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The Role of Extranuclear Signaling Actions of Progesterone Receptor in Mediating Progesterone Regulation of Gene Expression and the Cell Cycle

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Human progesterone receptor (PR) contains a motif that interacts with the SH3 domain of Src and mediates rapid activation of Src and downstream MAPK (Erk-1/-2) without relying on the transcriptional activity of the receptor. Here we investigated the role and intracellular location of this nontranscriptional activity of PR. Progestin activation of Src/MAPK occurred outside the nucleus with the B isoform of PR that was distributed between the cytoplasm and nucleus, but not with PR-A that was predominantly nuclear. Breast cancer cells stably expressing wild-type PR-B or PR-B with disrupting point mutations in the SH3 domain binding motif (PR-BΔSH3) that do not affect the transcriptional activity of PR, were compared for effects of progestin on endogenous target gene expression and cell proliferation. Progestin induction of the cyclin D1 gene, which lacks a progesterone response element, was dependent on PR activation of the Src/MAPK pathway, whereas induction of the Sgk (serum and glucocorticoid regulated kinase) gene that contains a functional progesterone response element was unaffected by mutations that interfere with PR activation of Src. Progestin induction of cell cycle progression was also abrogated in cells expressing PR-BΔSH3, and no effect of progestin on cyclin D1 expression and cell cycle was observed in the presence of PR-A. These results highlight the importance of PR activation of the Src/MAPK signaling pathway for progesterone-induced transcription of select target genes and cell cycle progression. (*Molecular Endocrinology* 21: 359–375, 2007)

PROGESTERONE PLAYS an important role in development, growth, and maintenance of female reproductive tissues, and its biological actions are mediated by the progesterone receptor (PR), a member of the steroid/nuclear hormone receptor superfamily. In the mammary gland, progesterone has both differentiation and proliferative effects and is essential for stimulating ductal side branching, and development of lobuloalveolar structures required for milk protein production during pregnancy (1–6). Progesterone and PR are also involved in mammary tumorigenesis and growth of mammary epithelial-derived cancer cells. PR knockout (PRKO) mice are less susceptible than wild-type mice to induction of mammary tumors in response to chemical carcinogens, and the synthetic progestin medroxyprogesterone acetate (MPA) induces mammary adenocarcinomas in female BALB/c mice (7, 8). In women, administration of combined estrogen and progestin hormone replacement therapy for postmenopausal symptoms increased the risk of breast cancer relative to women taking estrogen alone (9, 10). In PR-positive breast cancer cell lines, progestins have a biphasic effect on proliferation, inducing quiescent cells to enter the cell cycle and progress through one or multiple rounds of cell division followed by arrest in early G1 (11–15). Several G1-S phase cell cycle proteins are transiently up-regulated by progestins including c-fos, c-myc, and cyclins E and D1, leading to accumulation and increased activity of the Cdk4/Cyclin D1 complex and hyperphosphorylation of Rb (11–15). Growth arrest is associated with induction

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Abbreviations: CCND1, Cyclin D1; cdk, cyclin-dependent kinase; DBD, DNA binding domain; DCC-FBS, 5% fetal bovine serum that had been treated with dextran-coated charcoal; EGF, epidermal growth factor; ER, estrogen receptor; FRAP, fluorescence recovery after photobleaching; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; GRE, glucocorticoid response element; LBD, ligand binding domain; MMTV, mouse mammary tumor virus; MPA, medroxyprogesterone acetate; mPR, novel membrane G protein-coupled receptor; NF, nuclear factor; NLS, nuclear localization sequences; NTD, amino terminal domain; PI3K, phosphatidylinositol 3-kinase; PR, progesterone receptor; PRE, progesterone response element; PRKO, PR knockout; SDS, sodium dodecyl sulfate; Sgk, serum and glucocorticoid regulated kinase; SV, simian virus; YFP, yellow fluorescent protein.

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of cyclin-dependent kinase (cdk) inhibitors $p18^{INK}$ and p21, and decreased expression and activity of cdk complexes (13, 16, 17). Depending on the culture conditions, progestins can also promote sustained proliferation of human breast cancer cells (18), protect cells against apoptosis (19), and affect cell adhesion and migration (20).

Human PR is expressed from a single gene as two proteins, PR-A (molecular weight = 82,000) and PR-B (molecular weight = 98,000); sharing identical sequences in the ligand binding domain (LBD) and DNA binding domain (DBD) and part of the amino terminal domain (NTD), except for the first 164 amino acids that are missing in PR-A (21). The two PR isoforms have distinct functional activities *in vitro* and *in vivo*. Studies of transgenic and PR isoform selective knockout mice have shown that PR-B is more important for proliferative effects of progesterone in the mammary gland, whereas PR-A is the more important isoform for functional response to progesterone in the ovary and uterus (22–24). PR-B is generally a stronger transcriptional activator than PR-A (25), and microarray experiments have identified PR isoform selective target genes, as well as targets regulated by both receptors (26). Disruption of the normal ratio of PR-A to PR-B has been detected in human breast cancers, and PR-A rich tumors have worse disease-free survival rates and tend to develop resistance to tamoxifen (27, 28).

In addition to direct transcriptional effects mediated by nuclear PR, progestins can rapidly activate the Src/Ras/MAPK (Erk-1/-2) signaling pathway in breast cancer and mammary epithelial cells under conditions that correlate with progestin-induced proliferation or cell cycle arrest (15, 29–31). Progestins have also been shown to activate the Jak1/-2/Stat3 signaling pathway in a manner dependent on Src in C4HD mouse mammary tumor cells and to be correlated with progestin stimulation of growth (32). These effects of progestins on cell signaling pathways in the absence of transcription are dependent on conventional PR, suggesting PR has dual functions as a nuclear transcription factor and as an activator of cell signaling molecules. We previously defined the presence of a polyproline motif (amino acids 421–428) in the NTD of human PR that mediates direct interaction of PR with the SH3 domain of the nonreceptor tyrosine kinase, Src, and activates Src by an SH3 domain displacement mechanism (31). Point mutations (P422A, P423A, P427A) in this motif abrogated the ability of PR to interact with and activate Src in cell-free assays and within cells but had no detectable effect on other functional activities of PR including progestin binding, specific DNA binding and progestin-dependent transcriptional activation of progesterone response element (PRE) controlled reporter genes (31, 33). Conversely, mutations in the DNA binding domain or in a phosphorylation site, p294, which cripples transcriptional activity of PR, have no effect on rapid progestin activation of Src (15, 31, 33). Thus, the ability of PR to interact with Src appears to be separable from other functions of receptor and is dissociable by point mutations in the polyproline motif.

In the present study, we investigated the role of PR activation of Src through coupling with the SH3 domain, in mediating effects of progestin on cell functions. We also examined the intracellular location of this functional property of PR. Results show that progestin activation of the Src/MAPK signaling pathways occurs outside the nucleus with PR-B only and not with PR-A, and is required for progestin induction of cyclin D1 gene expression and cell cycle progression of breast cancer cells.

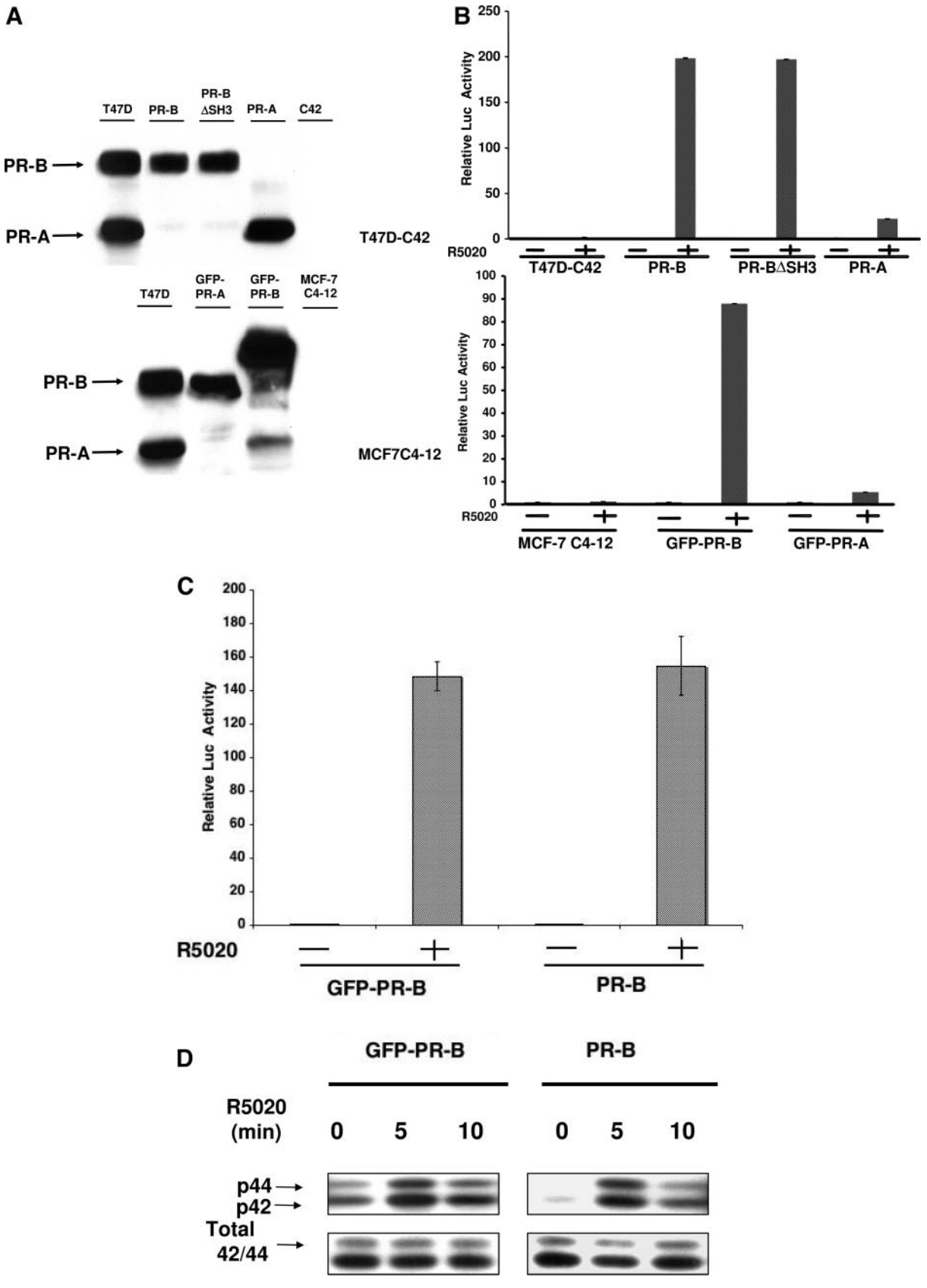
RESULTS

A and B Isoforms of PR Exhibit Differential Subcellular Localization and Ability to Mediate Progestin Activation of Src/MAPK

To determine cellular responses to progestin that require interaction of PR with the SH3 domain of Src, we have transiently or stably expressed wild-type PR, or PR bearing point mutations (ΔSH3) in the SH3 domain interaction motif into PR-negative cells. Target tissues for progesterone naturally express both PR-A and PR-B; thus, to study the function of each isoform independently also requires their ectopic expression in a PR null cell background. PR-negative variants of T47D (T47DY and T47DC42) and MCF-7 (MCF-7C4-12) breast cancer cells are attractive experimental systems for analysis of ectopically expressed PR as they should contain all the necessary cofactors and signaling pathways to mount an appropriate response to progestins. The T47DC42 (34) and MCF-7C4-12 (35) cells are also estrogen receptor (ER) negative, thus

Fig. 1. Stable Expression of PR in Breast Cancer Cells

A, *Upper panel*, Immunoblot analysis of T47DC42 breast cancer cells stably transfected with PR-B, PR-BΔSH3, or PR-A as compared with nontransfected T47DC42 and endogenous PR-A and PR-B in T47D cells. *Lower panel*, Immunoblot analysis of MCF-7C4-12 cells stably transfected with GFP-PR-B, or GFP-PR-A as compared with nontransfected MCF-7C4-12 cells and endogenous PR in the T47D cells. B, T47DC42 cells stably expressing PR-B, PR-BΔSH3, or PR-A in the *upper panel*, or MCF-7C4-12 cells stably expressing GFP-PR-B or GFP-PR-A in the *lower panel* were transduced for 24 h with adenovirus encoding (MOI = 5) an MMTV-Luc reporter. Cells were then treated with either vehicle (ethanol) or 10 nM R5020 for 24 h and cell lysates were assayed for luciferase activity. Values represent relative luciferase activity as the mean from three replicate experiments ± SEM. C, Same as B except analyzing R5020 induction of MMTV-luc in cells expressing GFP-PR-B or nontagged PR-B. D, Same as C except cells were treated for 5 or 10 min with 10 nM R5020 and MAPK activation was detected by immunoblot with a phosphospecific p42/p44 antibody *vs.* an antibody that detects total MAPK (Cell Signaling Technology, Beverly, MA).



enabling analysis of PR actions independent of ER, whereas T47DY cells have very low ER (36).

Wild-type PR-B, PR-BΔSH3 or a vector control expressing LacZ were stably transfected into these PR-negative breast cancer cells. Additionally, PR-A was stably transfected in T47DC42 cells and green fluorescent protein (GFP)-PR-A and GFP-PR-B were expressed in MCF-7C4-12 cells. Cells isolated from each group were selected based on a uniform immunostaining pattern of PR in the majority of cells (not shown), equivalent expression of PR protein as detected by immunoblotting (Fig. 1A), and function with respect to progestin-induction of gene transcription and rapid activation of the Src/MAPK signaling pathway. In T47DC42 cells, PR-A, PR-B, and PR-BΔSH3 proteins were all expressed at equivalent levels, slightly lower than that of endogenous PR in standard T47D cells, whereas no PR was detected in parental T47DC42 cells (Fig. 1A). GFP-PR-A and GFP-PR-B in MCF-7C4-12 were also expressed at similar levels as endogenous PR in parental T47D cells, but as expected the fusion proteins exhibited a slower mobility on sodium dodecyl sulfate (SDS) gels (Fig. 1A, *lower panel*). PR-B and PR-BΔSH3 expressed in T47DC42 cells mediated equivalent induction of a mouse mammary tumor virus (MMTV)-Luc reporter gene by the synthetic progestin R5020 (10 nM), whereas PR-A as expected exhibited a much lower transcriptional activity than PR-B (Fig. 1B). PR-B and PR-BΔSH3 expressed in T47DY cells exhibited the same properties and relationships as in T47DC42 cells (not shown). GFP-tagged receptors were fully functional with respect to R5020 induction of MMTV-Luc, (Fig. 1, B and C) and rapid activation of MAPK when compared directly with nontagged receptors (Fig. 1, B–D). Thus, as previously reported, the GFP tag did not measurably alter transcriptional or nontranscriptional activity of PR (37).

PR-A and -B have identical SH3 domain interaction polyproline motifs in their NTD, and we previously showed that both PR isoforms are capable of efficiently interacting with SH3 domains and activating Src tyrosine kinases in cell-free assay (31). However, our previous studies on progestin activation of Src within cells were done with the B isoform of PR only (31, 33). To determine whether PR-A in cells can mediate rapid progestin stimulation of Src kinase, steroid receptor-negative COS-7 cells were transiently cotransfected with human PR-B or -A along with c-Src. Kinase activity was assayed by immunoprecipitation of total c-Src followed by ^{32}P-ATP incorporation into enolase as a Src substrate (31). As shown in Fig. 2A, a short 2-min treatment with progestin stimulated an increase in Src kinase activity in cells expressing PR-B, but not in cells expressing PR-A. Similar results were observed in T47DC42 cells that stably expressed PR-A or PR-B using an ELISA to detect activated Src based on phosphorylation of tyrosine 418 in the activation loop (Fig. 2B). Activation of Src by short-term treatment with R5020 (2–5 min) was observed only in cells expressing PR-B and was similar to that stimulated by epidermal growth factor (EGF). Little or no activation of Src by R5020 was observed in cells expressing PR-A or PR-BΔSH3 (Fig. 2B). Consistent with the effect on Src, R5020 stimulated a rapid (5–10 min) activation of MAP kinase (Erk1/-2) in cells expressing PR-B, but had little to no effect in cells expressing PR-A or PR-BΔSH3 (Fig. 2C). Induction of MAPK activity by R5020 was blocked by the Src inhibitor PP2 indicating a dependency on Src for MAPK activation (Fig. 2C). It should be noted that progestin-induced activation of MAPK is more transient and less robust than that of EGF used as a positive control (Fig. 2C). The more transient response is likely due to progestins and EGF activating the Src/MAPK signaling pathway by a different mechanism and quenching by translocation of some cytoplasmic PR to the nucleus.

One possible explanation for the inability of PR-A to activate Src and MAPK within cells is a different intracellular localization than PR-B. To address this question, we examined localization of stably expressed GFP-PR-A and GFP-PR-B by direct fluorescence confocal microscopy in either live or fixed cells. GFP-PR-B in fixed MCF-7C4-12 cells, as analyzed by digital deconvoluted fluorescence microscopy, was found to be distributed between the nucleus and cytoplasm in the absence of hormone. Addition of progestin for 60 min (before fixation) caused an increased accumulation of GFP-PR-B in the nucleus, but substantial amounts remained in the cytoplasm after hormone treatment (Fig. 3A). In contrast, GFP-PR-A was almost exclusively nuclear in the presence and absence of progestin (Fig. 3A). To determine whether this different localization of PR-A and PR-B could be caused by the GFP, nontagged PR-A and PR-B expressed in T47DC42 cells were also analyzed by indirect immunofluorescence with the PR-specific monoclonal antibody, 1294. Nontagged PR-A was almost exclusively nuclear in the presence and absence of R5020, whereas nontagged PR-B exhibited a distribution between the nucleus and cytoplasm (Fig. 3B). Similar results were observed in COS-7 cells by transiently expressing nontagged PR-B or PR-A (Fig. 3C), except that cytoplasmic staining of PR-B was more punctate and some staining at the cell periphery was observed suggesting both cytoplasmic and membrane localization under these conditions. In cells stably expressing PR-B, a fairly homogeneous cytoplasmic staining was observed with no evidence of staining at the cell membrane. Nonetheless, the major difference between PR-A and PR-B under all conditions examined was a predominant nuclear localization of PR-A, whereas PR-B exhibited a distribution between nuclear and extranuclear sites.

We also analyzed localization of GFP-PR in live cells and quantitated the ratio of receptor distribution between the nucleus and cytoplasm. Cells were treated with vehicle (ethanol) or 10 nM R5020 for 2 h, and live cells were imaged using a laser confocal microscope (Zeiss LS510; Carl Zeiss MicroImaging Inc., Thornwood, NY). GFP fluorescence intensities of the whole

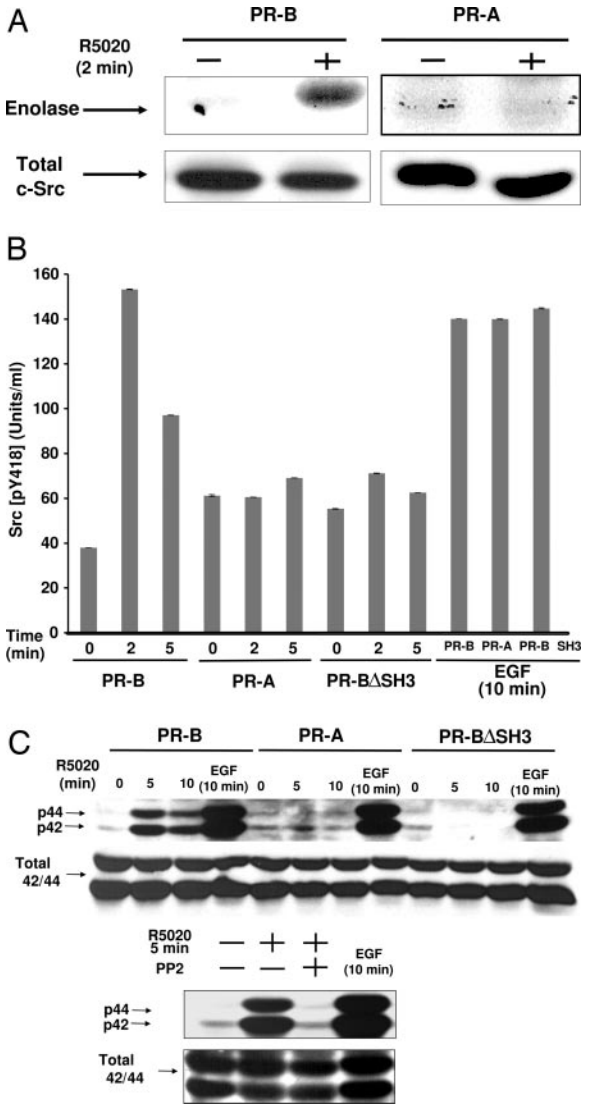


Fig. 2. PR-B But Not PR-A Mediates R5020 Activation of Src

A, COS-7 cells were transfected with PR-B or PR-A and Src kinase activity was measured before and after treatment of cells for 2 min with 10 nM R5020. Immunoprecipitated Src (*upper panels*) from cell lysates was assayed by ^{32}P incorporation into the substrate enolase followed by SDS-gel electrophoresis and autoradiography. Total Src protein (*lower panel*) was detected by immunoblot with a Src-specific antibody (327) antibody. B, T47DC42 cells expressing PR-B, PR-A or PR-BΔSH3 were treated with vehicle (ethanol), 10 nM R5020 for 2 and 5 min, or EGF (25 ng/ml) for 10 min. Activated Src [Src-pY (418)] was determined by a Src [pY418] ELISA kit (BioSource) as described in *Materials and Methods*. Data are average determination from three replicates ± SEM. C, *Upper panel*, T47DC42 cells expressing PR-B, PR-A or PR-BΔSH3 were treated with vehicle (ethanol), 10 nM R5020 for 2 and 5 min, or EGF (25 ng/ml) for 10 min. Activated MAPK (Erk-1/-2) was detected by immunoblot with phosphospecific p42/p44 antibody (Cell Signaling Technology) and total MAPK was detected with a pan antibody (Cell Signaling Technology). *Lower panel*, T47DC42 expressing PR-B were treated with 10 nM R5020 for 5 min in the presence or absence of the Src kinase inhibitor, PP2 (10 μM). Data are representative of at least three independent experiments.

cell and the nucleus (n = 15–20 cells) were measured and the percent nuclear fluorescence was calculated (Fig. 4). Consistent with fixed cells, GFP-PR-B was distributed between the nucleus and cytoplasm in the absence and presence of hormone, with 51.5 ± 8 and 65.7 ± 8% nuclear fluorescence intensities, respectively. In contrast, PR-A localized mainly to the nucleus in the absence of hormone with little change after hormone addition, exhibiting 87.5 ± 5 and 87.8 ± 7% nuclear fluorescence, respectively. GFP-PR-BΔSH3 displayed a similar distribution between the cytoplasm and nucleus as wild-type PR-B in the absence of progestin (Fig. 3B); however, a strong cytoplasmic-nuclear translocation occurred with hormone. In live cells, GFP-PR-BΔSH3 in the absence of hormone exhibited 60% (60.9 ± 0.9%) nuclear fluorescence and this increased to 87% (87.3 ± 8%) after hormone treatment (Fig. 4). To begin to address the mechanism underlying the distinct localization of PR-A and PR-B, we analyzed the mobility of GFP-tagged receptors stably expressed in living cells by the technique of fluorescence recovery after photobleaching (FRAP). Based on the half-time of recovery of a laser-induced bleached area over the cell nucleus, PR-B exhibited a faster mobility ($t_{1/2}$ of 2.5 ± 0.09 sec) than PR-A ($t_{1/2}$ of 4.7 ± 0.85) or PR-BΔSH3 ($t_{1/2}$ of 4.0 ± 1.05) in the presence of hormone (see Supplemental Figure 1, which is published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org), indicating a correlation between slower mobility and greater nuclear retention of PR-A. These data, taken together, indicate that localization outside the nucleus is required for PR to activate Src, and that differences in intracellular trafficking contribute to the greater cytoplasmic localization and nontranscriptional signaling activity of PR-B.

Progestin Activation of Src Is an Extranuclear Function of PR

To further address the role of PR localization in progestin activation of Src, the nuclear localization sequences (NLS) of PR were removed by deleting the second zinc-finger in the DNA binding domain and part of the hinge region (amino acids 591–644). These deletions are analogous to NLS mutants described previously in rabbit PR (38). Ectopically expressed PR-BΔnls and PR-AΔnls in COS-7 cells exhibited exclusive cytoplasmic localization in the presence and absence of hormone as shown by indirect immunofluorescence staining with a PR-specific antibody (Fig. 5A). The NLS PR mutants had no transcriptional activity with respect to progestin induction of a PRE luciferase controlled reporter gene (data not shown), indicating that if there is a small amount of nuclear PR it is not of functional consequence. PR-BΔnls mediated progestin-induced activation of Src to a similar extent as wild-type PR-B, whereas no progestin activation of Src was mediated by wild-type PR-A, unless it was forced to the cytoplasm by mutation of the NLS (Fig. 5A). Interestingly, a more pronounced activation of Src

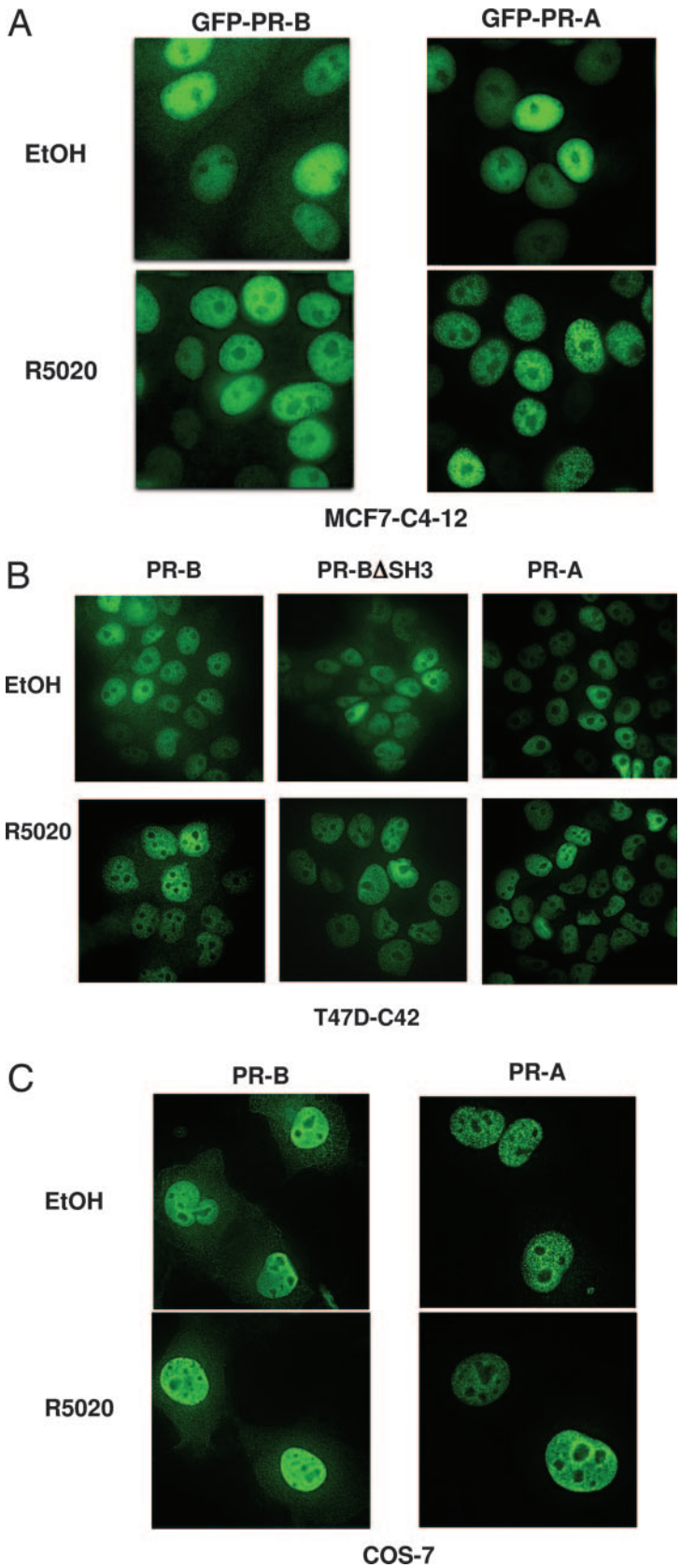


Fig. 3. Intracellular Localization of PR-A and PR-B

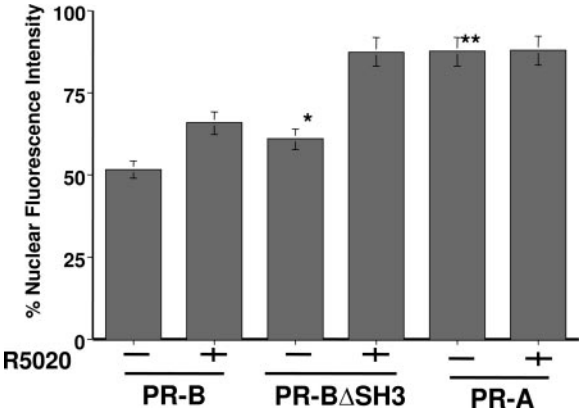


Fig. 4. Quantitation of Receptor Localization in Live Cells

T47D cells stably expressing GFP-PR-B, GFP-PR-BΔSH3, or GFP-PR-A were treated with ethanol or 10 nM R5020 for 2 h. Live cells were imaged as described in *Materials and Methods*. Fluorescence intensities of whole cells and nuclei were measured and percentage of nuclear fluorescence intensities were calculated. Values represent average percentage of nuclear fluorescence intensities from 15–20 cells. * and **, Statistically significant differences ($P < 0.05$) as determined by Student's *t* test between the indicated treatment groups as compared with unliganded PR-B.

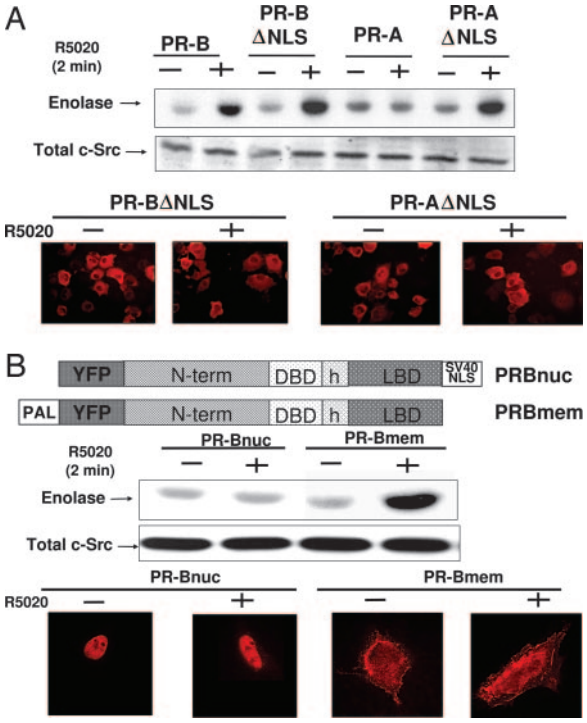


Fig. 5. Activation of cSrc Is an Extranuclear Function of PR

A, COS-7 cells were transiently transfected with PR-B, PR-A, or the same receptors with a deletion of the nuclear localization sequences (ΔNLS) as described in *Materials and Methods*. Cells were treated for 2 min with vehicle (−) or R5020 (10 nM) and Src kinase activity (*upper panels*) and total Src (*bottom panel*) were measured as Fig. 2A. *Lower panels* show immunofluorescence staining of PR-BΔNLS and PR-AΔNLS with a PR-specific antibody (1294) in the absence and presence of 10 nM R5020 (2 h). B, *Upper panel*, A schematic of PR constructs: PR-Bnuc with N-terminal YFP-tag and C-terminal 3XSV40 NLS: PR-Bmem with N-terminal YFP and palmitoylation (PAL) sequences from neuromodulin. Cells transiently transfected with PR-Bmem were treated for 2 min with vehicle (−) or R5020 (10 nM) and total Src protein levels (*lower panel*) and Src kinase activity were detected as in Figs. 2A and 5A. *Lower panels* are indirect immunofluorescence staining of PR with 1294 Mab in cells transfected with PR-Bnuc or PR-Bmem and then treated without or with R5020 for 2 h.

by progestin was not observed with either PR-AΔnls or PR-BΔnls as compared with wild-type PR-B, even though more receptors are present in the cytoplasm than wild-type PR-B. These results suggest that kinases and cell signaling machinery rather than the concentration of extranuclear PR are limiting for progestin-induced activation of Src (Fig. 5A).

In a reciprocal experiment, PR-B was forcedly targeted to the nucleus by tagging receptor with three SV40NLSs (PR-Bnuc). As verified by indirect immunofluorescence staining, PR-Bnuc showed predominantly nuclear localization in the presence or absence of hormone with little evidence of the cytoplasmic staining observed with native PR-B (Fig. 5B). As opposed to wild-type PR-B, PR-Bnuc failed to mediate rapid progestin activation providing further evidence that progestin activation of Src requires extranuclear localization of PR (Fig. 5B). We also attempted to forcedly target PR-B to the cell membrane by tagging it with palmitoylation sequences (PR-Bmem). Although PR-Bmem in COS-7 cells did not localize exclusively to the cell membrane, it exhibited a disproportionately higher localization in the cell membrane relative to cytoplasmic localization as compared with wild-type PR-B; yet, the magnitude of progestin activation of Src was comparable for PR-Bmem and wild-type PR-B (Fig. 5B). Because PR-Bmem is not exclusively membrane, we cannot determine whether it activates Src in the cytoplasm or in the cell membrane. However, these results provide further evidence that Src activation is mediated by PR outside the nucleus and that Src signaling complexes must be limiting whether PR

A, MCF-7C4-12 cells stably expressing GFP-PR-B or GFP-PR-A were treated with vehicle [ethanol (EtOH)] or R5020 (10 nm) for 2 h. Cells were then fixed and examined by a digital deconvoluted fluorescence microscope. Figures represent a single Z-section of each image. B, Same as A except T47DC42 stably expressing nontagged PR-B, PR-BΔSH3, or PR-A were treated with vehicle (ethanol) or R5020 (10 nM) for 2 h and PR was detected by indirect immunofluorescence with a PR-specific monoclonal antibody, 1294. C, Same as B except COS-7 cells were transiently transfected with PR-B or PR-A, and treated with vehicle (ethanol) or R5020 for 2 h.

is located in the cytoplasm or associated with the cell membrane.

Progestin Activation of Src Mediated by PR-SH3 Domain Interaction Is Sufficient to Induce Activation of a MAPK-Dependent Nuclear Transcription Factor, Elk-1

Because MAPK is capable of phosphorylating and activating certain nuclear transcription factor targets suggests that activation of the Src/MAPK signaling pathway may provide an alternate means for progesterone to regulate gene transcription independent of the direct nuclear transcriptional activity of PR. To test this possibility and determine whether transient activation of Src/MAPK by progestin is sufficient enough to be of functional consequence, we analyzed the effect of progestin on activation of a MAPK-dependent transcription factor target, Elk-1. The C-terminal transcriptional activation domain of Elk-1 fused to the heterologous DNA binding domain of GAL-4 translocates to the nucleus and mediates transcription of a GAL4-responsive reporter gene (7X UAS-Luc) in response to phosphorylation by MAPK (39). T47D cells stably expressing different forms of PR were cotransfected with Gal4DBD-Elk-1 and a 7XUAS-luciferase reporter. In cells expressing PR-B, treatment with R5020 induced an Elk-1-mediated transactivation that was nearly as efficient as that induced by EGF (Fig. 6). No progestin induction was observed in cells expressing PR-BΔSH3, or that lacked PR-B (Fig. 6). Progestin-dependent activation of Elk-1 was blocked by the Src kinase inhibitor, PP2, but not the inactive analog PP3, and by the MEK inhibitor U0126, indicating this response to progesterone is dependent on both Src and MAPK (Fig. 6). Consistent with the inability of PR-A to mediate activation of Src/MAPK, progestin-dependent Elk-1 activation was not observed in cells expressing PR-A (data not shown). Similar results of progestin activation of Elk-1 were extended to endometrium carcinoma cells (Hec-1B) cotransfected with different forms of PR and GAL4DBD Elk-1 (data not shown). Because the reporter gene lacks a PRE and has a DNA binding site only for the Elk-1 construct, enables the conclusion that progesterone induction is PRE independent via MAPK-dependent phosphorylation of Elk-1 (Fig. 6). These results show that the transient activation of Src and MAPK mediated by the SH3 domain interaction motif of PR, is sufficient to influence gene transcription through MAPK-dependent activation of other transcription factors.

Progestin Induction of the Endogenous Cyclin D1 Gene Is Dependent on PR-B Coupling with the SH3 Domain of Src

The Elk-1 results prompted us to determine whether endogenous target genes can also be regulated through cytoplasmic signaling pathways activated by PR through its SH3 domain binding motif. Two known target genes were analyzed, cyclin D1 (CCND1) that lacks a progesterone response element (PRE) (40–42), and Sgk (serum and glucocorticoid regulated kinase) that contains a functional glucocorticoid response element (GRE)/PRE that mediates glucocorticoid or progesterone induction of transcription (43–45). PR-negative T47DC42 cells stably expressing PR-A, PR-B, PR-BΔSH3, or LacZ as a vector control were treated with vehicle (ethanol) or 10 nM R5020 (6 h), RNA was prepared and cyclin D1 and Sgk mRNA levels were

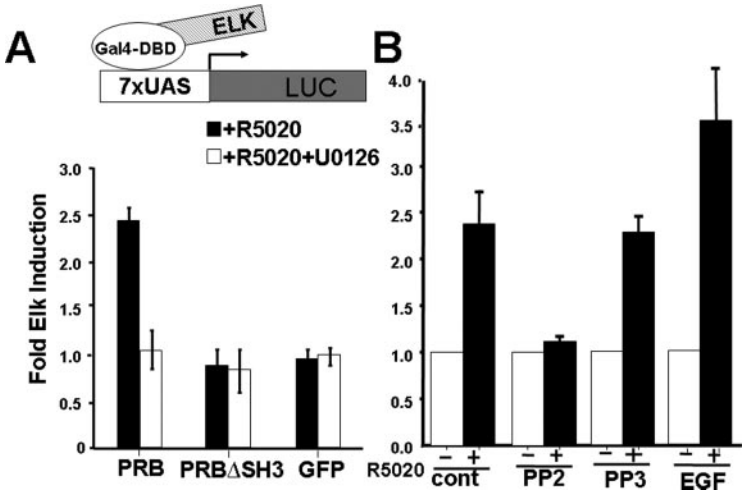


Fig. 6. Progestin-Induced Transactivation of Elk-1 Is Dependent on the SH3 Domain Binding Motif of PR, Src, and MAPK

T47DC42 cells stably expressing PR-B, PR-BΔSH3 or GFP (no PR vector control) were transiently transfected with Gal4DBD-Elk1 and a 7XUAS-Luc reporter gene and then treated with and without 10 nM R5020 for 16 h. A, Some cells were pretreated for 30 min with the MEK inhibitor UO126 (30 μM). B, Other cells were pretreated with the Src inhibitor PP2 (10 μM), or the inactive analog, PP3 (10 μM). Cells were also treated with EGF (50 ng/ml) as a positive control. Values represent fold R5020 or EGF induction of luciferase expression and are the mean of at least three independent determinations ± SEM.

determined by quantitative real time RT-PCR. In cells containing PR-B, R5020 induced a 4- to 5-fold stimulation of CCND1 expression, whereas no induction was obtained in cells lacking PR or expressing PR-BΔSH3 (Fig. 7A). In contrast, Sgk was induced 20- to 25-fold by progestin in the presence of either wild-type PR-B or PR-BΔSH3 (Fig. 7A). Both genes are primary transcriptional targets, as progestin induction was not inhibited by the protein synthesis inhibitor cycloheximide (Fig. 7B). To further investigate the role of signaling pathways, cells expressing PR-B were pretreated with UO126 or PP2 for 30 min before R5020. Both MAPK and c-Src inhibitors partially blocked R5020 induction of cyclin D1 gene expression. The phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 also attenuated progestin-induced activation of cyclin D1. gene expression. These inhibitors had no significant effect on progestin induction of Sgk (Fig. 7C). In cells expressing PR-A, treatment with R5020 did not induce expression of CCND1, but Sgk expression was induced, although to a lesser extent than mediated by PR-B. (Fig. 7D). These data indicate that progesterone regulation of cyclin D1 gene expression is mediated by PR activation of cell membrane/cytoplasmic signaling pathways that can converge upon and activate other nuclear transcription factor targets. The data with pharmacological inhibitors further indicates the involvement of multiple kinase cascades including Src/MAPK and PI3K/Akt signaling pathways.

Progestin Induction of Cell Cycle Progression Is Dependent on the Src SH3 Domain Binding Motif of PR

Because CCND1 plays a key role in cell cycle progression through G1-S, we asked whether PR activation of

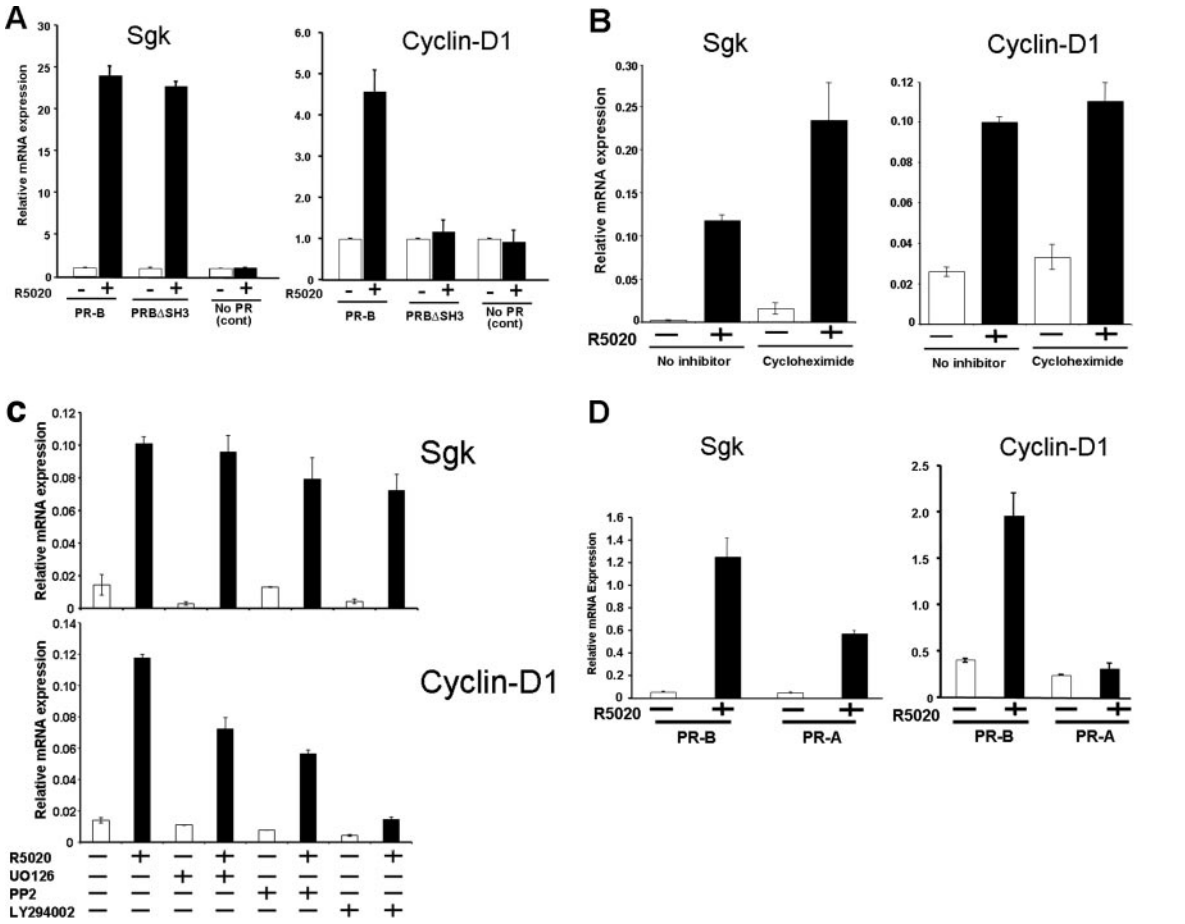


Fig. 7. Progestin-Induction of Sgk and Cyclin D1 Genes Is Mediated by Distinct Pathways

A, T47DC42 cells expressing PR-B, PR-BΔSH3, or no PR (Lac-Z as vector control) were treated with vehicle (−) or R5020 (10 nM) for 6 h, total RNA was prepared and analyzed for Sgk and Cyclin D1 mRNA by real time quantitative RT-PCR as described in *Materials and Methods*. Data were expressed as cyclin D1 or Sgk RNA relative to GAPDH RNA. B, Same as A, except that cells from T47DC42 cells expressing wild-type PR-B were treated with ± 10 nM R5020 for 6 h in the presence and absence of 10 μg/ml cycloheximide. C, Same as A, except cells expressing wild-type PR-B were pretreated for 30 min with the Src, inhibitor (PP2, 10 μM), the MEK inhibitor UO126 (30 μM), or the PI3K inhibitor LY 294002 (30 μm). D, Same as A except comparison of Sgk and cyclin D1 RNA expression was between cells expressing PR-B and PR-A. All values represent the mean of at least three independent experiments ± SEM.

signaling pathways through coupling with SH3 domain of Src and perhaps other signaling molecules has a role in the well-known biphasic effect of progestins on cell cycle progression. Quiescent T47DC42 expressing PR-B, PR-BΔSH3 or PR-A grown on 2.5% charcoal-stripped fetal bovine serum, were treated with R5020 and analyzed at different times by flow cytometry to determine the distribution of cells in phases of the cell cycle. In cells expressing PR-B, treatment with progestin for 24 h resulted in a substantial (70%) increase of cells in G2/M +S that declined slightly at 48 h of hormone treatment (Fig. 8A). By 72 h of treatment with R5020, the percentage of cells in G2/ M + S decreased below that of the starting cell population in the absence of hormone, whereas the percentage of cells in G1 phase increased (Fig. 8A). Progestin stimulation of cell cycle progression was substantially reduced in cells expressing PR-BΔSH3 or PR-A and no effect of progestin was observed in the absence of PR (LacZ control). The cell cycle arrest phase (72–96 h)

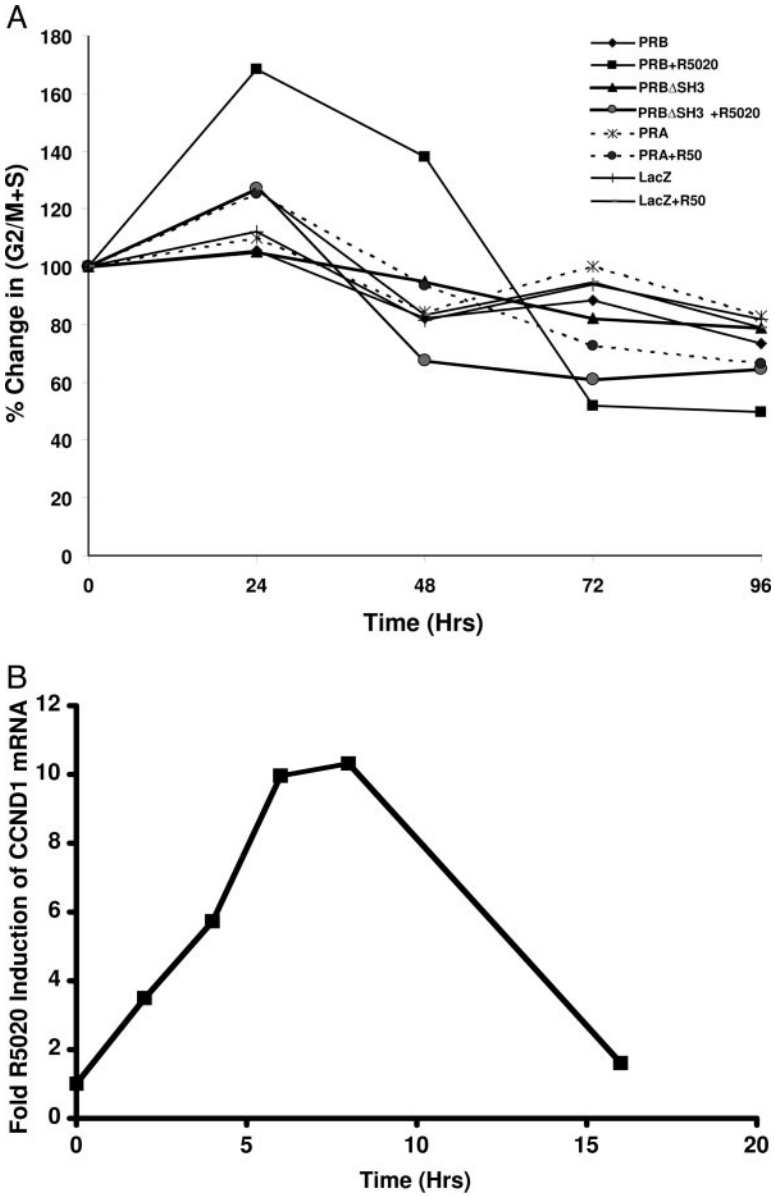


Fig. 8. Progestin-Induced Cell Cycle Progression Mediated by PR-B Is Dependent on the SH3 Domain Interaction Motif

A, T47DC42 cells stably expressing PR-B, PR-A, PR-BΔSH3 or no receptor (LacZ control) were treated for the times indicated with 10 nM R5020, harvested and subjected to cell cycle analyses by flow cytometry as described in *Materials and Methods*. The y-axis represents change in percentage of cells in G2M and S phase as compared with the zero time control. The figure shows a single representative experiment of three independent experiments. B, Time course of progestin induction of cyclin D1 mRNA. T47DC42 cells expressing PR-B and treated with ± 10 nM R5020 for indicated times were analyzed for expression of CCDN1 mRNA by real-time quantitative RT-PCR as described in *Materials and Methods*. Data were expressed as fold R5020 induction of cyclin D1 RNA expression after normalization to GAPDH RNA.

induced by R5020 was also diminished in cells expressing PR-A or PR-ΔSH3 as compared with wild-type PR-B. A time course of progestin induction of cyclin D1 mRNA expression showed that stimulation was first observed at 3 h, peaked at 8–10 h and declined thereafter (Fig. 8B). This transient induction of cyclin D1 gene expression correlated with the biphasic effect of progestin on inducing cell cycle progression followed by arrest in G1. Taken together, these results support the conclusion that PR activation of a Src/MAPK/cyclin D1 signaling pathway, independent of the transcriptional activity of PR, has a key role in mediating the effect of progesterone on the cell cycle in breast cancer cells.

DISCUSSION

Steroid receptors have been extensively characterized as ligand-dependent transcriptional activators that bind to hormone response elements in the regulatory regions of target genes and recruit coactivators that facilitate chromatin remodeling and assembly of the transcription complex (46, 47). More recently, they have been described to also function as mediators of rapid steroid activation of protein kinase signaling pathways independent of their transcriptional activity (48). An important question is the biological significance of the nontranscriptional activity of steroid receptors. To gain insights into this question, we have analyzed the influence of point mutations in PR that disrupts interaction with SH3 domain of Src and other signaling molecules without affecting transcriptional activity, on progesterone regulation of gene expression and induction of cell cycle progression.

Progesterone is known to induce transcriptional activation of cyclin D1, but this target gene lacks a PRE and the mechanism of regulation has not been well defined (15, 40–42). We show here that progestin induction of cyclin D1 gene expression is dependent on PR coupling with SH3 domain of Src. Induction occurred only with wild-type PR-B, not with PR-BΔSH3 or PR-A that are unable to activate Src or MAPK, and was sensitive to MAPK and Src inhibitors (Fig. 7). However, progestin induction was only partially blocked by the MEK inhibitor U0126 or the Src inhibitor (PP2), indicating involvement of Src/MAPK as well as other signaling pathways. Progestins have been reported to induce activation of the PI3K/Akt pathway in breast cancer cells (40) and we previously showed that PR binds to SH3 domains of a select group of other kinases including the p85 subunit of PI3K (31). Thus, mutations in the SH3 domain interaction motif of PR likely disrupt progestin activation of other signaling pathways. Indeed, the PI3K inhibitor LY294002 substantially reduced progestin induction of cyclin D1 gene expression suggesting an involvement of P13K/Akt (Fig. 7). The cyclin D1 promoter has regulatory elements for multiple transcription factors including specificity protein-1, cAMP response element binding protein, c-jun, nuclear factor (NF)-κB and Ets, any of which could be nuclear targets of progestin-activated signaling pathways (41, 49–51). Kurachi and colleagues (40) reported that MPA induction of cyclin D1 expression in breast cancer cells was dependent on activation of a PI3K/Akt/NFκBα nongenomic signaling pathway. Whether MPA stimulated binding of NFκBα to the cyclin D1 promoter was not explored. Based on chromatin immunoprecipitation assays, PR was reported to be recruited to the cyclin D1 promoter through tethering with c-jun bound to an AP-1 site (41). The tethered PR/c-jun complex itself could be a nuclear target of a progesterone activate membrane/cytoplasmic signaling pathways; an interesting possibility that has not been explored. Further studies will be needed to more completely define the mechanisms, signaling pathways and other transcription factor targets that mediate progestin induction of the cyclin D1 gene.

The Sgk gene contains a functional GRE/PRE in the upstream promoter, is inducible by either glucocorticoids or progestins, and glucocorticoid induction was shown to be dependent on GR binding to the GRE/PRE (43–45). By chromatin immunoprecipitation assay, we have detected a progestin-dependent PR recruitment to this region of the Sgk promoter indicating that progesterone regulation is also mediated by PR interaction with the GRE/PRE (unpublished observations from Melvin, V., V. Boonyaratanakornkit, and D. P. Edwards). Progestin induction of Sgk was mediated equally by wild-type PR-B and PR-BΔSH3 (Fig. 7A), and was insensitive to pharmacological inhibitors of Src, MAPK, and P13 kinase (Fig. 7C). Thus, rapid activation of Src/MAPK and other signaling pathways was of no consequence for progestin induction of Sgk. Whether PR activation of signaling pathways as a general rule is required for target genes lacking PREs, and not for genes regulated by direct PR binding with PREs, remains to be determined. Gene expression microarray experiments are currently being conducted with breast cancer cells expressing wt PR-B and PR-BΔSH3 to identify and categorize progesterone-regulated target genes in this manner. The ability of PR to regulate gene expression by activation of signaling pathways that converge on other transcription factors potentially provides a mechanism to expand the diversity of progesterone target genes.

Cyclin D1 is a key cell cycle regulatory protein that promotes G1-S phase transition and is an important mitogenic sensor linking intracellular signals to the cell cycle machinery (52, 53). Therefore, as a potential cellular consequence of progestin induction of cyclin D1 expression through PR activation of signaling pathways, we examined cell cycle regulation. Progestin stimulation of cell cycle progression was observed in cells expressing PR-B but was minimal in the presence of PR-BΔSH3 or PR-A that are not capable of activating Src/MAPK signaling (Figs. 2 and 6) or in-

ducing cyclin D1 gene expression (Fig. 7). Consistent with the biphasic effect of progestins on cell cycle to stimulate entry and progression followed by arrest in G1, induction of cyclin D1 mRNA was transient. Although the decline of cyclin D1 mRNA occurred earlier than the peak induction of cells in G2/M + S at 48 h, cyclin D1 protein levels stimulated by R5020 in breast cancer cells was reported to remain elevated out to 48 h (15). These results indicate that PR activation of signal transduction pathways by coupling with the SH3 domain of Src, or perhaps other signaling molecules, is involved in mediating the proliferative effects of progesterone on breast cancer cells through induction of key cell cycle regulatory genes. Cyclin D1 as a progesterone target gene regulated in this manner is of potential relevance because it is required for normal mammary gland development (54), is frequently overexpressed in breast cancer (55), its overexpression in mice promotes development of mammary tumors (56), and inactivation of cyclin D1 protects against mammary tumor formation induced by *neu* and *ras* oncogenes (57). Gene amplification accounts for only approximately 30% of the overexpression of cyclin D1 in breast cancer (58) suggesting that epigenetic factors such as progesterone up-regulation of gene expression also plays a role.

Alternative mechanisms for how progesterone induces rapid activation of Src and MAPK have been reported. The NTD of PR-B has been shown to physically associate with the LBD of unliganded ERα and it was proposed that unliganded ERα, not PR in the complex, directly activates Src in response to binding progesterone (59). However, progestin-induced activation of the Src/MAPK signaling pathway that leads to cyclin D1 expression and cell cycle progression was obtained here with T47DC42 cells that lack ERα. Thus, our results support the importance of the direct interaction of PR with SH3 domain of Src without the need for an intermediate interaction through unliganded ERα. A novel membrane G protein-coupled receptor (mPR) has been identified that binds progesterone and was reported to mediate progestin-induced oocyte maturation in sea trout and activation of MAPK and inhibition of cAMP production in PR-negative MDA-231 breast cancer cells and in human myometrial cells (60, 61). The relationship between mPR and conventional PR in mediating progestin-induced activation of the Src/MAPK/cyclin D1 signaling pathway observed here has not been explored. Whether mPR is expressed in T47D or MCF-7 cells is not known. If mPR is not expressed, our data would indicate that conventional PR alone is sufficient for response to progesterone and mPR plays no role. If mPR is expressed, this raises the possibility of a functional cooperation between mPR and conventional PR. More studies will be needed to determine the biological significance of mPR in mediating nongenomic actions of progesterone in breast cancer cells.

The A and B isoforms of PR have distinct transcriptional activities that can vary in a cell type- and promoter context-dependent manner (24). Here we show that PR-A and PR-B also have different activities with respect to progestin activation of signaling pathways. PR-B, but not PR-A, was able to mediate progestin activation of Src and MAPK (Fig. 2). PR-A also failed to mediate progestin induction of MAPK-dependent Elk-1 transactivation (Fig. 6), endogenous cyclin D1 expression (Fig. 7) and cell cycle progression (Fig. 8). The inability of PR-A to mediate some of these effects has been observed previously (29, 40). Migliaccio *et al.* (29) suggested that the region of the NTD unique to PR-B must be responsible for mediating activation of c-Src. However, the polyproline motif that interacts with SH3 domain of Src lies within a region of the NTD common to both PR isoforms. Our results suggest the inability of PR-A to activate Src is due to a different intracellular localization than PR-B. In both live and fixed cells, PR-B localized to both the nucleus and cytoplasm in the presence and absence of progestin, whereas PR-A was mainly nuclear (Figs. 3 and 4). Lim *et al.* (62) using transiently transfected GFP-PR-B and GFP-PR-A in different cell types also observed a more nuclear localization of PR-A. This difference was questioned to be due to a property of transiently expressed PR that might not be observed with endogenous PR, or with stably expressed receptors in replicating cells (62). Our data showing that the differential localization of PR-A and PR-B was similar whether receptors were expressed transiently or stably in replicating cells suggests this is a fundamental property of the PR isoforms. The mechanism responsible for differential subcellular localization of PR-A and PR-B is not known. PR-A and PR-B have been proposed to adopt distinct conformations that influence the NLS and nucleo-cytoplasmic shuttling (62). Consistent with this idea, the slower intranuclear mobility of PR-A than PR-B observed by FRAP assay (Supplemental Figure 1) suggested that PR-A may have unique interactions with other proteins, or the nuclear matrix, that promotes a more efficient nuclear retention.

Experiments that forcedly targeted PR to different intracellular locations provided evidence that PR activation of Src occurs outside of the nucleus. Deletion of the NLS of PR-A forcing it to localize to the cytoplasm, enabled PR-A to mediate progestin-induced activation of c-Src, whereas wild-type PR-A that is mostly nuclear does not activate Src (Fig. 5A). Conversely, PR-B forcedly targeted to the nucleus with multiple simian virus (SV) 40 NLS tags failed to mediate progestin activation of Src (Fig. 5B) as does native PR-B that is distributed between the cytoplasm and nucleus (Figs. 3 and 4). To determine whether the cell membrane or the cytoplasm is the compartment outside of the nucleus where PR activates signaling pathways, we attempted to target PR to the plasma membrane by tagging it with palmitoylation sequences. Although PRmem mediated progestin-induced activation of Src, it did not localize exclusively in the cell membrane, some cytoplasmic localization was also observed (Fig. 5B). Thus, Src activation could be mediated by either

PR in the membrane or in the cytoplasm; the distinction cannot made under these conditions. However, the fact that the magnitude of progestin-induced activation mediated by PRmem, which is disproportionately much higher in the cell membrane, was similar to that of wild-type PR-B, suggested the Src cellular signaling machinery is limiting and that only small amounts of PR are required in the membrane or the cytoplasm to generate a response.

Biochemical cell fractionation and immunocytochemistry experiments have provided evidence for association of a small subpopulation of cellular ER (both ectopically expressed and endogenous ER) with the plasma membrane in different cell types. Studies have further shown that ER associates with cell membranes through interaction with other proteins such as caveolin-1, striatin, Shc, or IGF-I receptor and through lipid modification of ER by palmitoylation (see review in Ref. 63). Similar studies of whether and how PR localizes to plasma membrane in mammalian cells have not been done. With breast cancer cells that stably express receptors, we found that localization of PR-B outside the nucleus was fairly homogenous throughout the cytoplasm with little evidence of PR in the plasma membrane (Fig. 3, A and B). However, some punctate cell membrane immunostaining was detected with transiently transfected PR-B in COS-1 cells (Fig. 3C). Further studies are needed to determine whether a subpopulation of PR in mammalian cells associates with the plasma membrane and whether Src activation is mediated by PR at the cell membrane or by transient interaction with signaling molecules in the cytoplasm.

MATERIALS AND METHODS

Materials

R5020 (Promegestone) was obtained from Dupont/New England Nuclear Products (Boston, MA). 1294/H9 is a mouse monoclonal antibody that recognizes both human PR-A and PR-B isoforms (64). MMTV-luciferase adenovirus was a gift from Sergio Onate, University of Pittsburgh and α327 is a mouse monoclonal antibody that recognizes total c-Src (gift from Dr. J. Brugge, Harvard Medical School).

Plasmid Constructions

PR-B cDNA were inserted into *Eco*RI and *Sma*I sites of pEGFP-C1 to create pGFP-PR-B. To create pGFP-PR-Bmpro and pGFP-PR-A, PR-Bmpro or PR-A cDNA were inserted in *Xho*I/*Eco*RI and *Eco*RI/*Pst*I sites of pEGFP-C2, respectively.

To create pYFPPR-Bnuc, the stop codon in PR-B cDNA was first mutated from TGA to TGT *in vitro* using Pfu-Turbo DNA polymerase (Stratagene, La Jolla, CA) in the presence of sense and antisense strand-annealing oligonucleotides with mutated sequences. Mutations were confirmed by DNA sequencing. PR-B cDNA with the stop codon mutated was then inserted in *Bsp*EI and *Xho*I sites of pEYFPnuc (Clontech, Palo Alto, CA) in-frame with 4XSV40NLS at the C terminus, generating a fusion PR-B protein with yellow fluorescent protein (YFP)-tagged at the N terminus and 4XSV40NLS at the C terminus. To create pYFP-PR-Bmem, wild-type PR-B with an intact stop codon was first inserted in *Bsp*EI and *Xho*I sites of pEYFPnuc (Clontech) in-frame with YFP at the N terminus (pEYFP-PR-B). An *Age*I-*Pst*I fragment containing palmitoylation sequences was excised form pEYFPmem and inserted into *Age*I-*Pst*I site at the N terminus of the YFP sequence of pEYFPR-B in-frame with the YFP sequence, generating a fusion protein of N-terminally YFP containing palmitoylation membrane insertion sequence tagged PR-B. Sequences were confirmed by DNA sequencing.

To generate pcDNAI-PR-BΔnls and pcDNAI-PR-AΔnls, sequences between amino acid 591–644 of PR-B or 427–480 of PR-A sequence in pcDNAI-PR-B or pcDNAI-PR-A were deleted by ExSite PCR-based site directed mutagenesis as described by manufacturer (Stratagene) using the following oligonucleotides as primers 5′-CTCGAGGTCAGAGTTGTGAGAGCACTGGATGCTGTTGCTC-3′ and 5′-GAAGACCTTACAGCTCCCACAGGTAAGGACACCA-3′. *Xho*I site was introduced and used for screening of PR deletion mutants. Mutated sequences were confirmed by DNA sequencing.

Cell Cultures

PR-negative T47DY (36), T47DC42 (34), and MCF-7C4-12 (35) breast cancer cells were provided by K. Horwitz (University of Colorado), V. Craig Jordan (Dana Farber), and Wade Welshans (University of Missouri), respectively. Human PR-B, PR-BΔSH3 or PR-A cDNA were inserted into *Xho*I and *Eco*RI sites of pZeoSV2(−) (Invitrogen, Carlsbad, CA). Cells were stably transfected with PR expression plasmids using Effectene as described by the manufacturer (QIAGEN, Valencia, CA). Cells transfected with GFP-tagged human PR-B, PR-BΔSH3, or PR-A in pEGFP-C2 (Clontech) were selected as single colonies in 1 mg/ml G418 (Invitrogen). Cells transfected with PR-B, PR-A, PR-BΔSH3 in pZeoSV2(−) (Invitrogen) were selected as single colonies in 250 μg/ml Zeocin (Invitrogen). For experiments, cells were maintained overnight in phenol red-free MEM in 5% fetal bovine serum that had been treated with dextran-coated charcoal (DCC-FBS) for 24 h before hormone treatment (10 nM R5020) in the same medium supplement with 0.2% DCC-FBS, 2.5% DCC-FBS or with 5% DCC-FBS.

Transient Transfection and Luciferase Assays

For R5020-mediated Elk activation experiments, cells stably expressing PR-B, PR-ΔSH3, or PR-A (5×10^5 cells per well in six-well dishes) were transiently transfected with Gal4DBD-Elk and 7XUAS-Luc reporter plasmids using Effectene as described by the manufacturer (QIAGEN) in MEM supplement with 5% DCC-FBS. After 18 h, cells were rinsed with serum-free medium, replaced with MEM supplemented with 0.2% DCC-FBS with ethanol (vehicle control) or 10 nM R5020, and incubated for additional 24 h. For R5020-mediated induction of MMTV-Luc, 5×10^5 cells per well in six-well dishes were transduced with MMTV-luciferase adenovirus at MOI = 5 in MEM supplement with 5% DCC-FBS. After 18 h, cells were rinsed with serum-free medium, replaced with MEM supplemented with 5% DCC-FBS with ethanol (vehicle control) or 10 nM R5020, and incubated for additional 24 h. Cells were then harvested and assayed for luciferase activities as described (65, 66). Luciferase activities were normalized to total cellular protein to generate relative luciferase units. Data represent average relative luciferase values from three independent experiments ± SEM.

SDS-PAGE and Immunoblotting

Proteins were separated by 8% SDS-PAGE and analyzed by immunoblotting as previously described (65, 66) and detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

Src Kinase Assays

COS-7 cells were plated at 1.1×10^6/100-mm dish in DMEM supplemented with 5% FBS and incubated overnight and transfected with plasmids indicated in the figures by an attenuated adenovirus-mediated procedure as previously described (31, 33). After transfection, cells were incubated for 24 h in phenol red-free DMEM supplemented with 5% DCC-FBS, followed by 48 h in DMEM supplemented with 0.2% DCC-FBS. Hormones were added for the times indicated and cells were lysed and c-Src kinase activity was assayed by immunoprecipitation using incorporation of α p-32-ATP into enolase as substrate as previously described (31).

Alternatively, Src kinase activity was determined by the BioSource Src [pY418] ELISA kit according to manufacturer's protocol (BioSource, Camarillo, CA). T47D C42 cells stably expressing PR-B, PR-BΔSH3, or PR-A were plated at 4.5×10^6/60 mm dish in MEM supplemented with 5% FBS overnight. Cells were then rinsed with serum-free medium and grown in MEM supplemented with 2.5% DCC-FBS for 48 h. Cells were rinsed once with serum-free medium, replaced with MEM supplemented with 0.2% DCC-FBS and incubated overnight. Four hours before hormone treatment, medium was replaced with fresh MEM supplemented with 0.2% DCC-FBS. Cells were treated with either ethanol control or 10 nM R5020 as indicated in the figure. Cell were lysed with Cell Extraction buffer [10 mM Tris, pH 7.4; 100 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM NaF; 2 mM sodium pyrophosphate; 20 mM sodium vanadate; 1% Triton X-100; 10% glycerol; 0.1% SDS; 0.5% deoxycholate; 1 mM phenylmethylsulfonyl fluoride; and protease inhibitors cocktail (Roche Applied Science, Indianapolis, IN)]. Diluted cell lysates with equal amounts of protein, along with activated Src-[pY418] standards were added to microtiter wells precoated with total Src antibody and incubated for 2 h at 4C. Wells were washed, replaced with an antibody that recognizes the phosphotyrosine [p418] in the activation loop of Src, and incubate for 1 h. Wells were then washed, replaced with antirabbit IgG-horseradish peroxidase for 30 min and followed by a stabilized chromogen for another 30 min. Absorbance intensities of stopped reactions were detected at 450 nm with a Molecular Devices ELISA plate reader. Relative Src-[pY418] concentrations were determined by using standard curve plotting absorbance at 450 nm against known concentrations of Src-pY[418]. Results represent average Src-[pY418] ± SEM from three replicate experiments.

Real-Time RT-PCR Analysis

Total RNA was isolated from cells using Versagene RNA purification kit with deoxyribonuclease treatment (Gentra Systems, Minneapolis, MN). Equal amounts of total RNA [500 ng for CCND1 and Sgk and 50 ng for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) per reaction] were analyzed by real-time PCR (ABI Prism 7700 sequence detector, Applied Biosystems) with TaqMan primers and probe for CCND1 gene (forward primer, 5′-GTCCTACTACCGCCTCACACG-3′; reverse primer 5′-GGGCTTCGATCTGCTCCTG-3′; VIC (Applied Biosystems) probe, 5′-AAGTGTGACCCAGACTGCCTCCGG-tetramethyl carboxyrhodamine) for Sgk gene (forward primer, 5′-CACAACAGCACAACATCCACC-3′; reverse primer 5′-GGCTGCTTATGAAGCACCTCA-3′; FAM (6-carboxy fluorescein) probe, 5′-TGGCACGCCGGAGTATCTCGC-tetramethyl carboxyrhodamine) and with a Taqman probe set for GAPDH from Applied Biosystems. Reactions were carried out under universal conditions using one-step reverse-transcription PCR reagents (Applied Biosystem), with the following cycling parameters: 48 C for 30 min, 95 C for 10 min, followed by 40 cycles of 95 C for 15 sec and 60 C for 1 min. Cycle threshold values were analyzed using the SDS 1.9 software (PE Applied Biosystems, Foster City, CA), and relative quantification of Sgk and CCDN1 expression was determined using the comparative cycle threshold method (ABI Prism 7700 SDS User Bulletin No. 2; Applied Biosystems). RNA values were normalized to endogenous GAPDH. Data represent average values from three independent experiments ± SEM.

FRAP Live Cell Imaging

Live cell fluorescence imaging was carried out with cells stably transfected with GFP-PR as previously described with minor modifications (67). Briefly, 2×10^6 cells were grown on glass-bottom Delta-T dishes (Bioptech Inc., Butler, PA) containing MEM supplemented with 5% DCC-FBS at 37 C for 16–18 h. The same medium with or without ligands was recirculated with a peristaltic pump during the analysis. FRAP analysis was carried out at 37 C on a Zeiss LSM 510 laser confocal microscope. A single z-section was imaged before and at 2-sec time intervals after bleaching. The bleach was carried out at 458 nm and at maximum power for 150 iterations of a rectangular area representing approximately 25% of the nuclear volume. Fluorescence intensities of regions of interest were obtained using LSM software and data were analyzed using Microsoft Excel.

For live cell quantitation of receptor localization, 4×10^6 cells were plated on 60-mm glass bottom dishes in MEM supplemented with 5% DCC-FBS overnight. Cells were treated with ethanol or 10 nM R5020 for 2 h and a single z-section of live-cell images from 15–20 cells per treatment group were collected under a Zeiss LSM 510 laser confocal microscope. Fluorescence intensities of the whole cell and nucleus were obtained using LSM software and data were analyzed using Microsoft Excel.

Indirect Immunofluorescence

Hela cells (4×10^4 cells) in four-well chamber slides were grown in DMEM supplemented with 5% DCC-FBS and transduced for 24 h with recombinant adenovirus vectors encoding wild-type PR-B, PR-A or PR-BΔSH3. For COS 7 cells, 5×10^4 cells were transiently transfected with various PR constructs using FuGENE 6 transfection reagent as described by the manufacturer (Roche Applied Science). Cells were then treated with ethanol or 10 nM R5020 for 2 h. After hormone treatment, cells were rinsed once with ice-cold PBS, fixed with 3.7% paraformaldehyde for 20 min at 4 C, and permeabilized with 0.5% Triton X-100 in PBS for 4 min. Cells were washed twice with PBS and blocked with 1% BSA in PBS for 1 h before incubating with primary antibody in 1% BSA-FBS (3.5 μg/ml of 1294/H9) for an addition hour. Cells were washed three times with PBS and incubated with secondary antibody at 1:1000 dilution (Alexa 568 conjugated with goat antimouse antibody, Molecular Probes, Eugene, OR). Cells were washed three times with PBS and 4′,6-diamidino-2-phenylindole (Molecular Probes) was added at 1:5000 dilution in the second PBS wash for nuclear staining. Slides were mounted using Vectashield reagent (Vector Laboratories, Burlingame, CA). Digital confocal images were collected using a fluorescence microscope with Delta Vision system (Applied Precision, Issaquah, WA).

Acknowledgments

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