing wt PR. We have recently expanded these results to other progestin-regulated gene products, including the signaling molecule, insulin-receptor substrate-1 (IRS-1), a PR-B regulated gene (A. Lee, personal communication). PR-null T47D cells lack IRS-1, as do cells expressing only the A isoform of PR, while cells expressing wt PR-B contain IRS-1 (Byron and Yee, unpublished results). Interestingly, however, cells expressing S294A PR-B are also devoid of IRS-1 protein and mRNA (Fig. 6C), indicating that unliganded PR may regulate the basal expression of certain genes via a mechanism that requires phosphorylation of Ser294. We are currently screening a large number of genes that may be regulated by PR in a ligand-independent, growth factor-dependent manner; examination of candidate genes

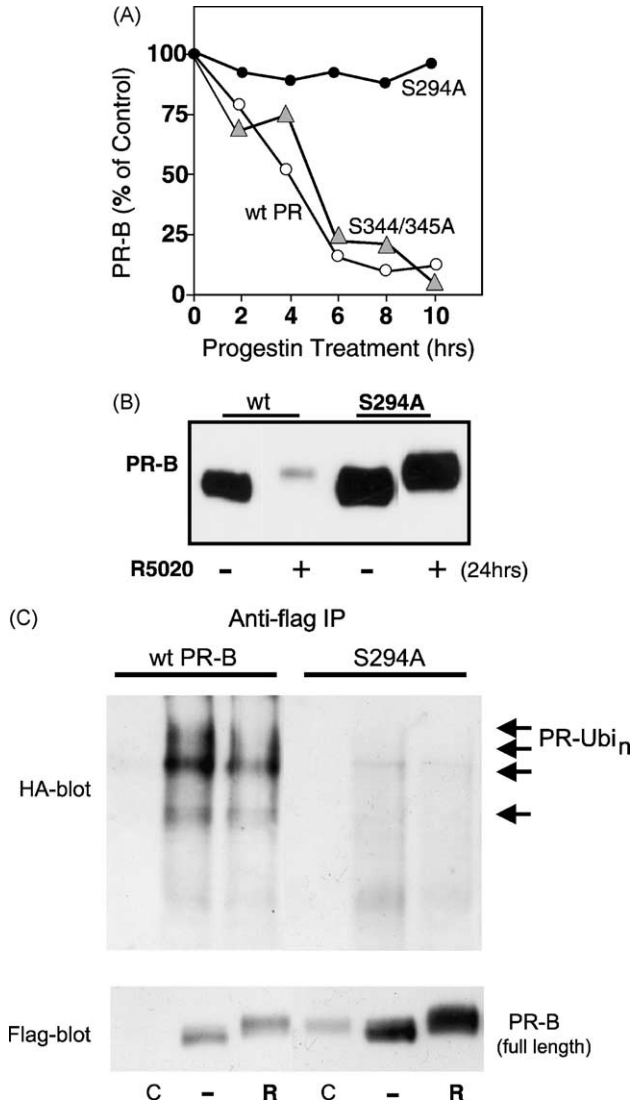

Fig. 5. Mutations of PR Ser294 blocks ligand-dependent downregulation. (A and B) S294A PR are highly stable. Wild-type PR-B (open circles), S344/345A (triangles), or S294A PR-B (closed circles) were expressed stably in T47D-Y (PR-null cells) as described [24], and treated without or with R5020 (10 nM) for 2–10 h (A; line graph) or 24 h (B; immunoblot). PR were visualized using monoclonal antibodies and quantitated using a phosphorimager. (C) S294A PR fail to be ubiquitinated. HeLa cells were transiently co-transfected with expression vectors encoding HA-tagged ubiquitin and either epitope-flag-tagged wt PR-B (wt-PR-B:flag, lanes 1–3) or epitope-flag-tagged S294A mutant PR (S294A:flag, lanes 4–6), and treated for 4 h without (lanes 2 and 5) or with R5020 (lanes 3 and 6). PR were immunoprecipitated using an anti-flag M2 affinity gel and visualized by immunoblotting with either HA- (upper blot) or flag-specific (lower blot) antibodies. Lanes 1 and 4: non-specific antibody and similar affinity gel. High molecular weight ubiquitinated forms of PR in immunoprecipitates from lysates of cells containing wt, but not S294A mutant PR-B, are indicated by arrows [24]. Adapted from Lange et al. [24].

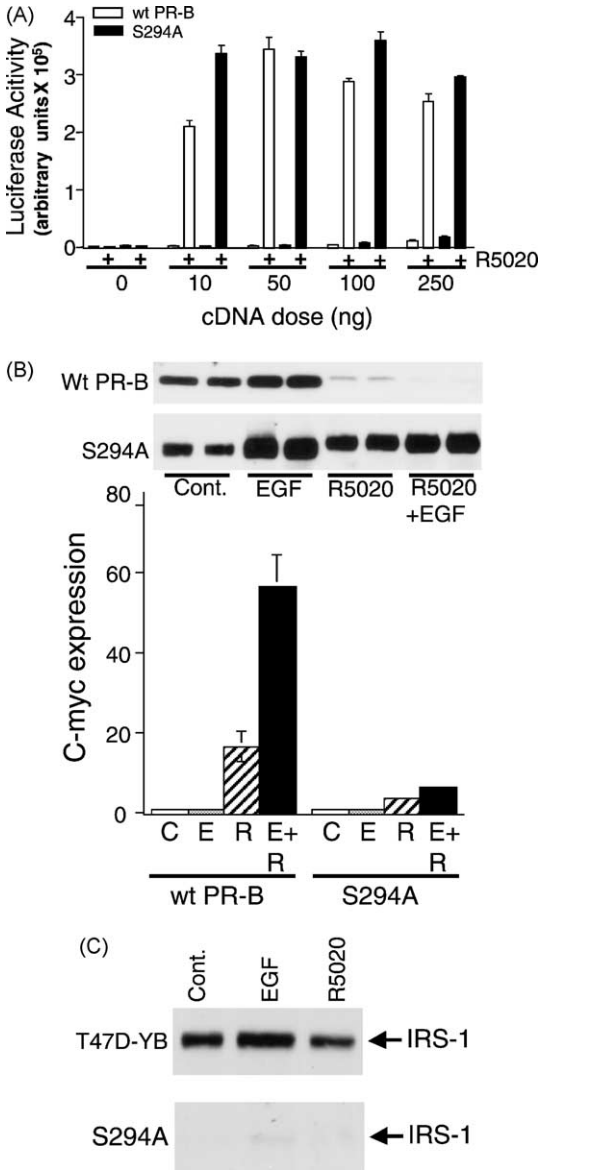

Fig. 6. Expression of wt and mutant PR. (A) S294A PR are functional in transient transfection assays. HeLa cells were transiently co-transfected with either wt or S294A PR-B and a PRE-luciferase reporter construct and then treated with R5020 for 24 h. Luciferase activity was measured in cell lysates as described [15]. (B) c-myc and PR expression are inversely related. Duplicate cultures of T47D-Y (PR-null) cells stably expressing either wt or S294A PR-B were treated with EtOH (control), EGF, R5020, or both agents for 6–12 h and PR and c-myc protein levels were measured using specific monoclonal antibodies as described [15]. The c-myc protein was quantitated using a phosphorimager and plotted as fold increase in total protein levels over control (the value of control is 1.0) as described [15]. (C) Wild-type but not S294A PR-B regulates basal expression of IRS-1 in T47D cells. T47D-Y (PR-null) cells stably expressing either wt PR-B or S294A PR were treated with EGF or R5020 for 24 h and IRS-1 was visualized in cell lysates using specific antibodies. T47D-Y cells are devoid of IRS-1 mRNA and protein (not shown). Results were repeated in several independently derived clones.

may reveal novel mechanisms of PR regulation of non-PRE containing gene promoters.

The idea that transcriptional activation and ubiquitin-mediated proteolysis are functionally linked by common regulatory elements is emerging as a potentially important

cellular control mechanism. This linkage most likely occurs at the transcriptional level. McNally et al. [51] reported a continuous exchange of liganded glucocorticoid receptors (GRs) with genomic targets and the nucleoplasmic compartment. Thus, the interaction of transcription factors with target sites in chromatin is a highly dynamic process. This exchange may provide liganded receptors the opportunity to interact with the cellular machinery required for their activation and subsequent degradation; indeed several points of interaction have been documented. The proteasome subunits, Sug1 and Sug2, interact with transcriptional activation domains [52–54] and Sug1 interacts with a subunit of the basal transcription factor, TFIIH [55]. The ubiquitin-protein ligases, hRPF1 [56] and E6-AP [57], have been shown to function as co-activators for liganded steroid hormone receptors, including PR. The E2 ubiquitin conjugating enzyme, Ubc9, is a positive regulator of GR activity [58]. Finally, histones are substrates for the ubiquitin–proteasome pathway and their ubiquitination correlates with increased transcriptional activity [59,60]. Most recently, ubiquitination has been shown to be required for transcriptional activity of the VP16 transactivation domain [61]. Thus, although their significance remains to be defined, it appears that complex interactions between regulatory molecules governing both transcription and ubiquitination/degradation exist.

Steroid hormone receptor transcriptional activity is presumably coupled to protein degradation by protein–protein interactions with co-regulatory molecules that also function in the ubiquitin–proteasome pathway. We favor a model whereby, in the presence of ligand, a co-activator(s) is recruited to steroid receptor complexes (see Fig. 9); this same factor either functions directly in the ubiquitin pathway, or associates with enzymes required for receptor ubiquitination [62]. Although some likely candidates exist [56,57], the binding of such a “coupling” factor(s) has not been demonstrated for human PR. However, the presence of a consensus “D-box” motif coincident with Ser294 hints at a mechanism involving protein–protein interactions. Thus, in human PR, it is possible that phosphorylation of Ser294 may induce the association of an analogous factor that is both an ubiquitin-ligase (or associated protein) as well as a transcriptional co-activator (see Fig. 9). Perhaps during tumor progression, breast cancer cells that still contain functional ER and PR find ways to circumvent steroid hormone receptor downregulation, allowing for their inappropriate activation by a continuous supply of growth factors. Alternatively, low levels of rapidly turning over receptors may be hypersensitive to growth factor stimulation.

8. Ser294 mediates PR nuclear association and hypersensitivity in response to EGF

Phosphorylation may facilitate PR nuclear localization [47]. Thus, it is possible that compartmentalization (i.e. following phosphorylation) of steroid hormone receptors within the cell may modulate the coupling of ligand-induced transcriptional activity and subsequent rapid protein degradation. Not surprisingly, therefore, a third functional correlate of S294A PR is related to its subcellular localization. Monoclonal antibodies to PR phospho-Ser294 [63] indicated that Ser294 phosphorylation increased following a 30–60 min exposure to R5020, as expected [64]. The time course of PR nuclear translocation is very similar to that of Ser294 phosphorylation, and is typically undetectable to modest after 30 min, but complete by 60 min of treatment with 10^{-8} M R5020 [47] (Fig. 7A). Confocal microscopy of intact T47D or HeLa cells stably expressing wt PR demonstrated that phospho-Ser294 PR was predominantly nuclear [47] (Fig. 7B). Thus, one possible explanation for loss of transcriptional activity of S294A PR is that it may fail to enter the nucleus following ligand binding. In fact, the opposite is true; S294A PR proteins persist in nuclei following their ligand-induced translocation and appear incapable of efficient nuclear export once liganded [47]. However, further imaging experiments using intact cells stably expressing either wt or S294A GFP-tagged PR clearly showed no major differences in PR subcellular localization in resting cells or cells treated with progestins (Fig. 7C). We showed previously that MAPK activation in response to MEKK1 or EGF also leads to robust phosphorylation of PR Ser294 [15] (Fig. 2). In contrast to the slow kinetics of ligand-induced Ser294 phosphorylation, this occurs within 5–10 min of EGF treatment and is MAPK dependent [47]. Thus, confocal microscopy revealed an additional novel role for Ser294 in ligand-independent regulation of PR nuclear association; wt PR, but not S294A PR rapidly (within 5 min) translocate in response to EGF (Fig. 7C); these events were inhibited by the MEK inhibitor (U0126) and repeated upon expression of constitutively active MEK1 (not shown), indicating that growth factor-mediated translocation of PR is truly MAPK dependent.

The functional significance of MAPK-dependent nuclear localization of unliganded PR is unknown. Although ligand-independent activation of PR transcriptional activity has been reported [65,66], it is generally quite modest compared to that elicited by progestin binding. We therefore reasoned that PR, once sequestered within the nucleus following growth factor receptor activation, might function more efficiently in response to steroid hormone ligands. That is, Ser294 phosphorylation may simply serve to rapidly localize PR to the correct subcellular compartment (i.e. the nucleus), resulting in a “hypersensitive” receptor. To test this idea, T47D or HeLa cells stably expressing either wt or mutant S294A PR were transfected with a PRE-driven luciferase reporter construct, serum-starved, and then treated with R5020 alone (in concentrations ranging from 10^{-12} to 10^{-8} M) or pretreated with EGF followed by R5020 (Fig. 8). At all R5020 concentrations, wt PR from EGF pretreated cells demonstrated significantly greater transcriptional activity compared to R5020 alone; the dose–response

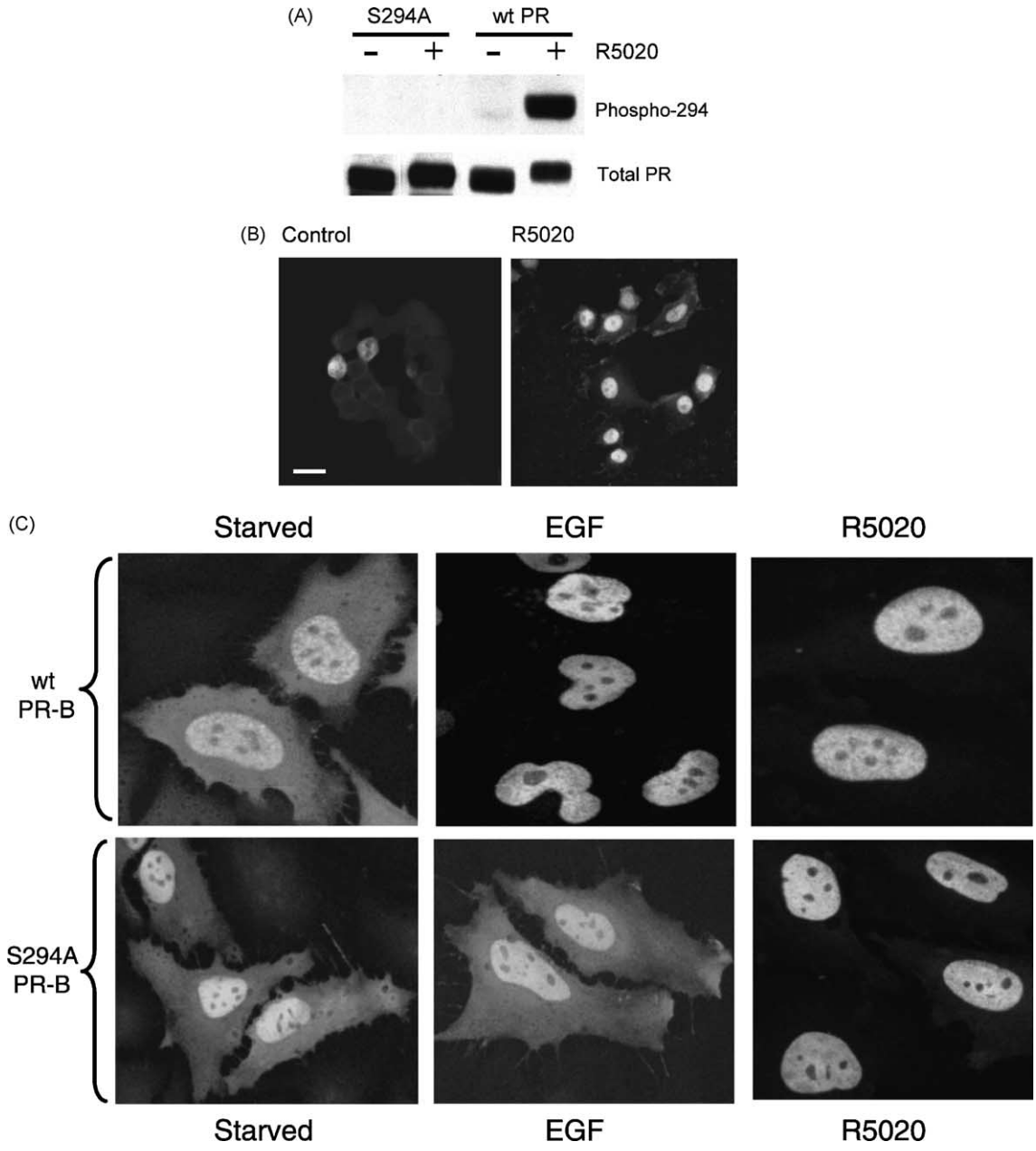

Fig. 7. Phosphorylation of PR Ser294. (A) R5020-induced phosphorylation of PR Ser294. T47D cells stably expressing wt or S294A PR-B were treated without or with R5020 (10 nM) for 2 h and PR-B protein levels in cell lysates (100 μg protein per lane) were measured by immunoblotting using either phospho-Ser294 (upper panel) or total (lower panel) PR-specific monoclonal antibodies. (B) Nuclear localization of phospho-Ser294 in T47D-YB cells. T47D-YB cells were cultured on cover-slips and treated for 2 h with EtOH vehicle (control) or R5020 (10 nM). Cells were fixed and subjected to IHC. Representative fields are shown from one of four independent experiments. T47D cells containing S294A PR-B were entirely negative (not shown). Scale bar: 20 μm. (C) Nuclear translocation of PR-B by EGF. HeLa cells stably expressing either wt GFP-PR-B or S294A GFP-PR-B were cultured on cover-slips, placed in serum-free medium (starved) for 24 h, and untreated or treated with either EGF (30 ng/ml) for 5 min or R5020 (10 nM) for 2 h. Cell cultures were fixed and PR were visualized using confocal microscopy as described [47]. Representative fields are shown from one of five independent experiments. Scale bars: 20 μm.

curve shifted to the left in the presence of EGF. S294A PR transcriptional activity was not appreciably altered under the same conditions. These results suggest that nuclear translocation in response to ligand binding (a normally slow process) may limit PR function at subphysiologic or "threshold" levels of steroid hormone ligand. Growth factors may bypass this required activation step by inducing rapid nuclear translocation, allowing for receptor activation by concentrations of ligand that are normally too low to stimulate receptor activity in the absence of other stimuli. This model demonstrates how steroid hormones may become extremely potent molecules in cells co-stimulated by growth factors. Overexpression of steroid hormone receptors in transient assays may mask these effects (Fig. 6A).

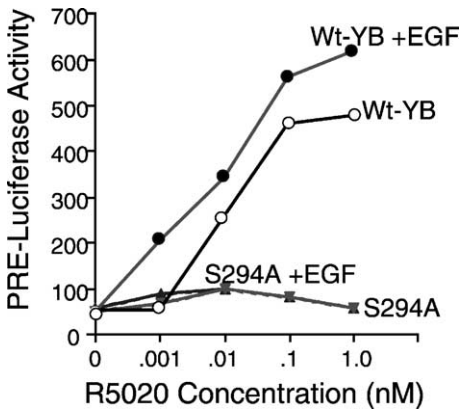

Fig. 8. Translocated PR are hypersensitive. HeLa cells stably expressing either wt or S294A PR were transiently transfected with a PRE-luciferase reporter construct and pretreated without or with EGF (30 ng/ml), followed by increasing concentrations of R5020 (10^{-12} to 10^{-8} M). Wild-type, but not mutant S294A PR-B were well activated by sub-threshold concentrations of R5020 following EGF pre-treatment.

9. Concluding remarks

Herein, we have illustrated how phosphorylation of a model steroid hormone receptor profoundly alters its function. Both steroid hormone and growth factor stimulation of cells leads to phosphorylation of the same sites in PR. Indeed, Ser294 appears to be a "hot-spot" for the regulation of several aspects of PR function by progestins as well as growth factors (Fig. 9). Progesterone can have either growth stimulatory or inhibitory effects depending on the target tissue (i.e. inhibitory in the uterus, proliferative in the breast) and the presence of other steroid hormones, such as estrogen [67,68]. Growth factors are likely to significantly influence PR regulation in settings where they are abundant, such as the developing breast [67,69] and during breast cancer progression [2,70]. For example, EGF potentiates the proliferative effects of progesterone and estrogen, and causes ductal side branching and lobuloalveolar development of mature mammary gland [69]. Furthermore, human clinical studies show that the addition of a progestin to hormone replacement therapy enhances markedly the risk of breast cancer relative to estrogen use alone [71]. Thus, it is important to define how these key signaling pathways interact in breast epithelial cells, where in addition to steroid hormones, IGF, EGF and heregulin are physiologically important mediators of normal breast cell growth and development [17], and are implicated in the loss of growth control that characterizes breast cancer tumor progression [2]. These pathways (steroid hormone receptors, kinase cascades, and the ubiquitin–proteasome complex) make excellent targets for chemotherapeutic intervention and may soon be exploited as part of future combination therapies.

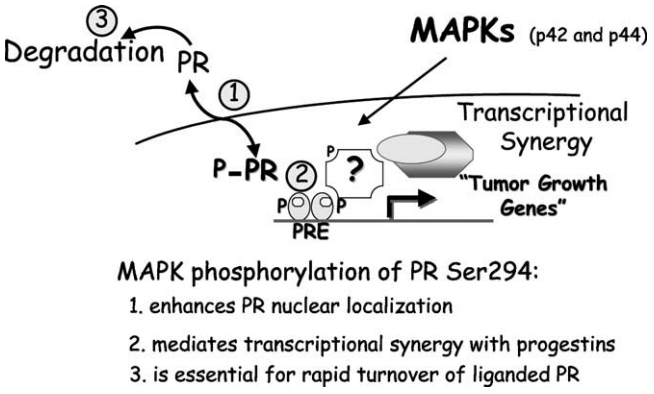

Fig. 9. Regulation of PR Ser294 by progestins and growth factors. Phosphorylation of PR Ser294 couples multiple PR functions, including nuclear localization (step 1) in the presence of growth factors, transcriptional synergy during MAPK activation (step 2), and rapid ligand-dependent PR downregulation by the ubiquitin pathway (step 3); PRs are degraded in the cytoplasm [47]. Phosphorylated PR may recruit regulatory molecules that function in one or more of these inter-connected processes (i.e. multiple PR functions may be coupled by one or more binding proteins that are part of a common cellular machinery).

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