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Mitogen-Activated Protein Kinase Regulates Nuclear Association of Human Progesterone Receptors

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Breast cancers often have increased MAPK activity; this pathway may drive breast cancer cell growth by targeting steroid hormone receptors. MAPK phosphorylates human progesterone receptors (PRs) on Ser294, thus regulating several aspects of PR activity. To study the role of PR Ser294 phosphorylation on subcellular distribution, we stably expressed wild-type (wt) or S294A (Ser294 to Ala) PR-B in several cell types. PRs phosphorylated on Ser294 were nuclear. Activation of MAPK induced Ser294 phosphorylation and rapid nuclear translocation of wt, but not S294A, PR-B; both receptors concentrated in the nucleus after progestin treatment. The MAPK kinase inhibitor, U0126, blocked epidermal growth factor but not progestin-induced Ser294 phosphorylation and translocation of wt PR, indicating a novel mechanism for nuclear localization. After progestin treatment, wt PR-B underwent ligand-dependent down-regulation, while S294A PR-B persisted in nuclei. Prolonged treatment with U0126 or the nuclear export inhibitor, leptomycin B, promoted nuclear accumulation of wt PR-B and blocked ligand-dependent PR down-regulation, suggesting that PR degradation occurs in the cytoplasm and requires MAPK-dependent nuclear export. Stabilization of PRs by leptomycin B also blocked PR transcriptional activity, indicating a link between nucleocytoplasmic shuttling, receptor stability, and function. These results support a regulatory role for MAPK in nuclear steroid hormone receptor subcellular localization and coupling to multiple PR functions. (*Molecular Endocrinology* 17: 628–642, 2003)

PROGESTERONE RECEPTORS (PRs) are members of a large family of nuclear steroid hormone receptor transcription factors that reside in both the nucleus and cytoplasm, but undergo continuous nucleo-cytoplasmic shuttling (1). This is a dynamic process, in which the majority of protein is nuclear in the absence of hormone. Closely related glucocorticoid receptors (GRs) also shuttle, but are largely cytoplasmic in the absence of ligand and translocate to the nucleus upon hormone binding. Regardless of their intranuclear location, ligand binding is thought to be required for PRs to interact with target genes. Although several transcription factors are known to be shuttling proteins, the functional significance of shuttling is not well understood. Shuttling could contribute to the specificity of gene regulation by allowing for communication with, or sequestration from, signaling pathways the protein mediators of which are compartmentalized. Recently, shuttling has been implicated in control of protein degradation by the ubiquitin-proteasome pathway (2).

Human PRs are substrates for the ubiquitin-proteasome pathway (3). After ligand binding, PR protein is rapidly and extensively down-regulated. In breast cancer cells, the majority of PRs are degraded 6–8 h after progestin treatment. Specific inhibitors of the 26S proteasome block this process, and ubiquitinated PR species accumulate in cells (3). Inhibitors of p42 and p44 MAPK also block PR down-regulation by progestins. PRs are known to be phosphoproteins and their ligand-induced phosphorylation has been well characterized (4, 5). We mapped the signal for ligand-dependent PR degradation to phosphorylation of PR Ser294 (3), a MAPK consensus site known to be predominantly phosphorylated in response to ligand binding (5). Mutation of Ser294 to alanine (S294A) resulted in highly stable PR incapable of progestin-induced ubiquitination and subsequent degradation by the 26S proteasome (3). Thus, in the presence of progestins, phosphorylation of PR on Ser294 by MAPKs mediates ubiquitination and targets PR for ultimate degradation by the 26S proteasome. It is un-

Abbreviations: cPR, Chicken PR; DMSO, dimethylsulfoxide; EGF, epidermal growth factor; ER, estrogen receptor α; GFP, green fluorescent protein; GR, glucocorticoid receptor; IHC, immunohistochemistry; LMB, leptomycin B; MEK, MAPK kinase; PR, progesterone receptor, PR-B, PR B-isoform; S294A PR-B, PR B-isoform containing a point mutation in which Ser294 is changed to Ala; NES, nuclear export signal; NLS, nuclear localization signal; PRE, progesterone-responsive element; RT, room temperature; STAT5, signal transducer and activator of transcription 5; wt, wild-type.

known whether PR ubiquitination and/or subsequent degradation occur in the cytoplasm or the nucleus.

The classic view of proteasome-mediated protein down-regulation predicts that ubiquitination serves to tag regulatory proteins for destruction by the multisubunit proteasome complex to rapidly attenuate the signal (*i.e.* transcriptional activity in the case of transcription factors). However, several recent studies suggest other exciting functional roles for ubiquitination and/or proteasome subunits. Ubiquitination has been shown to be required for transcriptional activity of the VP16 transactivation domain (6). In addition, elongation of mRNA transcripts by RNA polymerase II requires the 19S regulatory particle of the 26S proteasome (7). Human estrogen receptor-α (ERα) is ubiquitinated in the presence of ligand (8, 9), and an inverse relationship exists between protein stability and the transcriptional activity of ERα (10) and thyroid hormone receptors (11). Similarly, PRs that are capable of efficient turnover serve as strong transcriptional activators (12). Phosphorylation of PR Ser294 by agents that activate MAPK result in greatly heightened transcriptional activity in the presence of progestins and, simultaneously, augment PR down-regulation. In contrast, mutant S294A PRs stably expressed in breast cancer cells are incapable of undergoing ligand-dependent down-regulation (3, 12) and only weakly activate transcription (12). Thus, phosphorylation of PR on a unique serine residue (Ser294) by MAPK tightly couples PR transcriptional hyperactivity to PR protein down-regulation. This linkage perhaps ensures that highly active receptor transcription factors are destined for ultimate destruction. Considering that MAPK activities are often elevated in advanced breast cancers, loss of this functional coupling may contribute to altered gene expression and acquisition of steroid hormone resistance characteristic of breast cancer tumor progression. Although the mechanism(s) are unknown, steroid hormone receptor transcriptional activity is presumably coupled to protein degradation by protein-protein interactions with coregulatory molecules that also function in the ubiquitin-proteasome pathway (13, 14). Another possibility is that compartmentalization of steroid hormone receptors within the cell may modulate the coupling of ligand-induced protein degradation and transcriptional activity; phosphorylated receptors may be sequestered into or out of nuclear compartments during nucleo-cytoplasmic shuttling. The latter may explain why mutant S294A PRs are weakly active compared with wt receptors (12).

The effect(s) of phosphorylation on the cellular distribution of PRs are currently unknown. Herein, we studied the role of MAPK on PR nuclear localization by direct examination of intact cells stably expressing either wild-type (wt) or mutant S294A PR-B. PRs phosphorylated on Ser294 are nuclear, and treatment of cells with epidermal growth factor (EGF), a strong activator of MAPKs, results in Ser294 phosphorylation and rapid nuclear import of wt, but not mutant, PR, revealing a ligand-independent mechanism of PR nuclear association. Furthermore, PR nuclear export (during shuttling) is delayed by inhibition of MAPK, and blockade of nuclear export stabilizes PR in the presence of ligand and inhibits its transcriptional activity. These results suggest a functional role for MAPK in PR nucleo-cytoplasmic shuttling. Regulation of nucleo-cytoplasmic shuttling of PR by MAPK may serve to couple PR protein stability to regulation of transcriptional activity.

RESULTS

PRs Phosphorylated on Serine 294 Are Nuclear

Although PRs are basally phosphorylated on multiple serine residues including Ser294, progestin treatment results in additional rapid phosphorylation, predominantly on Ser294 and Ser345 (5). Due to ligand-induced down-regulation, PRs phosphorylated on Ser294 are relatively short-lived species (12). To analyze PR phosphorylated on Ser294 in the absence and presence of ligand, we used monoclonal antibodies specific to PR phospho-Ser294 (15). T47D cells stably expressing wt PR-B were untreated or treated with R5020 for 5 min to 2 h. Western blots indicated that the PR monoclonal antibody was highly specific for PR phoshorylated on Ser294 (Fig. 1A). A faint band of phospho-Ser294 wt PR-B was visible in the absence of ligand. R5020 induced robust Ser294 phosphorylation of wt PR-B compared with untreated controls or to mutant PR lacking Ser294 (S294A PR). Phosphorylation of Ser294 was detectable after 30 min and was maximal after 60 min.

To visualize the subcellular localization of phospho-Ser294 PR, cells expressing wt PR were treated with R5020 for 120 min, fixed, permeabilized, and subjected to immunohistochemistry (IHC) using antibodies to phospho-Ser294 PR (Fig. 1B). Confocal microscopy indicated that no staining is observed in cells expressing the phosphorylation-deficient S294A mutant. The majority of untreated cells expressing wt PR stained negative for phospho-Ser294 PR-B, although faint nuclear staining is apparent in some cells, as are slight rings or halos weakly outlining some nuclei. Controls demonstrated that this pattern is specific to PR phospho-Ser294 (see S294A control) and the primary antibody, suggesting that PR Ser294 is basally phosphorylated weakly, even in the absence of hormone. In contrast, Ser294-phosphorylated PRs were highly visible in the nuclei of R5020-treated cells. These data show that Ser294-phosphorylated PRs are predominantly nuclear. Because S294A PR can also be phosphorylated independently of ligand, we next sought to determine whether ligand-independent mechanisms result in similar findings.

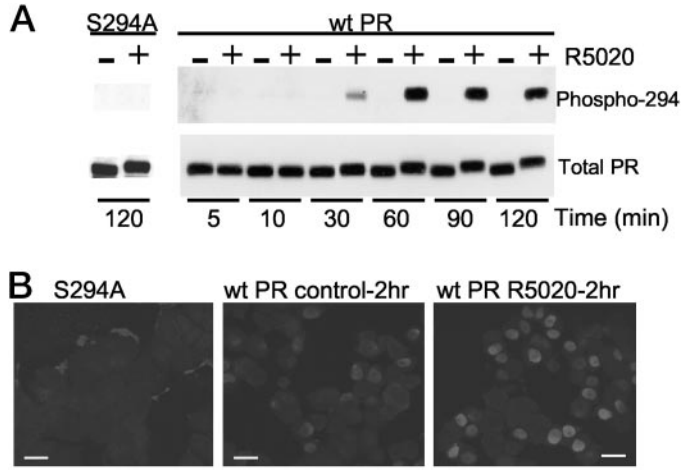

Fig. 1. Ligand-Induced Phosphorylation of PR Ser294 in T47D Cells

A, Time course of R5020-induced phosphorylation of PR Ser294. T47D cells stably expressing wt or S294A PR-B were treated without or with R5020 (10 nM) for 5 min to 2 h, and PR-B protein levels in cell lysates (100 μg protein/lane) were measured by immunoblotting using either phospho-Ser294 (*upper panel*) or total (*lower panel*) PR-specific monoclonal antibodies. Note that untreated T47D-YB and S294A cells express similar amounts of total PR-B. No other bands were visible using affinity-purified PR-specific monoclonal antibodies (15). B, Nuclear localization of phospho-Ser294 in T47D-YB cells. T47D-YB cells were cultured on coverslips 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 served as a negative control. *Scale bar*, 20 μm.

MAPK Mediates Nuclear Translocation of wt, But Not S294A Mutant, PR Proteins

To investigate the effect of Ser294 phosphorylation in the absence of progestins, we treated T47D cells with EGF, a strong activator of p42 and p44 MAPKs (Fig. 2A). Because progestins and estrogens have been reported to weakly activate MAPKs at very early (a few minutes) time points (16–18), we examined the ability of R5020 to activate MAPK. We did not observe MAPK activation by R5020 after 60 min of treatment (Fig. 2A), the time at which R5020-induced PR Ser294 phosphorylation is maximal (Fig. 1A). In contrast to EGF, R5020 treatment after 5–60 min did not activate MAPK (not shown), suggesting that ligand-induced Ser294 phosphorylation may not occur via MAPK-dependent pathways. EGF, however, induced persistent phosphorylation of PR Ser294 in T47D-YB cells. In contrast to R5020 (Fig. 1A), the effect was rapid, occurring within 5 min and becoming stable for at least 60 min (Fig. 2B).

We next compared the subcellular localization of PR after phosphorylation by EGF or R5020. T47D-YB cells were treated with EGF (5 min) or R5020 (60 min), and PRs were visualized by IHC. Surprisingly, EGF induced rapid nuclear translocation of wt PR-B comparable to that seen with R5020 alone (Fig. 2C). Translocation of cytoplasmic wt PR-B into the nucleus of EGF-treated cells occurred within 5 min, consistent with EGF-induced phosphorylation of PR Ser294 (Fig. 2B) and robust activation of p42/p44 MAPKs (Fig. 2A). In contrast, R5020-mediated nuclear translocation occurs over a much slower time course (*i.e*. $>$30 min and complete by 60 min), consistent with the time course of progestin-induced Ser294 phosphorylation (Fig. 1). We then analyzed EGF- and R5020-mediated translocation in cells expressing S294A mutant PR-B. Compared with T47D-YB cells, these cells express roughly equivalent amounts of PR protein in the absence of ligand (Fig. 1A). Both cell lines grow as associated clumps or colonies of cuboidal cell bodies having a relatively large nuclear-to-cytoplasmic ratio (Fig. 2C). Remarkably, the ability of EGF to translocate S294A PRs was entirely abrogated in S294A-expressing cells, whereas R5020-induced translocation was unaffected. Clearly, Ser294 is necessary for EGF-, but not R5020-, mediated nuclear translocation of PRs.

The EGF-induced nuclear translocation of wt, but not mutant, S294A PR (Fig. 2, C and D) was highly reproducible using multiple cell line models, including HeLa cells stably expressing either wt or mutant S294A green fluorescent protein (GFP)-tagged PR (Fig. 2D). These cells are large and flat and have abundant cytoplasm relative to T47D cells, allowing for clear visualization of the partitioning of PR proteins. This model system was one of several used by Lim *et al.* (19) to demonstrate the greater cytoplasmic distribution of human PR-B relative to PR-A isoforms. In contrast to wt GFP-PR-B, in which both EGF (5 min) and R5020 (60 min) efficiently translocated PR, mutant GFP-S294A PR-B failed to undergo nuclear translocation in response to EGF, but not R5020. This suggests that at least two independent mechanisms exist for nuclear translocation of PR; Ser294 phosphorylation is involved in growth factor-mediated, but not progestin-induced, nuclear import.

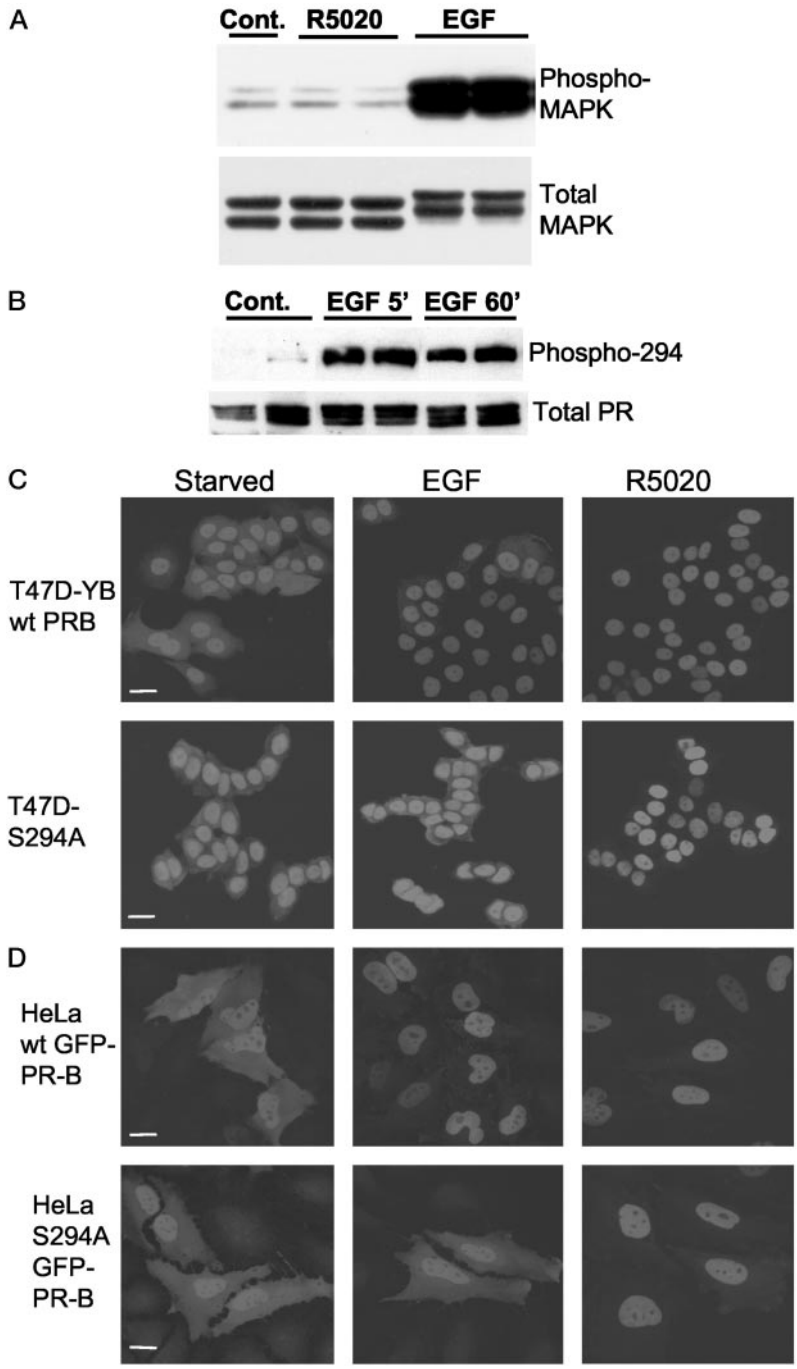

Fig. 2. Ligand-Independent Phosphorylation of PR Ser294 in T47D Cells

A, EGF activation of p42/p44 MAPKs in T47D cells. Duplicate cultures of T47D-YB cells containing wt PR-B were treated with R5020 (10 nM) for 1 h or EGF (30 ng/ml) for 5 min, and MAPK activity was measured using phospho-specific polyclonal antibodies. The same cell lysates were blotted with total MAPK-specific antibodies, indicating that equal levels of MAPK were present among gel lanes. MAPKs are equally responsive to EGF in S294A cells (12). B, EGF stimulates PR Ser294 phosphorylation. Duplicate cultures of T47D-YB cells were placed in serum-free medium for 24 h and untreated (Cont.) or treated with EGF (30 ng/ml) for 5 or 60 min, and PR-B protein levels in cell lysates (100 μg/lane) were measured by immunoblotting using phospho-Ser294 (*upper panel*) or total (*lower panel*) PR-specific monoclonal antibodies. C and D, EGF-induced nuclear translocation of wt, but not S294A, PR-B. T47D-YB cells (C) stably expressing wt PR-B or S294A PR-B or HeLa cells (D) stably expressing either wt GFP-PR-B or S294A GFP-PR-B were cultured on coverslips, 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 subjected to IHC (T47D cells) and confocal microscopy as described in *Materials and Methods*. Representative fields are shown from one of five independent experiments. *Scale bars*, 20 μm.

To further distinguish between two different translocation mechanisms, we tested the MAPK dependence of EGF- or R5020-induced PR nuclear translocation by pretreating cells with the MAPK kinase 1/MAPK kinase 2 (MEK1/MEK2) inhibitor, U0126 (Fig. 3). To clearly visualize nuclei, a dual-staining method was used in HeLa cells

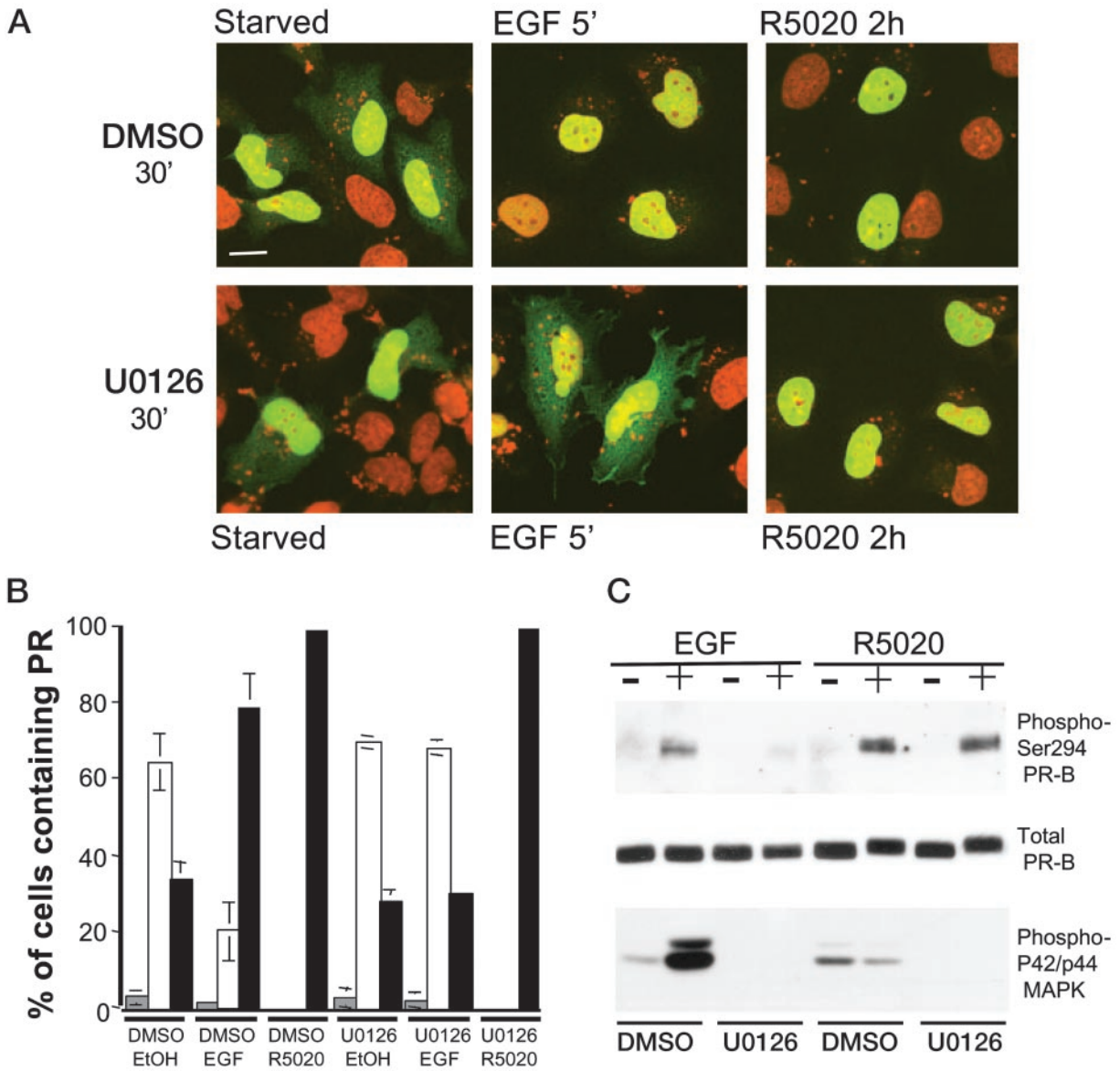

Fig. 3. MAPK Dependence of EGF-Induced PR Nuclear Translocation

A, Blockade of EGF-, but not R5020-, induced PR nuclear translocation with the MEK1/MEK2 inhibitor, U0126. HeLa cells cultured on coverslips for 24 h were transiently transfected with GFP-wt PR. Cells were washed and placed in serum-free medium for 24 h, pretreated with DMSO vehicle or U0126 (20 μM) for 30 min, and then treated with vehicle (starved), EGF (30 ng/ml) for 5 min, or R5020 (10 nM) for 2 h. Cells were counterstained with propidium iodide to indicate nuclei (*red*). GFP-PR-B in transfected cells (*green*) were visualized by direct fluorescence as described in *Materials and Methods*. Representative fields are shown from one of six independent experiments. Similar results were obtained using 10 μM U0126. *Scale bar*, 20 μm. B, PR-B localization in 100 cells per treatment condition**.** One hundred cells in each treatment group (in panel A) were scored for the presence of PR-B in the cell cytoplasm only (*shaded bars*), both cytoplasm and nucleus (*open bars*), and nucleus only (*solid bars*). U0126 blocked the effects of EGF on PR-B localization, but had no effect on R5020-induced translocation. *Error bars* represent the average of two independent experiments (1200 cells were counted). C, Blockade of EGF-, but not R5020-, induced PR Ser294 phosphorylation with the MEK1/MEK2 inhibitor, U0126. HeLa cells were treated as above (panel A) with vehicle (EtOH), EGF (30 ng/ml) for 5 min, or R5020 (10 nM) for 2 h, and cell lysates were blotted using PR Ser294 phospho-specific monoclonal antibodies (*upper panel*), total PR monoclonal antibodies (*middle panel*), or phospho-specific MAPK polyclonal antibodies (*lower panel*). U0126 pretreatment effectively inhibited p42/p44 MAPK activation in response to EGF. Results are representative of a total of four independent experiments.

expressing either wt or mutant S294A GFP-PR-B; propidium iodide-stained nuclei appear *red*, whereas GFP-PR-B fluoresce *green* (Fig. 3A). EGF-induced translocation of wt GFP-PR-B was fully blocked by pretreatment of HeLa cells with U0126. In contrast, R5020-mediated translocation was unaffected by inhibition of MAPKs. To quantify these results, 100 cells per treatment were scored for the subcellular location of PRs after control, EGF, and R5020 treatment, after pretreatment with dimethylsulfoxide (DMSO) vehicle or the MEK inhibitor (Fig. 3B). A few untreated cells contained PRs in the cytoplasm only, while the majority of resting cells contained both cytoplasmic and nuclear PRs; approximately 30% of control cells contained only nuclear PRs. EGF induced PR nuclear association in greater than 80% of cells, while 100% of cells treated with R5020 contained exclusively nuclear PRs. We conclude that MAPK inhibition completely blocks the effects of EGF, but not of R5020.

To examine the specificity of PR Ser294 regulation by EGF and R5020 in HeLa cells treated without or with the MEK1/MEK2 inhibitor, Western blots were performed using phospho-Ser294-specific monoclonal antibodies (Fig. 3C). Treatment of HeLa cells with either EGF (5 min) or R5020 (1 h) resulted in PR Ser294 phosphorylation, as in T47D cells (Figs. 1A and 2B). The MEK1/MEK2 inhibitor, U0126, blocked Ser294 phosphorylation in response to EGF, but not R5020. Western blots of total PR in the same cell lysates indicated that equal amounts of PR-B were present in each condition, and MAPK activity assays confirmed that U0126 inhibited p42/p44 MAPK activity (Fig. 3C). We did not observe ERK5 activation after 5–10 min of EGF treatment (not shown), indicating that the effects of U0126 were most likely due to blockade of p42/p44 MAPKs. Similar results were obtained using T47D cells (not shown). These results are consistent with activation of p42/p44 MAPKs by EGF, but not R5020, and the blockade of EGF- but not R5020-induced nuclear translocation of wt PR by U0126 (Fig. 3). The data also suggest that multiple protein kinases are capable of phosphorylating PR Ser294 and reveal the existence of a progestin-activated protein kinase (see *Discussion*).

Thus, there are at least two independent mechanisms for nuclear translocation of PR. One is ligand dependent (*i.e.* progestin-induced), independent of Ser294 phosphorylation, and does not require MAPK activation. The second is growth factor inducible, progestin independent, and requires phosphorylation of PR Ser294 by p42/p44 MAPKs. In addition to transcriptional synergy reported between growth factors and progestins at several genes (12, 20, 21), these results suggest a mechanism for ligand-independent actions of PR (22–24).

Liganded S294A PR-Bs Are Persistently Nuclear

Both wt and mutant PR proteins bind ligand and clearly undergo ligand-induced nuclear translocation, suggesting that Ser294 does not appear to be required for this event (Figs. 2 and 3). Both receptors interact specifically with a consensus progesterone-responsive element (PRE)-containing oligonucleotide in gel-shift assays (not shown) and are capable of activating transcription when transiently expressed (12). However, the mutant receptors are much weaker transactivators, perhaps because they are sequestered away from transcriptionally active nuclear compartments and/or chromatin. Ser294 is also required for ligand-dependent PR down-regulation (3). To visualize the location of stabilized PR over time, we completed a time course of R5020 treatment in intact cells (Fig. 4). T47D cells stably expressing wt or S294A mutant PRs were plated on coverslips and cultured in the absence of hormones. Cells were then treated with R5020 for 2–24 h, fixed, and permeabilized; PRs were visualized using monoclonal antibodies against human PRs (Fig. 4A). In the absence of hormone, cytosolic and nuclear PRs were visible in both cell lines; wt and mutant S294A PR-B appeared to be similarly localized in resting intact cells. These data were confirmed at early time points using semiquantitative fractionation studies (*inset*). Treatment of cells with R5020 for 2 h resulted in intense nuclear staining in both cell lines, due to complete translocation of both wt and S294A PRs upon hormone binding; there was no difference in the rate of ligand-induced nuclear import at early time points (not shown). PRs were nearly undetectable in cytosolic fractions of R5020-treated cells (*inset*). After 24 h of R5020 treatment, wt PRs were poorly visible, reflecting ligand-induced down-regulation. In contrast, intense nuclear staining of S294A PRs persisted after 24 h of R5020 treatment. These results demonstrate that liganded S294A PRs are nuclear, and highly resistant to down-regulation, as we reported using biochemical methods, after 10–12 h of progestin treatment (3, 12). Stable expression of either wt or mutant PRs in HeLa cells produced essentially the same results as in T47D cells (not shown).

Both wt and mutant S294A PR-Bs translocate into nuclei in the presence of progestin (Figs. 2 and 3). However, only mutant S294A PRs persist for long periods within this cellular compartment (Fig. 4A). To determine whether liganded wt PRs that are stabilized by pharmacological methods are also similarly nuclear, we blocked PR down-regulation using the proteasome inhibitor, lactacystin. We have shown by Western blotting that ligand-induced PR down-regulation is blocked by lactacystin, a highly specific inhibitor of the 26S proteasome (3). Consistent with this, lactacystin blocks R5020-induced down-regulation as measured by IHC and confocal microscopy (Fig. 4B). Wt PR-B in cells treated with R5020 for 18 h down-regulated as expected, but in cells pretreated with lactacystin (30 min) followed by R5020 (18 h), PR-B failed to down-regulate and remained entirely nuclear. Lactacystin alone had no effect on the subcellular localization of PR-B relative to untreated controls; PRs were visible in both the cytoplasm and nuclei of cells in the absence of added hormone. Thus, liganded wt PR-Bs stabilized by lactacystin resemble liganded mutant S294A PR-Bs.

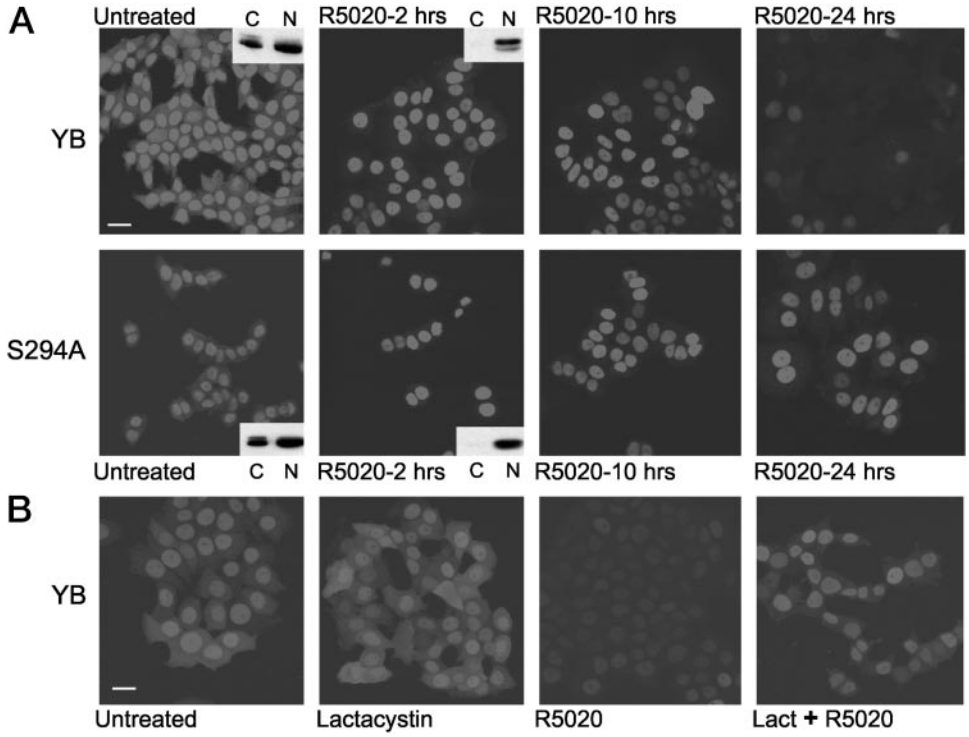

Fig. 4. Subcellular Distribution of wt and Mutant S294A PR-B in T47D Cells

A, Nuclear localization of S294A PR-B in intact cells. T47D-YB and S294A cells were cultured on coverslips, placed in medium containing charcoal-stripped serum, and untreated or treated with R5020 (10 nM) for 2, 10, and 24 h. Cells were fixed and permeabilized for IHC using PR-specific monoclonal antibodies as described in *Materials and Methods*. Negative controls (minus PR antibodies) indicated that immunostaining was specific to PR-B (not shown). Representative fields are shown from one of four independent experiments. *Scale bar*, 20 μm. *Inset,* Fractionation of cell extracts. T47D cells stably expressing either wt (T47D-YB) or S294A PR-B were treated without or with R5020 (10 nM) for 1–2 h, and cytosolic (C) and salt-extracted nuclear (N) fractions were prepared as described in *Materials and Methods*. Equal amounts of protein (100 μg) were loaded onto gels and immunoblotted using PR-specific monoclonal antibodies (56). B, Proteasome inhibitors block PR-B down-regulation. T47D-YB cells were cultured on coverslips, placed in medium containing charcoal-stripped serum, and pretreated with DMSO vehicle (Control) or lactacystin (10 nM) for 30 min before treatment with either EtOH vehicle (Control) or R5020 (10 nM) for 18 h. IHC was performed on fixed cells using PR-specific monoclonal antibodies. *Scale bar*, 20 μm.

MEK inhibitors also block ligand-dependent down-regulation of wt PR as measured by Western blotting, presumably by blocking phosphorylation of Ser294 by p42/p44 MAPK (3, 12). We used the MEK1/MEK2 inhibitor, U0126, as an additional means by which to stabilize wt PR-B in the presence of progestins and performed intact-cell imaging experiments (Fig. 5). In contrast to R5020 alone, pretreatment of T47D-YB cells stably expressing wt PR-B with U0126 resulted in intense and persistent nuclear staining of PRs up to18 h after R5020 exposure (Fig. 5A). Surprisingly, however, PRs appeared to accumulate in the nuclei of cells exposed to U0126 alone relative to vehicle-treated controls. This effect was not visible at early time points ($<$2 h), as is the nuclear translocation of PR after ligand binding or EGF treatment of cells (Figs. 2 and 3). Mutant S294A PR-B did not accumulate in nuclei during prolonged U0126 treatment (not shown; and see Fig. 5C). Thus, MAPK may regulate unidirectional PR shuttling out of the nucleus via phosphorylation of Ser294.

To more clearly visualize wt PR nuclear accumulation during prolonged MAPK inhibition, HeLa cells stably expressing GFP-tagged wt PR-B were treated without or with U0126 or R5020 (Fig. 5B). GFP-PR-Bs are largely cytoplasmic in this model, facilitating nuclear accumulation studies. PRs accumulated in the nuclei of HeLa cells treated for 10–18 h with U0126 relative to DMSO-treated controls. Although U0126-induced nuclear accumulation was somewhat less complete compared with short-term treatment with R5020 (1 h), these cells began to resemble progestin-treated cells. Essentially the same results were obtained using MCF-7 cells stably expressing GFP-PR-B (not shown). Fractionation studies confirmed these results in T47D cells stably expressing either wt or S294A PR-B (Fig. 5C). Cells were treated with or without U0126 for 18 h, or with R5020 for 1 h as a positive control, and cell lysates were fractionated into cytosolic (soluble) and nuclear (salt-extracted) fractions. Prolonged treatment with U0126 resulted in nuclear accumulation in cells expressing wt PRs, but not in

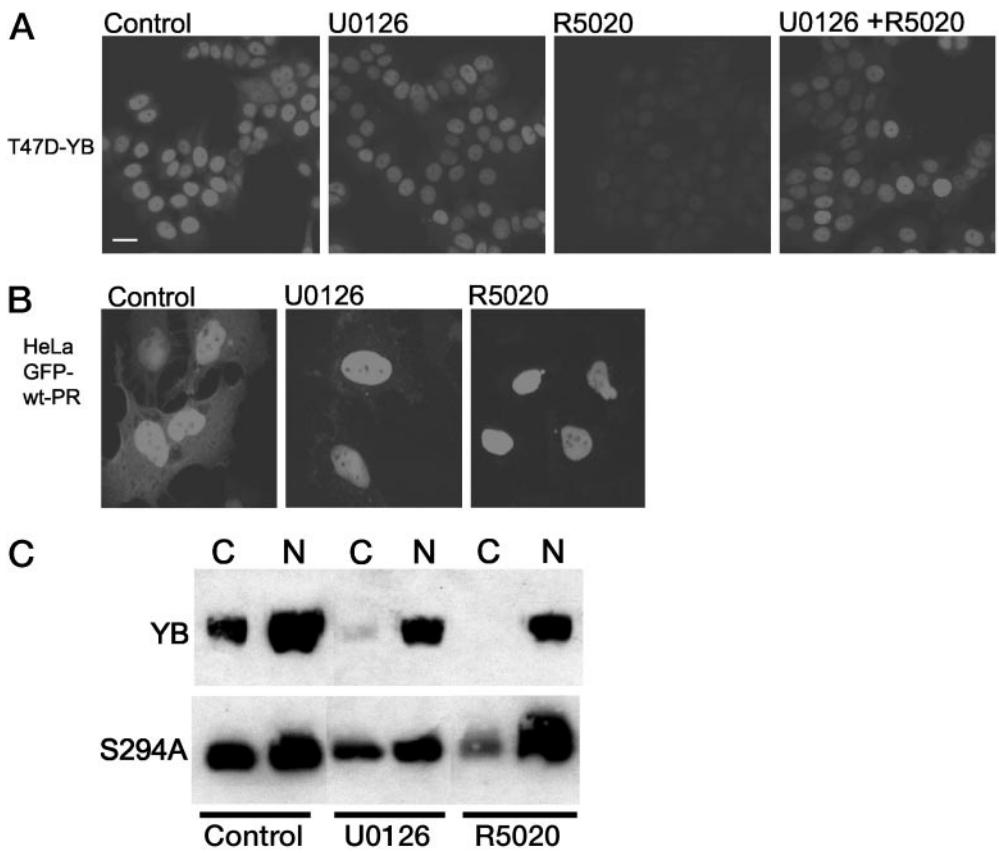

Fig. 5. Blockade of PR-B Down-Regulation by the MEK1/MEK2 Inhibitor

A, T47D-YB cells were cultured on coverslips, placed in serum-free medium, and pretreated with DMSO vehicle (Control) or U0126 (10–20 μM) for 30 min before treatment with either EtOH vehicle (Control) or R5020 (10 nM) for 18 h. IHC was performed on fixed cells using PR-specific monoclonal antibodies. *Scale bar*, 20 μm. B, HeLa cells stably expressing wt GFP-PR-B were treated as in panel A, except that R5020 was added 1 h before fixation as a positive control for nuclear association and PRs were visualized by direct fluorescence. Note that PR-B accumulated in cell nuclei in the presence of U0126 alone. *Scale bar*, 20 μm. C, T47D-YB or S294A cells were treated as in panel B and fractionated into cytosolic (C) or nuclear (N) fractions as described in *Materials and Methods*. Equal amounts of protein (150 μg) were loaded onto gels and immunoblotted using PR-specific monoclonal antibodies.

S294A PR-containing cells. These results suggest a novel role for MAPK in the nuclear export of unliganded PRs during nucleo-cytoplasmic shuttling and after ligand binding; PRs that are trapped in the nucleus (or undergo delayed nuclear export) may not be targeted to the 26S proteasome.

PR Proteins Are Degraded in the Cytoplasm

Our data suggests that PR stability is associated with nuclear retention or, conversely, that nuclear export may be required for receptor down-regulation. Nuclear export of ubiquitinated p53 is required for its destruction by the 26S proteasome within the cytoplasm (2). PRs may follow a similar pathway during ligand-induced down-regulation. To further explore this possibility, we expressed GFP-tagged wt PR-B in HeLa cells. To trap PR within nuclei, HeLa cells expressing GFP-wt PR-B were treated with or without leptomycin B (LMB), an inhibitor of exportin 1/CRM1-mediated nuclear export (25). LMB treatment resulted in the nuclear accumulation of unliganded PRs (Fig. 6A). These results demonstrate that unliganded PR-Bs are actively exported from the nucleus during shuttling.

We therefore tested the ability of LMB to block ligand-dependent PR-B down-regulation (Fig. 6, B and C). T47D-YB cells stably expressing wt PR-B were pretreated for 30 min without or with 15 nM LMB, and then treated without or with R5020 for 8 h. Inhibition of nuclear export effectively blocked ligand-induced PR down-regulation as measured by Western blotting (Fig. 6B). This concentration of LMB also stabilized p53 (Fig. 6B), a known substrate of the exportin 1/CRM1-pathway (26). We confirmed these results in T47D-YB cells by IHC and confocal microscopy (Fig. 6C). LMB treatment stabilized R5020-bound PR for at least 18 h and blocked PR down-regulation.

PR stability and transcriptional activity are inversely related (12); stabilization of PR either by mutation of Ser294, by MEK inhibitors, or by blocking the activity

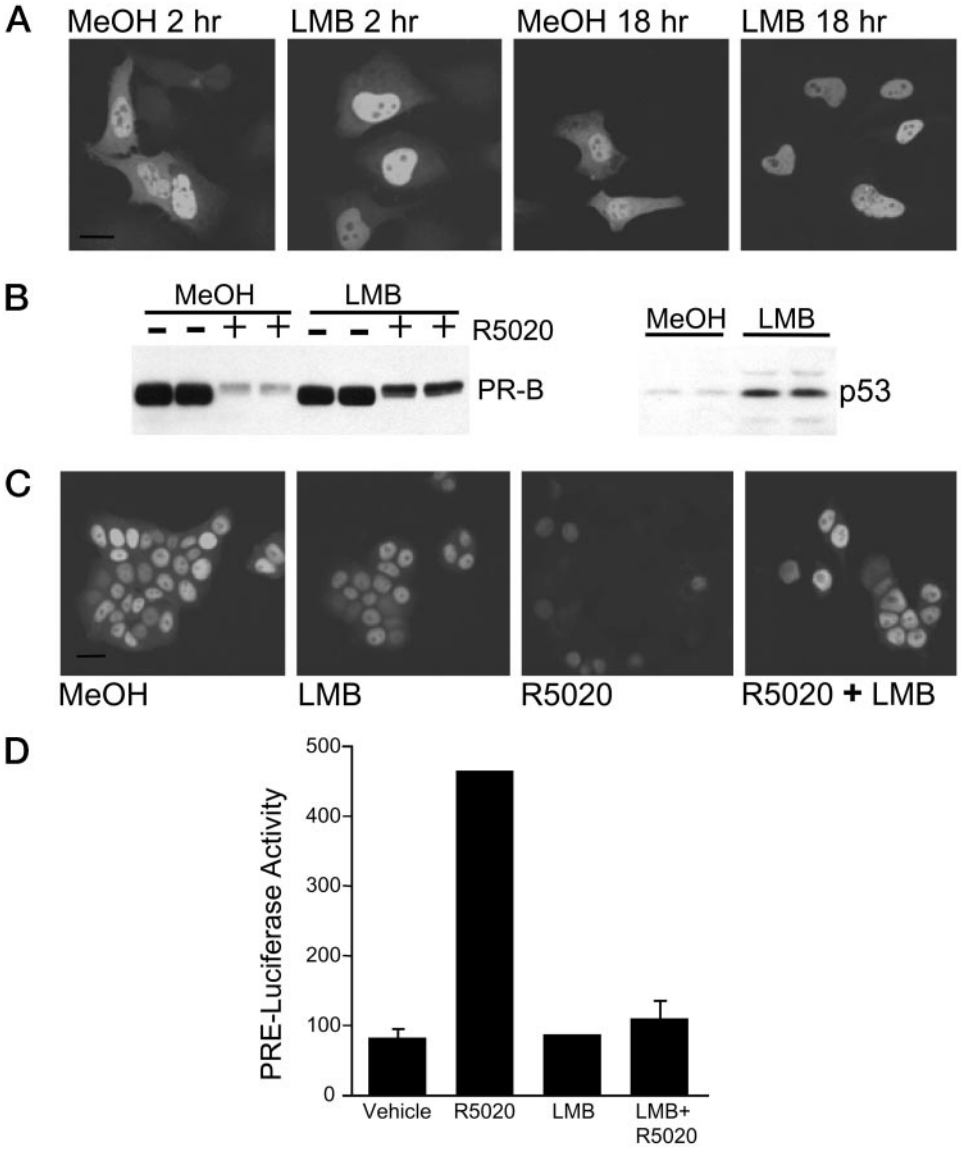

Fig. 6. LMB Blocks Ligand-Induced PR-B Down-Regulation

A, Nuclear accumulation of PR in LMB-treated cells. HeLa cells were cultured on coverslips and transiently transfected with wt GFP-PR-B. Cells were treated with MeOH vehicle or LMB (15 nM) for 2 or 18 h, and PRs were visualized by direct fluorescence confocal microscopy. *Scale bar*, 20 μm. B, Duplicate cultures of T47D-YB cells were pretreated with MeOH vehicle or LMB (15 nM) for 30 min and then without or with R5020 (10 nM) for 8 h, and PR-B was detected in cell lysates by immunoblotting (100 μg protein/lane) using PR-specific monoclonal antibodies (56). P53 protein levels were measured in cell lysates from duplicate cultures of T47D-YB cells treated without (MeOH) or with LMB for 5 h by immunoblotting using specific antibodies (*right panel*). C, T47D-YB cells were cultured on coverslips, placed in medium containing charcoal-stripped serum, and pretreated with MeOH vehicle or LMB (15 nM) for 30 min before treatment with either EtOH vehicle or R5020 (10 nM) for 18 h. IHC was performed on fixed cells using PR-specific monoclonal antibodies. *Scale bar*, 20 μm. D, Duplicate cultures of HeLa cells were transfected with wt PR-B and a PRE-luciferase reporter construct as described in *Materials and Methods*. Cells were treated as in panel C and harvested for determination of luciferase activity in cell lysates. *Error bars* represent the mean $\pm$ the range; experiments were repeated three times.

of the 26S proteasome blocked transcriptional synergy on a PRE-containing promoter induced by progestin and agents that activate MAPKs. To test whether stabilization of PR by LMB also suppresses PR function, we assayed PR transcriptional activity in response to R5020 on a PRE-driven luciferase promoter construct (Fig. 6D). HeLa cells were cotransfected with wt PR-B and treated with vehicle, R5020 alone, LMB alone, or with both agents as in Fig. 6, B and C. Luciferase assays indicated that LMB alone had no effect on basal transcription levels but blocked R5020-induced transcription. These results provide additional support for the paradoxical finding that PR stability is inversely related to transcriptional activity,

and reveal an important link between PR localization, protein stability, and transcriptional activity.

DISCUSSION

Our results suggest that MAPKs play a dual role in PR subcellular trafficking. In the absence of ligand, MAPKs aid the rapid nuclear association of PR via Ser294 phosphorylation in response to growth factors, but appear to have little role in ligand-induced nuclear import. However, during nucleo-cytoplasmic shuttling, and after ligand binding, phosphorylation of PRs on Ser294 by MAPK promotes nuclear export and cytoplasmic degradation. Mutant S294A PRs or wt PRs in lactacystin, U0126, or LMB-treated cells appear to be stabilized because they fail to be exported from the nucleus. Paradoxically, we find that stabilized PR that are trapped within the nucleus are transcriptionally impaired, suggesting that perhaps PR must undergo rapid and dynamic nucleo-cytoplasmic shuttling to function most efficiently.

We have shown that EGF can translocate human PR in breast cancer cells (Figs. 2 and 3), in a manner that is indistinguishable from R5020, suggesting that EGF may regulate PR transcriptional activity independently of its natural ligand. What is the significance of PR phosphorylation in response to growth factor receptor stimulation? At first glance, there appears to be little involvement with regard to unliganded receptors, at least at PRE-containing promoters (12). Indeed, historically, mutation of phosphorylated residues to nonphosphorylatable amino acids has produced fully functional PR capable of activating transcription upon progestin treatment in transient expression assays (27, 28). We were the first to demonstrate a clear role for PR Ser294 phosphorylation in mediating both ligand-dependent PR down-regulation (3) and transcriptional synergy between progestins and agents that activate MAPKs (12). However, the latter effect of MAPK is entirely progestin dependent. Interestingly, a constitutively active PR-B mutant receptor lacking the entire C-terminal hormone-binding domain is highly sensitive to MAPK-mediated transcriptional activation (not shown), demonstrating a clear separation between ligand- and phosphorylation-dependent contributions to transcriptional activity. Both events are probably necessary for maximal receptor activation. The importance of Ser294 phosphorylation is illustrated by the fact that S294A PR-B is about 10 times less active in the presence of progestins compared with wt PR-B when stably expressed in T47D PR null cells (12). Thus, PR phosphorylation, when linked to ligand-binding, may be a major mechanism to enhance transcriptional activity. The identity of the progestin-stimulated protein kinase is unknown, but must be independent of MEK1/MEK2, as inhibitors of this pathway still allow for progestin-induced Ser294 phosphorylation (Fig. 3C).

With regard to liganded PR, EGF and other agents that activate MAPKs synergize with progestins on several promoters, including mouse mammary tumor virus (29, 30), c-*fos*, and $p21^{waf1}$ (21), and c-*myc* (12). Because some of these genes lack PREs, transcriptional synergy may occur via nonclassical mechanisms involving PRs tethering to simian virus 40 protein 1 (Sp1; Ref. 31), signal transducer and activator of transcription 5 (STAT5; Ref. 21), or other factors. Thus, growth factors may serve to sequester PRs within the nucleus, promoting phosphorylation-dependent interactions with other regulatory molecules that may mediate transcriptional synergy in the presence of ligand. Evidence for activity of unliganded human PR has been scarce, but is growing (Figs. 2 and 3). Unliganded PRs activate activator protein 1 activity in human endometrial adenocarcinoma cells in the absence of any outside stimuli, an effect reversed by progestin (24). The role of phosphorylation in PR-mediated activator protein 1 activation is unknown. However, unliganded PR-Bs, but not S294A, PR-Bs induce basal expression of the insulin receptor substrate 1 protein in human breast cancer cells; PR null T47D cells lack insulin receptor substate 1 (Byron, S., C. A. Lange, and D. Yee, unpublished results). New breast cancer cells expressing inducible human PR have now been used to define genes regulated by unliganded PR-A (32). Labriola *et al.* (33) most recently found that heregulin could fully activate unliganded PR and phosphorylate Ser294 in breast cancer cells, leading to its nuclear localization, DNA binding, and PRE-dependent transcriptional activity. Ligand-independent regulation of human PR by EGF (Figs. 2 and 3) or heregulin is reminiscent of dopaminergic regulation of chicken progesterone receptors (cPRs). Dopamine promotes nuclear translocation of cPR (22). Interestingly, a serine residue in cPR is essential for dopamine, but not progesterone-dependent activation of cPR. Dopamine regulates reproductive behavior in rodents by this PR-dependent, but progesterone-independent, mechanism (23, 34). Thus, it seems receptor phosphorylation can substitute for ligand in specific settings (Fig. 3C). We propose the existence of a similar mechanism for unliganded PR activation by growth factors; this is a topic for further studies.

EGF and PR may collaborate during the coordinated regulation of normal cell growth in the developing breast. Indeed, cross-talk between these two signaling pathways is well documented (35–37). Progestins induce up-regulation of type I growth factor receptor tyrosine kinases, including EGF receptor, as well as several other growth factor receptors (38). Thus, progestins greatly increase the sensitivity of breast epithelial cells to locally acting mitogens that, in turn, modulate PR function (37). This bidirectional regulation may become reestablished and/or altered during breast cancer progression, as growth factor receptors become overexpressed in advanced-stage breast cancer (39, 40). Growth factors are known to contribute to steroid hormone receptor down-regulation (12,

41). An inverse correlation exists between EGF receptor and ERα mRNA (42). Hyperactivation of MAPKs reversibly down-regulates ERα mRNA and protein levels in breast cancer cells expressing constitutive Raf kinase (41). Thus, cross-talk with growth factor-initiated pathways probably contributes to the loss of steroid hormone receptor expression in a subset of hormone-resistant breast cancers. However, paradoxically, the majority of hormone-resistant breast cancers contain apparently functional receptors (43). Perhaps during tumor progression, breast cancer cells find ways to circumvent steroid hormone-dependent receptor down-regulation, allowing for their dysregulation by a continuous stream of signaling from overexpressed growth factor receptors. Although activation of MAPKs by EGF translocates PRs (Figs. 2 and 3), EGF treatment alone is insufficient to down-regulate PRs; however, it augments PR down-regulation in the presence of progestins (12).

How might MAPKs mediate nuclear association of PRs? Phosphorylation of PRs may induce their interaction with proteins that shuttle between the cytoplasm and the nucleus. For example, progestins induce the nuclear localization of STAT5, tethered to PRs (21). Thus, cytokine activation of STAT5 (and MAPK) may similarly translocate PRs. Additionally, like other steroid hormone receptors, PRs interact with a variety of heat shock proteins, chaperones (44), and immunophilins (45). These proteins play a role in protein trafficking (46). We are currently investigating the identity of proteins that interact with PR in a Ser294-phosphorylation-dependent manner. What domain(s) of PR mediate nuclear import? Similar to other steroid hormone receptors, PRs contain a bipartite nuclear localization signal (NLS) encompassing the hinge region and second zinc finger of the DNA-binding domain, with separable constitutive and ligand-dependent components (47). Thus, PR were initially predicted to diffuse into the cytoplasm and undergo constant and active transport back into the nucleus via the action of both NLS components (47). However, the NLS is involved in both nuclear import and export of PRs. Guiochon-Mantel *et al.* (48) showed that the PR NLS could mediate nuclear shuttling/export when fused to β-galactosidase, indicating that a single element may mediate movement in both directions. Both wt and S294A PR-Bs translocate to the nucleus in response to progestin (Figs. 2–4), whereas only wt PR-B responds to EGF (Figs. 2 and 3). Perhaps the NLS functions dominantly, whereas a separate latent domain is sensitive to growth factor activation of MAPKs. This may explain why S294A PR-Bs are not exclusively cytoplasmic in resting and/or serum-starved cells (Fig. 2).

How does PR exit the nucleus? Although sequences homologous to nuclear export signals (NES) have been found in both N- and C-terminal regions of PRs (49), it is unclear whether these represent functional NES. Our data suggest that phosphorylation of PRs on Ser294 both enables constitutive import of unliganded PRs, and allows for nuclear export of liganded PRs, perhaps via diffusion or by unmasking a latent NES; this most likely involves protein-protein interactions. Indeed, we have recently found that S294A PR-Bs are tightly associated with nuclear matrix protein component(s) (Olsen, A., and C. A. Lange, unpublished results). We are currently investigating the identity of proteins that interact with PRs in a Ser294 phosphorylation-dependent manner. The p53 tumor suppressor protein contains multiple NES; the function of at least one is dependent on its dephosphorylation (2). PR heterodimers in cells coexpressing wt PRs and NLS-deleted mutant PRs were able to exit the nucleus in the presence of LMB, suggesting that the CRM1 pathway is not involved (49). In contrast, we show LMB-induced accumulation of PRs in the absence of ligand, and blockade of ligand-induced PR down-regulation, suggestive that PR export is, at least in part, CRM1 mediated (Fig. 6); the PR contains at least two leucine-rich putative NES consensus sequences found in many exportin/CRM1 substrates ($LX_{1-3}LX_{2-3}LXL$). Possible explanations for these differences include the use of different cell line models, coexpression of modified PR molecules (NLS-deleted PR), and different LMB concentrations and/or treatment conditions (49). Thus, CRM1-mediated PR nuclear export may represent one of many alternate pathways used by PRs depending on the cellular conditions (discussed below). Nuclear export of glucocorticoid receptors (GR) has also been shown to be LMB sensitive (50) or insensitive (51) in different model systems.

Nonetheless, it is becoming clear that there are numerous mechanisms by which steroid receptors may enter and exit from the nucleus (Refs. 1, 47–49, and 51 and Fig. 3). Why do cells utilize multiple shuttling mechanisms? Perhaps they are needed to allow the cell to respond appropriately to a wide variety of signaling combinations. Prolonged nuclear retention of GR after initial ligand-induced translocation limits receptor down-regulation in the cytoplasm, possibly modifying responses to secondary stimuli (51). PRs also undergo cytoplasmic degradation (Fig. 6). Thus, growth factors may confine PRs to the nucleus to temporarily prevent degradation, thereby prolonging the transcriptional response to multiple and complex signaling inputs. EGF alone does not appreciably activate unliganded PRs (12, 21). PRs localized to nuclei in response to growth factors may thus function more efficiently in the presence of progesterone, before their rapid cytoplasmic degradation.

MAPK couples multiple functions of human PRs via phosphorylation of Ser294 (3, 12). How is the regulation of nucleo-cytoplasmic shuttling linked to both PR transcriptional activity and degradation? Several recent reports support a dual role for ubiquitination in both transcriptional activation and subsequent destruction of a growing number of transcription factors (2, 6, 11, 12), including several members of the steroid receptor superfamily (10–12). How this process is

linked to nuclear export is unclear. However, linkage of ubiquitination of a protein to its nuclear export provides an additional means of separating activated transcription factors from chromatin to attenuate gene regulation. Gottifredi and Prives (52) suggest that, in the case of p53, monoubiquitination occurs in the nucleus, whereas polyubiquitination and destruction occur in the cytoplasm. This scenario fits a model in which monoubiquitination is required for transcriptional activation (6). This modification may also lead to the exposure or unmasking of NES located on the transcription factor itself or associated shuttling and/or chaperone molecules. A chaperone-like enzyme, termed $CDC48^{UFD1/NPL4}$, selectively recognizes ubiquitinated proteins and removes them from oligomeric protein complexes during nuclear targeting (53). It is also possible that there is a direct link between nuclear pores and the proteasome complex (52), providing a physical connection between export and degradation.

In sum, phosphorylation of PR by MAPKs is likely to contribute to the regulation of PR shuttling by increasing rapid nuclear association independently of ligand and, paradoxically, regulating some aspect(s) of PR nuclear export (or diffusion into the cytoplasm). How can MAPKs accomplish both tasks? A growing literature suggests that cytoplasmic and nuclear populations of MAPKs may mediate differential functions in signal transduction (54). Similarly, PRs likely interact with different subsets of signaling molecules depending on their subcellular location; this is a topic for further exploration. We previously reported that phosphorylation of Ser294 mediates both ligand-dependent PR down-regulation (3) and transcriptional synergy in the presence of progestins and agents that activate MAPKs (12). How these events are coupled (*i.e.* aside from Ser294 phosphorylation) is unclear. However, linkage of nucleo-cytoplasmic shuttling to receptor activity and degradation ensures that hormonal responses are tightly controlled. Hyperactivation of genes by unliganded PR could lead to deleterious effects on cells. In breast cancer cells, lesions leading to loss of this coupling mechanism are predicted to result in abnormally stabilized PR that may be resistant to steroid hormone-based treatments but remain highly responsive to growth factor-induced signaling events. We are currently testing this exciting hypothesis.

MATERIALS AND METHODS

Cell Lines and Reagents

Monoclonal T47D-YB human breast cancer cells engineered to stably express PR-B were previously described (55). Monoclonal T47D-YB-S294A cells (S294A) containing mutant PR-B with serine 294 replaced by alanine were engineered by stable transfection of mutant S294A PR-B into PR-negative T47D-Y cells as previously described (3). T47D-YB and S294A cells were routinely seeded at 1×10^6 cells per dish, cultured in 10-cm dishes, and incubated in 5% CO_2 at 37 C in a humidified environment as described (55). HeLa and MCF-7 cells expressing green florescent protein (GFP) tagged-PR were engineered by stable transfection of wt or mutant PR-B cloned into the pEGFP-N3 expression vector (CLONTECH Laboratories, Inc., Palo Alto, CA), at the *Eco*RI (5′) and *Kpn*I (3′) cloning sites. Stably transfected cells were maintained in 400 μg/ml neomycin analog G418 (Life Technologies, Inc., Gaithersburg, MD). For experiments involving steroid hormone (R5020, 10 nM) or EGF (30 ng/ml) treatments, cultures were placed in serum-free media for 18–24 h before growth factor addition. Phospho-specific and total p42/p44 MAPK antibodies were purchased from New England Biolabs, Inc. (Beverly, MA). The MEK1/2 inhibitor (U0126) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Horseradish peroxidase-conjugated secondary antibodies were obtained from Collaborative Biomedical Products Inc. (Bedford, MA). R5020 was obtained from NEN Life Science Products (Boston, MA). Lactacystin was purchased from Calbiochem (La Jolla, CA). LMB was purchased from Sigma (St. Louis, MO). For IHC studies, the anti-PR monoclonal antibody, AB-8, was purchased from NeoMarkers (Fremont, CA). For immunoblotting studies, the anti-PR monoclonal antibodies, AB-52 and B-30, were produced in the Horwitz laboratory (56). Phospho-294-specific PR antibodies were a kind gift of D. P. Edwards (15). The p53 antibody (Pab 240; sc-99) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell Fractionation

For comparison of PR subcellular distribution, cytosolic and nuclear extracts were prepared essentially as described by Smith *et al.* (57), except that cells were lysed by 10 strokes in hand-held glass-glass homogenization tubes (instead of by Dounce homogenization). Protein concentrations in supernatants were determined by the method of Bradford (Life Technologies, Inc.), and equal amounts of protein were loaded onto gels for immunoblotting as described below.

Immunoblotting

For detection of PR-B, MAPKs, or p53 in whole-cell lysates, cells growing in 10-cm dishes were washed twice in 4 ml of PBS and lysed by scraping in RIPA buffer [10 mM sodium phosphate (pH 7.0), 150 mM NaCl, 2 mM EDTA, 1% (wt/vol) Nonidet P-40, 0.1% (wt/vol) sodium dodecyl sulfate, 1% sodium deoxycholate, 20 μg/ml aprotinin, 50 mM sodium fluoride, 200 μM Na_3VO_4, 0.1% (vol/vol) β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride]. Lysates were clarified by centrifugation for 10 min at maximum speed in a refrigerated microfuge. Soluble proteins in clarified lysates were quantified by the method of Bradford (Life Technologies, Inc.) and equal amounts of protein were resolved by SDS-PAGE (10% acrylamide for p53 or MAPK; 7.5% for PR), transferred onto nitrocellulose filters, and immunoblotted with specific antibodies.

Transient Transfections

HeLa cells were seeded at 100,000–300,000 cells per well onto 22-mm coverslips in 6-well multidishes. After 24 h, cells at 50–60% confluence were transiently transfected with 1 μg plasmid DNA (empty vector control or PR-B-containing vector) and 5 μg LipofectAmine in Opti-MEMI reduced serum medium, according to the manufacturer's instructions (Life Technologies, Inc.). Reduced serum medium was replaced with MEM containing 5% charcoal-stripped fetal bovine serum 6–8 h after transfection. Duplicate cultures of cells were treated with 10 nM R5020 or EtOH vehicle control before fixation (described below).

IHC and Confocal Microscopy

Cells were seeded onto coverslips in six-well multidishes. After the appropriate treatments, cells were washed, fixed by incubation in 1 ml of 3.7% paraformaldehyde in PBS for 20 min at room temperature, and permeabilized by incubation in 1 ml PBS containing 0.5% Triton X-100 for 5 min at room temperature (RT). Coverslips were incubated with primary antibody against PR (NeoMarkers Ab8) diluted (1:500) in 100 μl PBS containing 1% BSA for 1 h at RT. After five washes in PBS, coverslips were exposed to secondary antibody (fluorescein isothiocyanate-conjugated goat anti-mouse; Santa Cruz Biotechnology, Inc.) diluted 1:1000 in 100 μl PBS containing 1% BSA for 30 min at RT. Coverslips were mounted on slides using ProLong Antifade (Molecular Probes, Inc., Eugene, OR). For analysis of GFP-PR expression in cells stably expressing PR, 2–3 d posttransient transfection and/or after hormone treatment, cells were fixed in 3.7% paraformaldehyde as above, and coverslips were mounted and subjected to direct fluorescence imaging using a MRC-1024 confocal laser microscopy system (Bio-Rad Laboratories, Inc., Hercules, CA) with a $\times$60 oil immersion objective (Olympus Corp., Lake Success, NY). Samples were excited at 488 nm and analyzed at an emission of 522. Serial 1-μm sections were collected and digitized fluorescent images were analyzed using Adobe Photoshop (Adobe Systems, Inc., San Jose, CA).

Transcription Assays

HeLa cells were seeded at 300,000–400,000 cells per well in six-well multidishes. After 24 h, cells at 70–80% confluence were transiently transfected with 0.5 μg plasmid DNA (empty vector control or PR-B-containing vector), 1 μg PRE-2X-TATA-luciferase reporter plasmid, and 10 μg Renilla plasmid (as an internal control for transfection efficiency), using 4 μl LipofectAmine in Opti-MEM reduced serum medium, according to the manufacturer's instructions (Life Technologies, Inc.). Reduced serum medium was replaced with MEM starvation media without serum 6–8 h after transfection. Duplicate cultures of cells were treated with 10 nM R5020 or EtOH vehicle control, or without or with LMB (10 nM) 18–24 h before harvest in 1$\times$ lysis buffer and measurement of luciferase/Renilla activity as described by the manufacturer (Promega Corp., Madison, WI).

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