

# Activation of the Src/p21<sup>ras</sup>/Erk pathway by progesterone receptor via cross-talk with estrogen receptor

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**The molecular mechanisms by which ovarian hormones stimulate growth of breast tumors are unclear. It has been reported previously that estrogens activate the signal-transducing Src/p21<sup>ras</sup>/Erk pathway in human breast cancer cells via an interaction of estrogen receptor (ER) with c-Src. We now show that progestins stimulate human breast cancer T47D cell proliferation and induce a similar rapid and transient activation of the pathway which, surprisingly, is blocked not only by anti-progestins but also by anti-estrogens. In Cos-7 cells transfected with the B isoform of progesterone receptor (PR<sub>B</sub>), progestin activation of the MAP kinase pathway depends on co-transfection of ER. A transcriptionally inactive PR<sub>B</sub> mutant also activates the signaling pathway, demonstrating that this activity is independent of transcriptional effects. PR<sub>B</sub> does not interact with c-Src but associates via the N-terminal 168 amino acids with ER. This association is required for the signaling pathway activation by progestins. We propose that ER transmits to the Src/p21<sup>ras</sup>/Erk pathway signals received from the agonist-activated PR<sub>B</sub>. These findings reveal a hitherto unrecognized cross-talk between ovarian hormones which could be crucial for their growth-promoting effects on cancer cells.**

**Keywords:** estradiol receptor/progesterone receptor/progestins/proliferation/signal transduction

## Introduction

Ovarian hormones stimulate cell proliferation in several female tissues (Clarke and Sutherland, 1990), in particular in the mammary gland, where estrogens and progesterone increase alveolar formation and ductal branching, and are required for full lobulo-alveolar development (Warner *et al.*, 1978). A positive role for progestins in mammary gland cell proliferation has been observed in a variety of systems, including the mouse mammary gland (Bresciani, 1968), mammary tumors of rodents (Kiss *et al.*, 1986; Manni *et al.*, 1987), primary culture of human mammary epithelial cells (Longman and Buehring, 1987) and human

breast cancer lines (Braunsberg *et al.*, 1987; Hisson and Moore, 1987).

In spite of this widely acknowledged importance of steroids as mitogens, the mechanisms by which they stimulate cell proliferation have not been deciphered yet. Regulation of growth factor production or stimulation by steroids of expression of genes required for cell division have been proposed (Weisz and Bresciani, 1993; Dickson and Lippman, 1995). Among the possible targets are the so-called early genes, such as *c-fos* and *jun-B*, but estrogens also induce *c-myc* expression, and this could be related to sustained growth (Weisz and Bresciani, 1993). In addition to early genes, cell cycle-controlling genes, such as cyclin D1, are also regulated by steroid hormones and could be targets of estrogens and progesterone (Musgrove *et al.*, 1994). The activity of the G1 cyclins could also be influenced through an effect of ovarian hormones on cyclin-dependent kinase (cdk) inhibitors (Musgrove *et al.*, 1995; Planas-Silva and Weinberg, 1997; Prall *et al.*, 1997). Moreover, estrogens could also participate in proliferation in an indirect way by inducing the production of growth factors (Dickson and Lippman, 1995). Recently, participation of the Src/Ras/MAP kinase pathway in estrogen-induced growth has been postulated (Di Domenico *et al.*, 1996; Migliaccio *et al.*, 1996).

Targeted disruption of the estrogen receptor  $\alpha$  (ER $\alpha$ ) gene clearly demonstrates a role for this receptor in the post-natal development of the uterus and the mammary gland, as homozygous ER $\alpha^{-/-}$  female mice exhibit hypoplastic uteri and undeveloped mammary glands, with only vestigial ducts present at the nipples (Lubahn *et al.*, 1993; Korach, 1994). On the contrary, PR $^{-/-}$  mice have normally developed uteri at puberty but respond to repeated administration of estrogens with a dramatic hyperplasia and inflammation of the uterus, suggesting that progesterone receptor (PR) is involved in controlling the proliferative response to estrogens and in preventing uterine inflammation (Lydon *et al.*, 1995). Progesterone is the actual proliferative hormone in the post-puberty growth of the mammary gland (Lydon *et al.*, 1995). While both stromal and epithelial cells participate in mammary gland development, it is the PR of epithelial cells which is essential for lobulo-alveolar development (Humphreys *et al.*, 1997). It is very likely that the effect of PR is mediated through induced expression of cyclin D1, since a disruption of this gene yields a very similar mammary phenotype, namely lack of post-puberty development (Fantl *et al.*, 1995; Sicinski *et al.*, 1995).

Steroids, in addition to regulating gene transcription (Beato *et al.*, 1995), produce rapid, non-transcriptional responses which in some cases are reminiscent of those evoked by peptide growth factors, suggesting an interaction of steroids with cell surface receptors (Pappas *et al.*, 1995). Vitamin D rapidly activates phospholipase C of

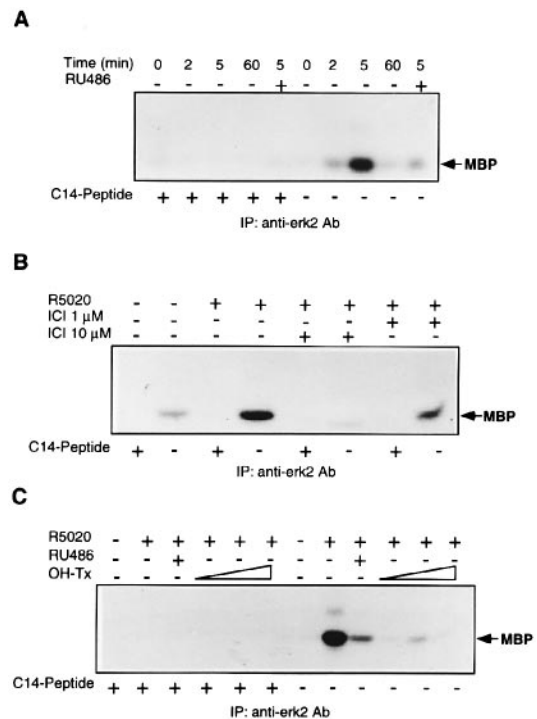
intact enterocytes (Lieberherr *et al.*, 1989), and estrogen stimulates adenylate cyclase in different cells (Aronica *et al.*, 1994). Progesterone stimulates oocyte maturation in *Xenopus* by a mechanism involving activation of protein kinases (Sagata *et al.*, 1989; Muslin *et al.*, 1993). Progestins also activate calcium influx in sperm (Blackmore *et al.*, 1990) and induce tyrosine phosphorylation of a protein (Mendoza *et al.*, 1995).

We have found that in MCF-7 mammary tumor cells, estradiol stimulates Src kinase and the MAP kinase pathway very rapidly (Di Domenico *et al.*, 1996; Migliaccio *et al.*, 1996). To investigate whether the proliferative activity of progestins is mediated by a similar mechanism, we have used T47D mammary tumor cells which are rich in PR (Chalbos *et al.*, 1982). Here we report that progestins rapidly and reversibly stimulate the transducing pathway c-Src/p21<sup>ras</sup>/Erk-2 (Schlessinger, 1993) in these cells. Surprisingly, the activation of this pathway by progestins not only requires the PR<sub>B</sub> but also ligand-free ER, as shown in T47D cells treated with anti-estrogens and in Cos-7 cells transfected with the appropriate receptors. In contrast to ER, PR<sub>B</sub> does not interact with c-Src, but it interacts with ER via its N-terminal region which is absent in PR<sub>A</sub>. Interestingly, a PR point mutant ineffective in transcriptional activation still activates the signaling transduction pathway. The relevance of this activation to cell proliferation is suggested by experiments with c-Src inhibitors of different specificity. Our data show that in addition to cross-talk with peptide growth factors (Ignar-Trowbridge *et al.*, 1992; Zhang *et al.*, 1994) and neurotransmitter receptors (Power *et al.*, 1991), steroid receptors can also cross-talk with each other.

## Results

### Progestin stimulation of Erk-2 activity in T47D cells

In order to study the effect of progestins on the MAP kinase pathway, we used T47D mammary carcinoma cells, which contain ER and constitutive high levels of PR (Figure 2C). To reduce the levels of activated ER, the cells were maintained for 1 week in the presence of charcoal-treated serum and in the absence of phenol red, a substance with a weak estrogenic activity (Berthois *et al.*, 1986). The Erk-2 activity in T47D cells treated for different times with 10 nM of the synthetic progestin R5020 was evaluated by immunoprecipitation of cell lysates with anti-Erk-2 antibody, followed by measurement of the myelin basic protein (MBP) phosphorylating activity in the immunoprecipitates. R5020 treatment resulted in a rapid stimulation of Erk-2 activity which was already detectable after 2 min, reached maximal values after 5 min and returned to normal levels after 60 min. The anti-progestin RU486 (1 mM) inhibited the stimulatory effect of the agonist treatment, indicating that binding to the PR is required for the progestin effect. No MBP phosphorylation was detectable in the control immunoprecipitates with the same anti-Erk-2 antibody but in the presence of an excess of the Erk-2 peptide (C14 peptide), against which this antibody has been raised (Figure 1A). Five minutes of R5020 treatment, in addition to stimulating Erk-2, also enhanced Erk-1 activity weakly, measured on



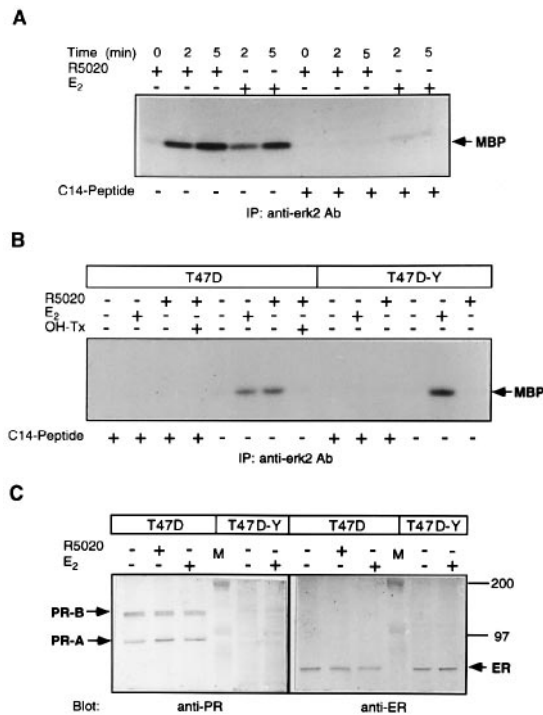
**Fig. 1.** Activation of Erk-2 in progestin-treated T47D cells. (A) Cells were left untreated or were treated for the indicated times with 10 nM R5020 in the absence or presence of the anti-progestin RU486 (1  $\mu$ M). Cells were also not treated or treated for 5 min with R5020 in the absence or presence of 1 or 10  $\mu$ M ICI 182,780 (B) or increasing concentrations (0.1, 1 and 10  $\mu$ M) of OH-tamoxifen (C). Cell lysates were immunoprecipitated using anti-Erk-2 antibody in the absence or presence of the C14 peptide against which the antibody had been raised. The immunoprecipitates were assayed for Erk activity using MBP as a substrate.

proteins immunoprecipitated from lysates by anti-Erk-1 antibody using MBP as a substrate. This effect is also inhibited by the anti-progestin (not shown). Surprisingly, the stimulatory effect of the progestin on Erk-2 was inhibited by different anti-estrogens. The pure steroidal anti-estrogen ICI 182,780 strongly inhibited the progestin induction at a concentration of 1  $\mu$ M, while 10  $\mu$ M ICI 182,780 reduced the Erk-2 activity even below the values observed prior to hormone induction (Figure 1B). The non-steroidal anti-estrogen OH-tamoxifen abolished Erk-2 stimulation by R5020 at concentrations of 0.1–10  $\mu$ M (Figure 1C).

A comparison of the effects of estradiol and progestin on Erk-2 activity in T47D cells showed a similar stimulation by the two steroids, detectable after 2 and 5 min of hormone treatment (Figure 2A). It is perhaps more relevant that the progestin R5020 stimulated Erk-2 activity in T47D but not in T47D-Y cells (Figure 2B), which lack PR but are endowed with high ER levels (Sartorius *et al.*, 1994) (Figure 2C). This finding confirms that Erk-2 activation by the progestin requires the PR. Estradiol stimulated Erk-2 activity in T47D as well as in T47D-Y cells (Figure 2B), showing that the estrogen effect is independent of PR.

### Progestins increase GTP-bound p21<sup>ras</sup> and stimulate c-Src activity in T47D cells

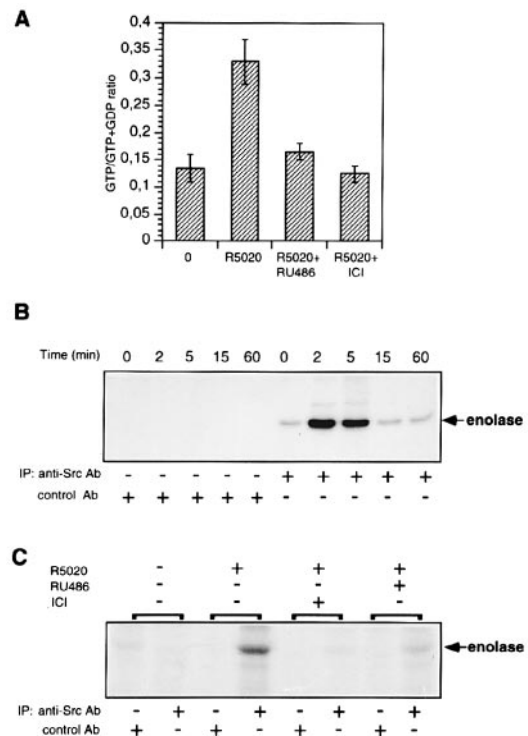
We next analyzed the effect of progestins on steps of the signal transduction pathway upstream of MAP kinases,



**Fig. 2.** Activation of Erk-2 in progestin- and estrogen-treated T47D and T47D-Y cells. (A) T47D cells were treated for 2 and 5 min with 10 nM of either estradiol (E<sub>2</sub>) or R5020. (B) T47D cells were treated for 2 min with either E<sub>2</sub> or R5020 in the absence or presence of 0.1 μM OH-tamoxifen; T47D-Y cells were treated with either E<sub>2</sub> or R5020. Cell lysates were immunoprecipitated using anti-Erk-2 antibody in the absence or in presence of the C14 peptide, and the immunoprecipitates were assayed for Erk activity using MBP as a substrate. (C) Western blot with anti-hPR or anti-ER (H222) antibodies of lysates from T47D and T47D-Y cells left untreated or treated with either E<sub>2</sub> or R5020. M indicates the marker lane. Molecular weights on the right are expressed in kDa. The arrows on the left indicate the positions of PR<sub>A</sub>, PR<sub>B</sub> and ER.

such as p21<sup>ras</sup> and c-Src. We measured the guanine nucleotides bound to p21<sup>ras</sup> in [<sup>32</sup>P]orthophosphate-labeled T47D cells treated for 4 min with 10 nM R5020, in the absence or presence of anti-progestins or anti-estrogens, and calculated the GTP:GTP + GDP ratio using a PhosphorImager. R5020 treatment increased the GTP:GTP + GDP ratio 2.6-fold, and this increase was strongly reduced or abolished by the addition of either the anti-progestin RU486 or the anti-estrogen ICI 182,780 (Figure 3A). These findings demonstrate that progestin activates p21<sup>ras</sup> by a mechanism that probably requires both PR and ER.

Treatment of T47D cells with 10 nM R5020 for different times enhanced c-Src kinase activity on enolase as measured in cell lysates immunoprecipitated with monoclonal 327 anti-c-Src antibody (Figure 3B). The activity was stimulated 2 and 5 min after hormone addition and decreased to uninduced levels thereafter. Immunoprecipitates obtained with a control antibody were devoid of c-Src kinase activity (Figure 3B). T47D cells were also treated with 10 nM R5020 for 2 min in the presence of either 1 μM of the anti-progestin RU486 or 10 μM of the anti-estrogen ICI 182,780 (Figure 3C). The stimulatory effect of R5020 on the c-Src kinase activity was almost completely abolished by the anti-progestin, indicating a requirement for PR occupancy by the agonist. As in the case of Erk-2



**Fig. 3.** Effect of progestins on p21<sup>ras</sup> and c-Src activity in T47D cells. (A) Cells were radiolabeled with [<sup>32</sup>P]orthophosphate and incubated for 4 min in the absence or presence of 10 nM R5020 either alone or together with 1 μM RU486 or 10 μM ICI 182,780. Nucleotides bound to p21<sup>ras</sup> were analyzed. The ratio GTP/GTP + GDP averaged from two experiments is shown. (B) Cells were left untreated or were treated with 10 nM R5020 for the indicated times. (C) Cells were not treated or treated for 2 min with R5020 in the absence or presence of either RU486 or ICI 182,780. In (B) and (C), lysates were incubated with either anti-c-Src 327 antibody or control antibody. The immunoprecipitates were assayed for Src kinase activity using acid-treated enolase as a substrate.

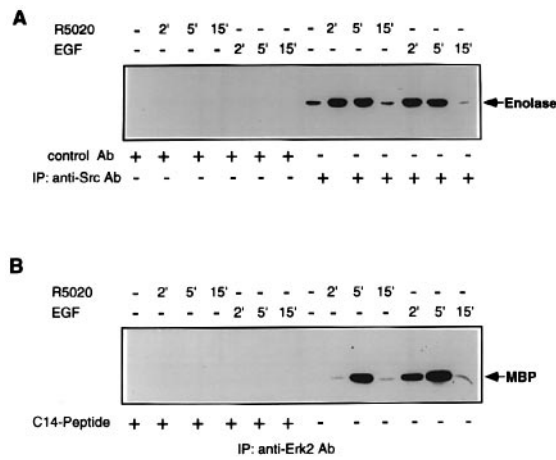
and p21<sup>ras</sup>, stimulation of c-Src activity by R5020 was strongly reduced by the pure anti-estrogen ICI 182,780, suggesting that progestin activation of the entire pathway requires ER.

### Comparison of c-Src and Erk-2 responses to EGF and progestin

To gain an impression of the relative magnitudes of activation of the signaling cascade by progestin and by a classical growth factor, T47D cells were stimulated with either 10 nM R5020 or 100 ng/ml epidermal growth factor (EGF), and the effects on c-Src and Erk-2 followed (Figure 4A and B). The growth factor and the hormone stimulated c-Src and Erk-2 with similar intensities and kinetics. We conclude that progestin activation of the signaling pathway is comparable with that following growth factor treatment.

### Effect of tyrosine kinase inhibitors on progestin-stimulated cell growth and Erk activation

It is known that activation of the c-Src/Ras/Erk pathway can lead to cell growth (Schlessinger and Ullrich, 1992). We explored the role of c-Src in the progestin stimulation of Erk-2 kinase activity and the link between the signal transduction pathway activation and cell growth stimulation using two c-Src kinase inhibitors, genistein (Akiyama and Ogavara, 1991) and PP1, a new, specific Src family

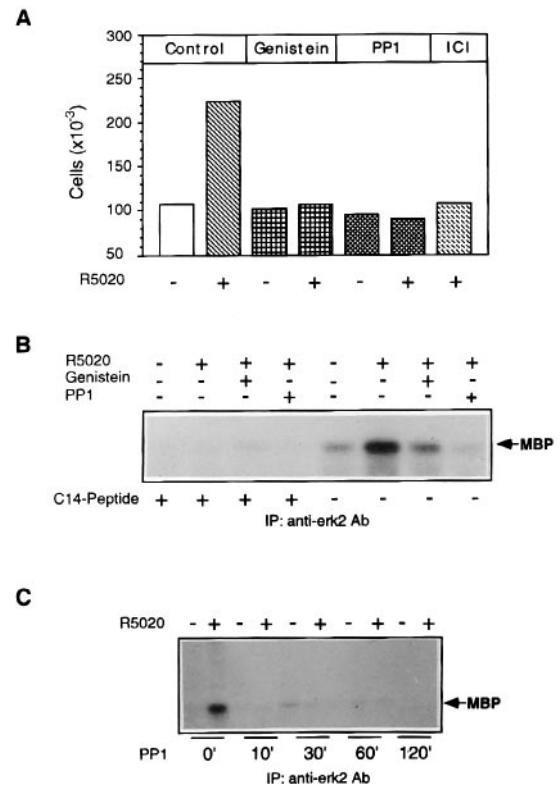


**Fig. 4.** Effect of EGF and the progestin R5020 on c-Src and Erk-2 of T47D cells. Cells were left untreated or were treated for the indicated times with 10 nM R5020 or 100 ng/ml EGF. Cell lysates were either incubated with control or anti-c-Src antibodies and the precipitates assayed for c-Src activity using enolase as a substrate (A) or immunoprecipitated with anti-Erk-2 antibodies in the presence or absence of the C14 peptide and the precipitates assayed for Erk-2 activity using MBP as a substrate (B).

kinase inhibitor (Hanke *et al.*, 1996). The inhibitors were added to the medium together with R5020, and cell growth was followed for 4 days. Both inhibitors prevented cell growth stimulation by progestin to the same extent as the anti-estrogen ICI 182,780 (Figure 5A). To assay the effect of the two inhibitors on Erk-2 activity, cells were pre-incubated with either genistein or PP1 for 18 h and Erk-2 activity measured after 5 min of progestin treatment. Both inhibitors reduced progestin stimulation of Erk-2 activity, but the effect of PP1 was stronger, leading to a reduction of Erk-2 activity below the level in uninduced cells (Figure 5B). The specificity of the action of PP1 on c-Src is supported by the fact that 10 min and 18 h of PP1 treatment are equally effective in abolishing Erk-2 stimulation by R5020 (Figure 5B and C). These results suggest a causal role of c-Src stimulation by progestin on both Erk-2 activation and cell proliferation.

**Progestin stimulation of Erk-2 and c-Src activities in COS-7 cells transiently transfected with hormone receptors**

The inhibitory effect of anti-progestins and anti-estrogens on R5020 activation of the Src/p21<sup>ras</sup>/Erk pathway suggests that progestin action is mediated by PR but also depends on antagonist-free ER. To test this notion, Cos-7 cells, which lack ER and PR, were transfected with either pSG5-HEGO plasmid encoding the human ER (Tora *et al.*, 1989a), pSG5-hPR<sub>B</sub> encoding the human PR<sub>B</sub> (Kastner *et al.*, 1990) or with both plasmids, and the effects of progestins on MAP kinase activity were assayed. Both receptors appear to be co-expressed at a similar level (Figure 6C). Figure 6A shows that in cells transfected with either ER or PR<sub>B</sub> expression vectors individually, progestin treatment for 5 min did not significantly affect the basal level of Erk-2 activity. In contrast, in cells co-transfected with both receptors, R5020 enhanced Erk-2 activity. The stimulation of c-Src activity by R5020 also required the simultaneous expression of both receptors (Figure 6B). These findings demonstrate that the progestin

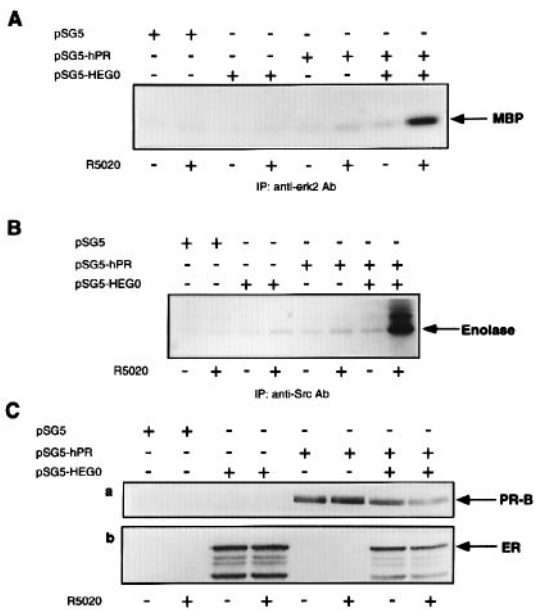


**Fig. 5.** Effect of tyrosine kinase inhibitors on progestin-induced cell growth and Erk-2 activity in T47D cells. (A) T47D cells were left untreated or were treated for 4 days in the absence or presence of 10 nM R5020 alone (control) or in the presence of either 40 μM genistein, 10 μM PP1 or 10 μM ICI 182,780, then counted. (B) Cells left untreated or treated for 18 h with 40 μM genistein or 10 μM PP1 were incubated in the absence or presence of R5020 for 5 min. Cell lysates were immunoprecipitated by anti-Erk 2 antibodies and assayed for Erk-2 activity. Reduction of PP1 treatment from 18 h to 120, 60, 30 or even 10 min did not affect the inhibition by PP1 of Erk-2 activity stimulated by the progestin (C).

effects on the signaling pathway in Cos cells require not only binding to the PR but also expression of ER. They also support the interpretation that the inhibitory effect of anti-estrogens on the progestin stimulation of c-Src, p21<sup>ras</sup> and Erk-2 in T47D cells (Figures 1–3) is a consequence of their binding to ER and the consequent blockade of ER function.

**Progestin- and estradiol-induced association of ER with c-Src in Cos-7 cells transiently transfected with hER and hPR<sub>B</sub> cDNA**

To investigate the molecular basis of the ER requirement for progestin action on the signaling pathway, we transfected Cos cells with ER and PR<sub>B</sub> expression vectors, treated the cells with R5020 or estradiol and immunoprecipitated the cell lysates with anti-ER, anti-PR or anti-c-Src antibodies. Proteins from each immunoprecipitate were blotted separately with anti-ER, anti-PR and anti-c-Src antibodies (Figure 7A–C). In the immunoprecipitates obtained with anti-ER antibody (Figure 7A), we detected PR<sub>B</sub> independently of the hormonal treatment, whereas c-Src was detected only when cells were treated for 2 min with either progestin or estradiol. This finding is consistent with the notion that, in the presence of hormones, c-Src interacts with ER pre-associated with PR, forming a stable



**Fig. 6.** Effect of progestin on Erk-2 and c-Src in Cos-7 cells transfected with hER and hPR<sub>B</sub> cDNA. (A and B) Cos-7 cells were transfected with either empty pSG5 vector, pSG5-hPR<sub>B</sub>, pSG5-HEG0 or both the pSG5-hPR<sub>B</sub> and pSG5-HEG0 vectors. Thereafter, cells were left unstimulated or were stimulated with 10 nM R5020. Lysates from cells stimulated for 5 min were incubated with anti-Erk-2 antibody (A) whereas lysates from cells stimulated for 2 min were incubated with anti-c-Src antibodies (B). The immunoprecipitates were assayed for Erk-2 (A) or c-Src (B) activity, respectively. (C) Panels a and b show blots of lysates from the experiments in (A) and (B) with anti-PR or H222 anti-ER antibodies, respectively.

ternary complex, but could also be explained by the existence of two independent complexes containing ER associated with either PR<sub>B</sub> or c-Src. Immunoprecipitation by anti-PR antibody (Figure 7B) showed association of ER with PR in the absence and presence of hormones, and failed to detect c-Src associated with PR<sub>B</sub>. Furthermore, when proteins immunoprecipitated by anti-c-Src antibodies were analyzed (Figure 7C), c-Src was found associated with ER only in the presence of hormones, while no association with PR<sub>B</sub> was found, independently of the hormonal treatment. Therefore, the results of immunoprecipitation with anti-c-Src and anti-PR antibodies do not support the existence of a stable ternary complex including PR<sub>B</sub>, ER and c-Src.

The experiments with Cos cells co-transfected with PR<sub>B</sub> and ER expression vectors leave open the possibility that PR<sub>B</sub> is not found associated with c-Src because it is competed by ER. To test this possibility, Cos cells were transfected separately with either hER or hPR<sub>B</sub> expression vectors and stimulated by estradiol or progestin, respectively. Cell lysates were precipitated with anti-ER (Figure 7D) or with anti-PR (Figure 7E) antibodies, and proteins in the precipitates were analyzed by blotting with anti-c-Src antibodies and either anti-ER (Figure 7D) or anti-PR (Figure 7E) antibodies. PR<sub>B</sub> was not found associated with c-Src in the absence of ER (Figure 7E), thus excluding a competition between the two hormone receptors for association with c-Src. In contrast, ER still associated with c-Src after estradiol treatment, in the absence of PR<sub>B</sub> (Figure 7D). Moreover, progestin in the absence of its receptor did not induce ER-c-Src association (Figure 7D),

confirming that progestin-induced ER-c-Src association requires PR.

To explore further the functional significance of the interaction between ER and c-Src, we investigated the effect of anti-hormones. The anti-estrogen ICI 182,780 prevented the association of ER and c-Src induced by estradiol (Figure 7F). Furthermore, the stimulation of the interaction between ER and c-Src observed 2 min after R5020 treatment was prevented by either the anti-estrogen or the anti-progestin RU486 (Figure 7F). The observation that in this experiment there is less ER-c-Src complex in the presence of anti-hormones than in their absence is probably due to some residual estradiol still present in the medium in spite of the charcoal treatment of the serum.

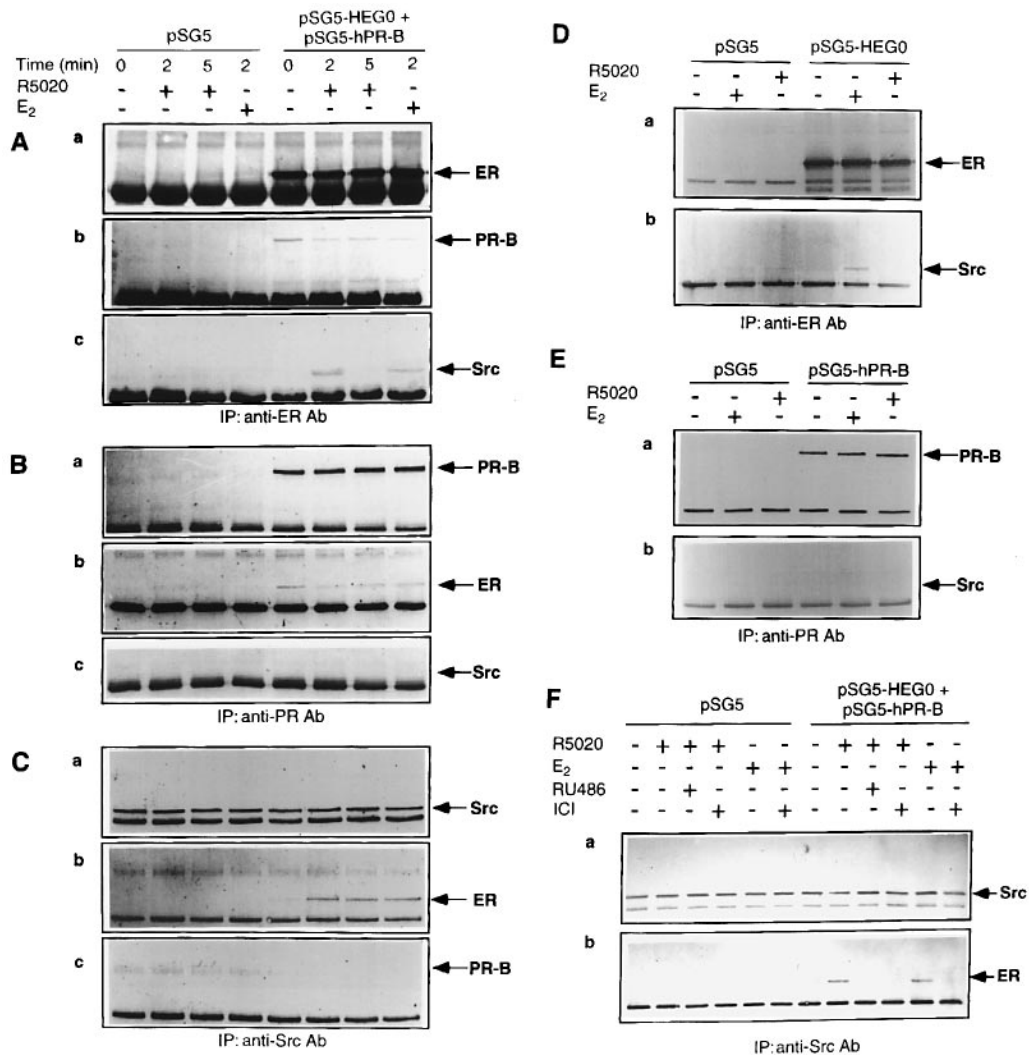
### Interaction between PR, ER and c-Src in T47D cells

We next attempted to define more precisely the interaction between PR and ER using immunoprecipitation and gene transfection experiments. When T47D cell lysates were immunoprecipitated by anti-PR antibodies, which recognize both the A and B forms of PR (Figure 8A, panel a), as in Cos cells (Figure 7B) the co-immunoprecipitation of ER was observed before as well as after treatment with progestin or estrogen (Figure 8A, panel b). The association between PR and ER appeared to be independent of ligand as it was not inhibited by anti-progestins or anti-estrogens (Figure 8A, panel b). As in transfected Cos cells (Figure 7B, C and E), PR did not associate with c-Src in T47D cells (Figure 8A, panel c). As expected from the experiment with Cos cells (Figure 7A), when anti-ER antibodies were used to immunoprecipitate T47D cell lysates, PR was co-precipitated in the absence or presence of progestin or estradiol as well as in the presence of antagonists, whereas co-precipitation of c-Src depended on treatment of the cells with either progestins or estrogens (Figure 8B, panel c). The interaction of ER with c-Src was inhibited by anti-progestins and anti-estrogens. The experiments with T47D cells presented in Figure 8A and B confirm that, as in Cos cells, only anti-ER antibodies immunoprecipitate the three proteins, ER, PR and c-Src.

The hormone dependence of ER-Src interaction was also verified in T47D cells. The time course of the progestin effect showed that prior to hormone induction there is no association of ER and c-Src and that equivalent amounts of c-Src are precipitated by anti-c-Src antibodies at different times after hormone induction (Figure 8C, panel a). Following 2 and 5 min of progestin treatment, ER co-immunoprecipitated with c-Src. This association was no longer detectable thereafter (Figure 8C, panel b). Interestingly, the kinetics of the association between c-Src and ER are similar to those of the induction of c-Src activity in T47D cells stimulated by R5020 (Figures 3B and 4A). This finding also supports a functional role for the interaction of ER with c-Src for the progestin induction of c-Src activity.

### Definition of ER and PR domains involved in signaling and receptor interactions

Transfection experiments were also used to identify the ER domain involved in the functional association between this receptor and c-Src. Cos cells transfected with pSG5-HEG19 synthesized an ER mutant lacking the N-terminal half, including part of the DNA-binding domain (Tora



**Fig. 7.** Interaction between PR, ER and c-Src in Cos-7 cells transfected either with both hER and hPR<sub>B</sub> cDNA or separately with hER cDNA or hPR<sub>B</sub>cDNA. (A–C) Cos-7 cells were transfected with either empty pSG5 vector or both the pSG5-HEG0 and pSG5 hPR<sub>B</sub> vectors. Cells were left untreated or were treated with either 10 nM R5020 for 2 and 5 min or 10 nM estradiol (E<sub>2</sub>) for 2 min. Cell lysates in (A–C) were immunoprecipitated with H222 anti-ER, anti-PR and anti-c-Src antibodies, respectively. Each immunoprecipitate was blotted with anti-ER, anti-PR or anti-c-Src antibodies, and the expected position of the proteins in the blot is indicated by an arrow. (D and E) Cos-7 cells were transfected with either empty pSG5 vector, pSG5-HEG0 or pSG5-hPR<sub>B</sub>. Cells were left untreated or were treated with E<sub>2</sub> or R5020 for 2 min, and lysates were incubated with either anti-ER (D) or anti-PR (E) antibodies. The immunoprecipitates were blotted with H222 anti-ER and anti-c-Src in (D) and with anti-PR and anti-c-Src in (E). (F) Cos cells transfected with either empty pSG5 vector or both the pSG5-HEG0 and pSG5-hPR<sub>B</sub> vectors were left untreated or were treated for 2 min with 10 nM R5020 in the absence or presence of either 1 μM RU486 or 10 μM ICI 182,780, or for 2 min with 10 nM E<sub>2</sub> alone or together with 10 μM ICI 182,780. Cell lysates were immunoprecipitated with anti-c-Src antibodies and blotted with anti-c-Src and anti-ER antibodies.

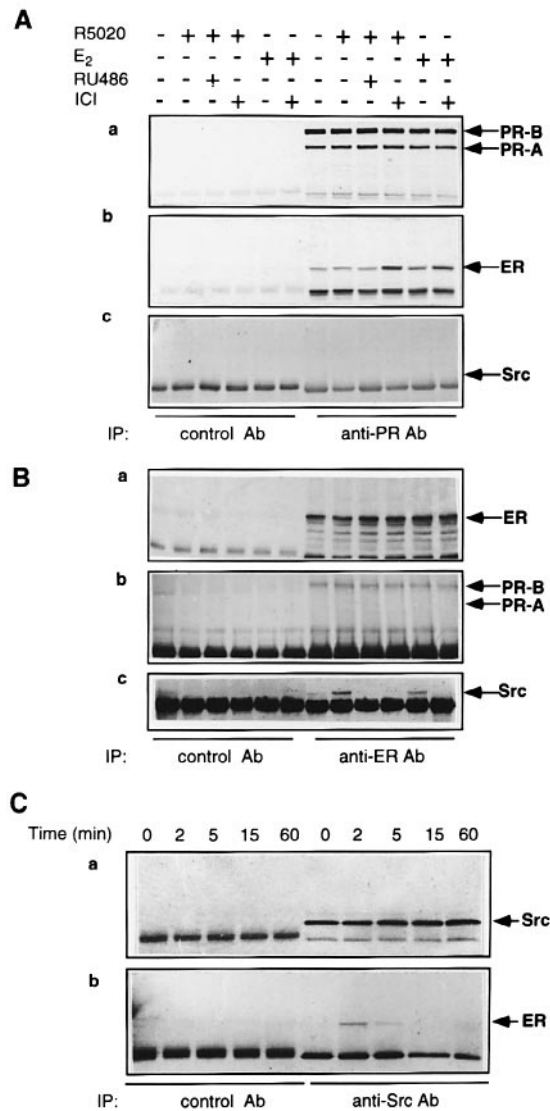
*et al.*, 1989b). This truncated ER was immunoprecipitated by anti-c-Src antibody (Figure 9A, panel a) and stimulated c-Src kinase activity in the presence of estrogens to a similar level as the wild-type ER (Figure 9A, panel b). In contrast, Cos cells transfected with pSG5-HEG15 synthesized a mutant ER lacking the C-terminal half including the ligand-binding domain (Kumar *et al.*, 1987). This ER mutant did not associate with c-Src and did not stimulate c-Src activity (data not shown). Therefore, ER association with c-Src and stimulation of c-Src activity requires the C-terminal half of ER.

Interestingly, only isoform B of PR (PR<sub>B</sub>) but not isoform A (PR<sub>A</sub>) which lacks the first 168 amino acids of PR<sub>B</sub>, associated with ER (Figure 8B, panel b). Moreover, when expression vectors for the two isoforms of PR were transfected in Cos cells, only PR<sub>B</sub> and not PR<sub>A</sub> activated

c-Src kinase activity in response to R5020, although both isoforms bind the hormone with equal affinity (Figure 9B). These results identify the N-terminal portion of PR<sub>B</sub> as the domain responsible for the association with ER, and demonstrate that this association is essential for c-Src activation.

***In vitro ER-PR interaction experiments***

The interaction between the two hormone receptors was analyzed further by *in vitro* experiments. A direct protein-protein interaction was observed by ‘far Western blotting’ between the immobilized, renatured ER and partially purified recombinant PR<sub>B</sub> (Figure 9C, panel a). In pull-down experiments, the GST-HEG14 (an ER mutant lacking the N-terminal half and the DNA-binding domain) bound recombinant PR<sub>B</sub>, identifying the C-terminal half



**Fig. 8.** Interactions between PR, ER and c-Src in T47D cells. (A and B) T47D cells were treated with 10 nM R5020 in the absence or presence of either 1  $\mu$ M RU486 or 10  $\mu$ M ICI 182,780 and with 10 nM estradiol alone or with ICI 182,780. (A) Cell lysates were immunoprecipitated with anti-PR antibodies and then blotted with either anti-PR (a), H222 anti-ER (b) or anti-Src antibodies (c). (B) Cell lysates were immunoprecipitated with polyclonal anti-ER antibodies and then blotted with either H222 anti-ER (a), anti PR (b) or anti-Src (c) antibodies. (C) T47D cells were either not stimulated or stimulated with R5020 for the indicated times. Cell lysates were immunoprecipitated with control or anti-c-Src antibodies and blotted with anti-c-Src (a) and anti-ER (b) antibodies.

containing the estrogen-binding domain as one region interacting with PR<sub>B</sub> (Figure 9C, panel b).

#### **The progestin effect on c-Src activation is not mediated by transcriptional effects of PR<sub>B</sub>**

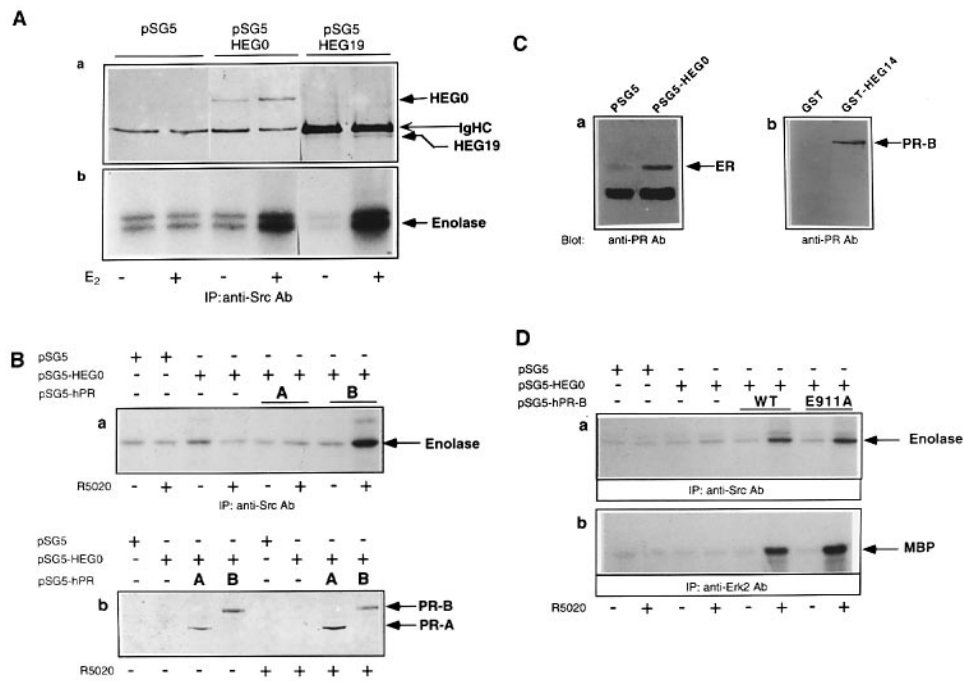
To exclude the possibility that the effect of progestins could be mediated by a nuclear transcriptional effect, we used a mutant of hPR<sub>B</sub>, E911A, carrying a glutamic acid to alanine substitution at residue 911, which binds hormone normally but is transcriptionally inactive (Gong *et al.*, 1997). When expressed in Cos cells together with ER, hPR<sub>B</sub>-E911A enhanced c-Src and Erk activities in response to progestins as efficiently as the wild-type PR<sub>B</sub> (Figure

9D, panels a and b). This shows that progestins exert their effects on the Src/p21<sup>ras</sup>/MAP kinase pathway independently of their genomic actions.

## **Discussion**

We report that in T47D cells the progestin R5020 activates the mitogenic c-Src/p21<sup>ras</sup>/MAP kinase signal transducing pathway which is known to be stimulated by different growth factors in different systems (Davis, 1993; Schlessinger, 1993). Recently, it has also been reported that this pathway is activated by estradiol in cell lines derived from human mammary cancer and human colon carcinoma (Di Domenico *et al.*, 1996; Migliaccio *et al.*, 1996). In addition, estradiol activation of MAP kinase activity has been observed recently in an osteoblast-like cell line (Endoh *et al.*, 1997) as well as in neuroblastoma cells (Watters *et al.*, 1997). The progestin stimulates c-Src and Erk-2 activity of T47D cells with a magnitude and kinetics similar to those of EGF. It requires its own receptor for activation of the pathway, as the anti-progestin RU486 inhibits the progestin stimulation of c-Src, p21<sup>ras</sup> and Erk-2. This notion is supported further by the finding that T47D-Y cells, which contain ER but lack PR, do not respond to R5020 with Erk-2 stimulation whereas they are estradiol responsive. This finding also excludes the possibility that spillover of the progestin on the ER is responsible for the R5020 activation of the signaling pathway. To our great surprise, in addition to the PR, the ER is also required for progestin stimulation of the signaling pathway. This was suggested initially by anti-estrogen inhibition of the stimulatory effect of R5020 on the single steps of the pathway, and subsequently was corroborated by the requirement of co-transfection of ER with PR in Cos-7 cells in order to observe the stimulatory progestin effect.

Aspects of the general mechanism of progestin activation have been analyzed. Immunoprecipitation experiments in T47D and Cos-7 cells expressing ER alone or both ER and PR show ligand-dependent association of ER with c-Src. In contrast, association of c-Src with PR is undetectable under all the experimental conditions used: in T47D cells and in Cos cells transfected with both PR and ER or PR alone, in the absence or presence of agonists. The association of ER with c-Src is stimulated not only by estradiol but also by R5020, with kinetics similar to c-Src stimulation by progestins in T47D cells. This and the finding that the association is prevented not only by anti-estrogens but also by anti-progestins, which also prevent progestin activation of c-Src/p21<sup>ras</sup>/MAP kinase, indicate a functional linkage between ER-c-Src association and progestin or estradiol activation of the pathway. It is noteworthy that in contrast to progestins, estradiol does not require the presence of the PR to activate the pathway, as shown by its ability to stimulate Erk-2 in T47D-Y cells. This is apparently due to the fact that ER, unlike PR, is able to interact with c-Src. Therefore, the ER-c-Src association is crucial for triggering the signaling pathway activation by both progestins and estradiol. Use of the HEG19 (Tora *et al.*, 1989b) and HE15 (Kumar *et al.*, 1987) ER mutants allows localization of the domain involved in activation of c-Src to the C-terminal portion of the ER.



**Fig. 9.** ER and PR domains involved in signaling and receptor interactions. **(A)** Cos cells were transfected with either empty pSG5 vector, pSG5-HEG0 or pSG5-HEG19. Cells were untreated or treated with estradiol for 2 min, and lysates were incubated with anti-c-Src antibodies. The immunoprecipitates either were blotted with anti-ER antibodies (a) or incubated with enolase to assay the c-Src activity (b). **(B)** Cos-7 cells were transfected with either empty pSG5 vector or pSG5-HEG0 with or without pSG5-hPR<sub>A</sub> or pSG5 hPR<sub>B</sub> vectors. Cells were untreated or treated for 2 min with R5020. Lysates were incubated with anti-c-Src antibody and the precipitates were assayed for Src kinase activity (a). Lysate aliquots were blotted with anti-PR antibodies (b). **(C)** ER-PR interaction was studied *in vitro*: lysates from Cos cells transfected with either empty pSG5 vector or the pSG5-HEG0 vector were precipitated with anti-ER antibodies and submitted to 'far Western blotting' with recombinant PR<sub>B</sub> (a); interaction of PR<sub>B</sub> overexpressed in Cos-7 cells with GST alone and GST-HEG14 was analyzed (b). **(D)** Cos-7 cells were transfected with either empty pSG5 vector or pSG5-HEG0 alone or with either pSG5 hPR<sub>B</sub> wild-type (WT) or pSG5 hPR<sub>B</sub>E911A (911A) vectors. Cells were untreated or treated for 2 or 5 min with R5020 to assay c-Src and Erk-2 activities, respectively. Cell lysates were incubated with anti-c-Src (a) and anti-erk-2 (b) antibodies and the precipitates were assayed for Src kinase and Erk-2 activity.

The receptors for estrogens and progestins are found to be associated in T47D and Cos-7 cells. This association is necessary for activation of the MAP kinase pathway by progestins. The A isoform of PR, PR<sub>A</sub>, identical to PR<sub>B</sub> except for the absence of the N-terminal 168 amino acids (Kastner *et al.*, 1990), neither associates with the ER nor transmits the progestin stimulation to the signaling pathway. These experiments, in addition to identifying the domain of PR<sub>B</sub> responsible for the association, demonstrate that this association is functional.

Our analysis of proteins immunoprecipitated by different antibodies, in different cells, under different conditions do not provide evidence for the existence of a stable ternary complex including PR, ER and c-Src. The results, rather, are consistent with the existence of at least two separate pools of ER complexes, one constitutive, containing PR<sub>B</sub>, and the other ligand-induced, containing c-Src. We can speculate that ER and PR exist in two conformations at equilibrium, one inactive, ER<sub>i</sub> and PR<sub>i</sub> (in the absence of ligand or in the presence of anti-hormones), and the other active, ER<sub>a</sub> and PR<sub>a</sub> (in the presence of agonists). If only PR<sub>i</sub> and ER<sub>i</sub> form a complex and only ER<sub>a</sub> interacts with c-Src, then ER<sub>a</sub>, once formed, either by binding of estradiol or by contact with the progestin-charged PR<sub>a</sub>, would leave the complex and interact with c-Src. The decrease in the levels of co-immunoprecipitated receptor in the presence of agonists when Cos cell lysates are incubated with anti-ER or anti-PR antibodies (Figure 7A and B, panel b) and the parallel

association with c-Src of the ER (Figure 7C, panel b) are consistent with our model. However, this decrease is not evident in agonist-stimulated T47D cells, probably due to the large excess of PR over ER.

An interesting question raised in this report is whether the progestin activation of the signaling pathway is associated with the ability of PR to activate transcription. For this purpose, we have employed a new PR mutant which binds ligand but is transcriptionally inactive (Gong *et al.*, 1997). This mutant is still capable of activating c-Src and MAP kinase in response to R5020, indicating that the signaling pathway can be activated independently of transcriptional activation by the same ligand. The reason for this independence might be that there are different sites of action of the steroid involved in the two processes. In addition to the main function of steroid hormone receptors localized in the nucleus and responsible for the transcriptional effects, a small fraction of receptors might be localized outside the nuclei in the vicinity of the cytoplasmic membrane poised to trigger the pathway which transduces signals from the membrane to the nucleus.

In spite of accumulated evidence showing that ER plays a central role in cell proliferation both in the presence and absence of its ligand (Vignon *et al.*, 1987; Auricchio *et al.*, 1995; Pietras *et al.*, 1995), the mechanisms by which steroids exert their proliferative action are obscure. Anti-estrogens prevent activation of the stimulatory effect of R5020 not only through the signaling pathway but also at



the level of cell proliferation (Figure 5A), suggesting that the progestin effect on proliferation of T47D cells not only involves PR but also ER. This also indicates that the same mechanism could be responsible for stimulation of both the signaling pathway and cell proliferation. This possibility is supported further by the observed effect of tyrosine kinase inhibitors on both Erk-2 activation and cell proliferation, suggesting that the progestin stimulates proliferation through signaling pathway activation. The same signal transduction pathway has been proposed previously to participate in estradiol-stimulated cell proliferation (Di Domenico *et al.*, 1996), and a role for c-Src in the induction of mammary tumors has been postulated (Guy *et al.*, 1994). Use of cells transfected with dominant-negative mutants or microinjected with antibodies to suppress specifically single steps of the signaling pathway should be used to verify whether steroid activation of the c-Src/p21<sup>ras</sup>/MAP kinase pathway really triggers cell proliferation.

Evaluating the significance of the interactions described here requires identification of targets of MAP kinase signal activated by the ovarian hormones in breast cancer cells. One possibility would be that ER itself is activated by MAP kinase phosphorylation (Kato *et al.*, 1995), thus creating a positive feed-back loop for agonist-dependent or ligand-independent effects of ER. Another possibility is modulation of AP1 activity, as estrogens are known to induce *c-fos* and *c-jun* gene expression (Weisz and Bresciani, 1993). Activation of the MAP kinase pathway could serve to potentiate these effects either by acting directly on c-Fos or c-Jun, or by regulating the expression of the corresponding genes (Deng and Karin, 1994; Derijard *et al.*, 1994). A recent report shows that the estrogen-induced *c-fos* early gene transcription is mediated by MAP kinase activation (Watters *et al.*, 1997). Alternatively the targets could be genes involved in cell cycle control. Mice lacking the cyclin D1 gene show abnormal development of the mammary gland (Fantl *et al.*, 1995; Sicinski *et al.*, 1995), and both estrogens and progestins are known to induce cyclin D1 gene expression in breast cancer cells (Musgrove *et al.*, 1993; Altucci *et al.*, 1996) although no classical hormone-responsive elements can be identified in the 5'-flanking region of the gene (Herber *et al.*, 1994). Activated p21<sup>ras</sup> and MAP kinases (Lavoie *et al.*, 1996) induce cyclin D1 expression (Filmus *et al.*, 1994; Lovec *et al.*, 1994; Albanese *et al.*, 1995) and activation of *c-raf-1* leads to an accumulation of cyclin D1 and a repression of the p27<sup>kip1</sup> cdk inhibitor (Kerkhoff and Rapp, 1997). Therefore, it is possible that the observed c-Src/p21<sup>ras</sup>/Erk pathway activation by steroids could mediate the hormonal induction of this key cell cycle gene. This would provide a rapid pathway for ovarian hormones to potentiate the effects of growth factors acting through membrane receptors. However, a recent report has questioned this possibility (Lukas *et al.*, 1996).

In conclusion, for the first time we observe that progestins stimulate the c-Src/p21<sup>ras</sup>/MAP kinase signaling pathway. This stimulation occurs in T47D and transfected Cos-7 cells when progestins bind to the B isoform of its own receptor which is pre-associated with ER. Progestin occupancy of the receptor induces association of ER with c-Src, which activates the pathway. This unorthodox cross-talk between steroid hormones could have a role in

modulating cancer cell proliferation. Experiments are under way to establish the cellular localization of the steroid receptors involved in c-Src activation as well as the molecular interactions along this novel signaling pathway, and the role of this pathway in mammary cell proliferation.

## Materials and methods

### Materials

Anti-p21<sup>ras</sup> rat monoclonal antibodies (clone Y 13-259), 327 mouse anti-Src monoclonal antibodies, anti-mouse IgG and anti-rat IgG goat antibodies were from Oncogene Science Inc. (Manhasset, NY). Anti-Erk-1 and anti-Erk-2 rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA). H222 rat monoclonal antibodies against ER were a generous gift from Abbott (Abbott Park, IL). Rabbit anti-ER polyclonal antibodies were from Sigma (St Louis, MO). Mouse anti-PR monoclonal antibodies were from Stressgene Inc. (Canada). All electrophoresis reagents were from Bio-Rad (Richmond, CA). BA-85 nitrocellulose was from Schleicher & Schuell (Dassel, Germany). Anti-mouse, anti-rat and anti-rabbit IgG alkaline phosphatase (AP) conjugates were from Promega (Madison, WI). Protein G-Sepharose beads, sodium orthovanadate, enolase, MBP, HEPES, PIPES, phenylmethylsulfonyl fluoride (PMSF), polyethyleneimine cellulose plates, guanosine and adenosine nucleotides were from Sigma; human recombinant EGF was from Boehringer Mannheim Biochemica (Germany). OH-Tamoxifen and ICI 182,780 were a gift from Zeneca (Basiglio, Italy). R5020 and RU 486 were a gift of Roussel-Uclaf (France). All reagents for cell culture media, including fetal calf serum (FCS) were from Gibco (USA). All other reagents were of analytical grade.

### Cell culture

Human breast cancer cells T47D and T47D-Y were grown routinely in 10% CO<sub>2</sub> in air atmosphere using plastic Petri dishes (100 mm) in RPMI-1640 medium supplemented with phenol red, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), insulin (0.2 U/ml) and 10% FCS. Prior to experiments, subconfluent cells were maintained for 3–4 days in the same medium, without phenol red, containing 10% FCS and treated twice with charcoal-coated dextran (CSS) prepared using a method described previously (Di Domenico *et al.*, 1996). Cells were passaged every 7 days and media changed every 2 days. Cos-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented as described above.

### Erk immunoprecipitation and activity assay

Cell lysates were prepared as described previously (Migliaccio *et al.*, 1996) and incubated with anti-Erk antibodies, and the pellets were assayed for MBP (0.3 mg/ml) phosphorylation using [ $\gamma$ -<sup>32</sup>P]ATP. Reactions were stopped by addition of 2× SDS-PAGE sample buffer and analyzed by SDS-PAGE (13.5% acrylamide gel) followed by autoradiography.

### p21<sup>ras</sup>-bound nucleotide analysis

T47D cells were maintained in phosphate-free medium for 16 h and labeled with 0.5 mCi/dish of [<sup>32</sup>P]orthophosphate for 3 h before treatment with 10 nM progestin for 4 min in the absence or presence of either anti-progestin or anti-estrogen. Cell lysates were immunoprecipitated with anti-p21<sup>ras</sup> antibody (Y13-259) and nucleotides were analyzed by PEI-cellulose chromatography according to a procedure reported previously (Migliaccio *et al.*, 1996).

### Immunoprecipitation of c-Src and c-Src kinase assay

Cell lysates were prepared as described above, incubated with ~1 µg/ml mouse monoclonal anti-c-Src antibodies (clone 327) overnight at 4°C, then added to an equal amount of goat anti-mouse IgG antibodies and incubated for an additional 30 min. At the end of the incubation, 40 µl of a 50% suspension of protein G-Sepharose were added and incubation continued for an additional 30 min. The samples were centrifuged and pellets washed with 1 ml of lysis buffer four times and used for c-Src kinase assays or eluted by Laemmli SDS-PAGE sample buffer and run on electrophoresis.

The activity of c-Src kinase was assayed using acidified enolase (0.5 mg/ml) as a substrate. The incubation was performed as previously reported (Di Domenico *et al.*, 1993)

**Immunoprecipitation of estradiol and PRs**

Cell lysates were prepared as described above, incubated with ~0.5 µg/ml of anti-PR mouse monoclonal antibodies (SR-1110), 1 µg/ml of anti-ER rabbit polyclonal antibodies (Sigma, St Louis, MO) or rat monoclonal antibodies (H222), then added to an equal amount of goat anti-mouse or rabbit anti-rat IgG antibodies, respectively, together with 40 µl of a 50% suspension of protein G-Sepharose, and incubated overnight at 4°C. At the end of the incubation, the samples were centrifuged and pellets washed with 1 ml of lysis buffer four times, eluted by Laemmli SDS-PAGE sample buffer and run on electrophoresis.

**Electrophoresis and immunoblotting**

Samples were submitted to SDS-PAGE (10–13.5% acrylamide, acrylamide:bis-acrylamide ratio 37.5:1). At the end of the run, proteins from gel slabs were electrophoretically transferred onto nitrocellulose filters at 25 V overnight at room temperature using a transfer buffer containing 50 mM Tris, 380 mM glycine, 0.1% SDS and 20% methanol. The filters were soaked in 10 mM phosphate-buffered saline (PBS), 0.1% Tween-20 pH 7.2 (PBST-buffer) containing 3% bovine serum albumin (BSA) to block non-specific binding sites (blocking solution) and incubated for 2 h. They were then incubated for at least 2 h with anti-PR or anti-ER antibodies (1 µg/ml in blocking solution) and washed at least three times for 10 min with PBST buffer. After washing, the filters probed with anti-PR were incubated with AP-linked anti-mouse IgG antibodies and the filters probed with anti-ER were incubated with AP-linked anti-rat IgG antibodies (1:4000 dilution in PBST containing 1% BSA) for 45 min at room temperature. Finally, filters were washed again as described above and protein-antibody complexes revealed according to the manufacturer's instructions.

**Transfection of Cos-7 cells with ER and human PR cDNAs**

The Cos-7 cells were grown routinely in 75 cm<sup>2</sup> plastic flasks in DMEM supplemented with 5% FCS. Five µg of pSG5-HEG0 or pSG5-HEG19 (Tora *et al.*, 1989b), or 10 µg of pSG5-HE15 (Kumar *et al.*, 1987), pSG5-hPR, form A and B (Kastner *et al.*, 1989) or pSG5-hPR-E911-A (Gong *et al.*, 1997) were, separately or in association, transiently transfected into Cos-7 cells by electroporation (220 V, 250 µF) using the Gene Pulser apparatus provided with a capacitance extender (Bio-Rad). As a control, parallel Cos-7 aliquots were also transfected with 5–15 µg of pSG5 vector alone. After 24 h, medium was changed to phenol red-free DMEM supplemented with 5% charcoal-stripped FCS for 48 h.

**In vitro ER-PR interaction**

Lysates from Cos-7 cells transfected with pSG5-hPR-B cDNA were incubated in the presence of 10 nM R5020 with glutathione-Sepharose loaded with GST alone or GST-HEG14 (domains D, E and F of hER). The proteins were eluted with 5 mM glutathione, analyzed by SDS-PAGE and blotted with anti-PR antibody. HEG0 from transfected Cos-7 cells was immunoprecipitated with H222 mAb, analyzed by SDS-PAGE and transferred onto a nitrocellulose filter. After denaturation/renaturation in 6–0.187 M guanidine hydrochloride, the filter was incubated with recombinant hPR-B and blotted with anti-PR antibodies. Histidine-tagged recombinant hPR was expressed in Sf9 insect cells infected with appropriate baculovirus vectors and purified through a Ni column as described previously (Russmann *et al.*, 1997).

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