

Non-transcriptional action of oestradiol and progestin triggers DNA synthesis

Gabriella Castoria, Maria Vittoria Barone^{1,2}, Marina Di Domenico, Antonio Bilancio, Donatella Ametrano, Antimo Migliaccio and Ferdinando Auricchio³

Istituto di Patologia Generale e Oncologia, Facoltà di Medicina e Chirurgia, II Università di Napoli, Largo S. Aniello a Caponapoli, 2, 80138 Napoli and ¹Fondazione Sen. G. Pascale, Istituto Nazionale per lo Studio e la Cura dei Tumori, Via Semmola, 3, 80131 Napoli, Italy

²Present address: Dipartimento di Biologia e Patologia Cellulare e Molecolare 'L. Califano', Via Pansini, 5, 80131 Napoli, Italy

³Corresponding author
e-mail: ferauric@unina.it

G. Castoria and M. V. Barone contributed equally to this work

The recent findings that oestradiol and progestins activate the Src/Ras/Erk_s signalling pathway raise the question of the role of this stimulation. Microinjection experiments of human mammary cancer-derived cells (MCF-7 and T47D) with cDNA of catalytically inactive Src or anti-Ras antibody prove that Src and Ras are required for oestradiol and progestin-dependent progression of cells through the cell cycle. The anti-tumoral ansamycin antibiotic, geldanamycin, disrupts the steroid-induced Ras–Raf-1 association and prevents Raf-1 activation and steroid-induced DNA synthesis. Furthermore, the selective MEK 1 inhibitor, PD 98059, inhibits oestradiol and progestin stimulation of Erk-2 and the steroid-dependent S-phase entry. The MDA-MB231 cells, which do not express oestradiol receptor, fail to respond to oestradiol in terms of Erk-2 activation and S-phase entry. Fibroblasts are made equally oestradiol-responsive in terms of DNA synthesis by transient transfection with either the wild-type or the transcriptionally inactive mutant oestradiol receptor (HE241G). Co-transfection of catalytically inactive Src as well as treatment with PD98059 inhibit the oestradiol-dependent S-phase entry of fibroblasts expressing either the wild-type oestrogen receptor or its transcriptionally inactive mutant. The data presented support the view that non-transcriptional action of the two steroids plays a major role in cell cycle progression.
Keywords: cell cycle progression/non-transcriptional action/oestradiol/progestin/Raf-1

Introduction

Breast cancer is one of the most widespread malignancies in Western society, but our scant knowledge of the mechanisms responsible for the hormone-dependent cell growth has hindered innovative approaches to therapy. The classic model of steroid transcriptional action (Evans, 1988; Beato *et al.*, 1995, Mangelsdorf *et al.*, 1995; Parker

and White, 1996) has not yet provided exhaustive insights into steroid action on cell proliferation. Therefore, a new model is needed on which to base the future analysis of the proliferative action of steroids (Gorski, 1997).

Mammary gland development is regulated by steroid receptors. Targeted disruption of the oestrogen receptor α (ER α) gene affects the development of the gland in homozygous ER α -/- female mice (Lubahn *et al.*, 1993; Korach, 1994). Mice lacking progesterone receptor (PgR) show lack of post-puberty development of the mammary gland (Lydon *et al.*, 1995). Furthermore, the ER plays a role in cell proliferation not only in the presence of oestradiol, but also in its absence, apparently as a consequence of cross-talk between growth factors and steroid receptors (Vignon *et al.*, 1987; Auricchio *et al.*, 1995; Pietras *et al.*, 1995). The putative targets of proliferative steroid action include early genes such as *fos*, *jun-B* and *myc* (Weisz and Bresciani, 1993), cell cycle-controlling gene products, such as cyclin D1 (Musgrove *et al.*, 1993), cyclin-dependent kinase inhibitors (Planas-Silva and Weinberg, 1997) and growth factors (Dickson and Lippman, 1995). Disruption of the cyclin D1 gene yields a mammary phenotype similar to that observed in mice lacking PgR (Fantl *et al.*, 1995; Sicinski *et al.*, 1995), supporting the view that the effect of PgR is mediated through cyclin D1 expression. The initial targets of steroids leading to modulation of expression and/or activity of these molecules have not yet been identified.

We recently reported that oestradiol activates the signal-transducing Src/Ras/Erk_s pathway in human mammary cancer-derived cell lines, MCF-7 and T47D, as well as in the human colon cancer-derived cell line, Caco-2 cells (Migliaccio *et al.*, 1993, 1996, 1998; Di Domenico *et al.*, 1996). This activation requires ER and Src interaction. Progestins also activate the same pathway in T47D cells through cross-talk between PgR-B and ER (Migliaccio *et al.*, 1998). The Src/Ras/Erk_s signalling pathway is a well-known target of growth factors. Its activation by ligands of 'nuclear' receptors such as steroids was an unexpected finding. Activation of this pathway triggers different cellular responses such as proliferation or differentiation (Cantley *et al.*, 1991; Marshall, 1994; Downward, 1997). Activation of Src has been observed in a large number of human breast and colon carcinomas (Rosen *et al.*, 1986; Ottenhoff-Kalff *et al.*, 1992), and the importance of Src in tumorigenesis has been tested directly in the mammary epithelium (Ottenhoff-Kalff *et al.*, 1992). Activation of Src results in an induction of mammary tumours in transgenic mice (Guy *et al.*, 1994). Constitutively active Ras mutants have been found in 25–30% of all cancers, including breast cancer (Kasid and Lippman, 1987).

Therefore, we addressed the role of the Src/Ras/Erk_s

pathway in steroid-stimulated DNA synthesis by combined use of molecules selectively interfering with the different steps of the pathway. Our data prove that such a role exists.

Both the inhibition of steroid-stimulated S-phase entry by steroid antagonists and the lack of response to oestradiol in cells derived from human breast cancer which do not express ER demonstrate the requirement for steroid receptors for hormone-dependent DNA synthesis. Moreover, NIH 3T3 fibroblasts respond to oestradiol in terms of DNA synthesis only after transient transfection with the human receptor ER α . Interestingly, such a response is also observed when the cells are transfected with a transcriptionally inactive mutant form of receptor (ER Δ 250–303). In addition, fibroblasts transfected with either ER or its transcriptionally inactive mutant use the same proliferative pathway of human mammary cancer-derived cell lines, when stimulated by oestradiol. Taken together, our findings support the view that the non-transcriptional action of oestradiol and progesterin is responsible for the mitogenic activity of these steroids.

Results

Microinjection of Src K⁻-expressing plasmid inhibits the steroid-stimulated DNA synthesis of target cells

These experiments address the role of Src in steroid-induced S-phase entry of target cells. Different human mammary cancer-derived cell lines were used: the MCF-7 cell line, representing a standard model to study oestradiol-regulated cell multiplication (Levenson and Jordan, 1997), and the T47D cell line, constitutively expressing the two PgR isoforms (PgR-A and -B), which can be utilized to study progesterin activity independently of oestrogens (Chalbos *et al.*, 1982; Horwitz *et al.*, 1982). Both cell lines were maintained for 4 days in the absence of phenol red, a substance with a weak oestrogenic activity, and in the presence of charcoal-treated serum to simulate hormone depletion conditions. The cells were then analysed for bromodeoxyuridine (BrdU) incorporation into newly synthesized DNA by *in situ* immunofluorescence analysis. A very low number of cells incorporated BrdU into DNA under these conditions (~8 and 5% for MCF-7 and T47D cells, respectively; see Figure 1A and B). A time course of BrdU incorporation was then followed after 10 nM oestradiol or progesterin R5020 stimulation of MCF-7 and T47D cells, respectively (not shown). A large number of cells incorporated BrdU in DNA, with a peak at 24 h after hormone addition to the medium (~70 and 60% of MCF-7 and T47D cells, respectively; see Figure 1A and B).

The mutant form of Src, with Lys295 changed to methionine (Src K⁻), has been utilized previously to demonstrate the requirement for a functional Src tyrosine kinase in platelet-derived growth factor (PDGF) (and other growth factors) signalling *in vivo* (Twamley-Stein *et al.*, 1993; Roche *et al.*, 1995; Barone and Courtneidge, 1996), as well as Src involvement in epithelial cell scattering (Boyer *et al.*, 1997). To investigate whether steroids stimulate DNA synthesis of MCF-7 and T47D cells by an Src-dependent mechanism, the nuclei of target cells were microinjected with a construct expressing Src K⁻. Six hours later, 10 nM oestradiol or progesterin R5020 were added to the medium together with BrdU, and 24 h after

hormone addition the cells were fixed and stained. Several independent microinjections were performed and multiple coverslips were analysed. To compare the results from different experiments, the number of BrdU-positive cells expressing Src K⁻ were compared with the number of BrdU-positive non-microinjected cells from the same coverslip. Data were then pooled and statistically analysed (see the legend of Figure 1). The results expressed in Figure 1A and B show that microinjection of Src K⁻ inhibits the steroid-stimulated entry into S-phase of both MCF-7 (Figure 1A) and T47D (Figure 1B) target cells by 70%. Figure 1A1 and B1 are representative fields of one experiment. The cells expressing Src K⁻ protein showed diffuse cytoplasmic staining with the anti-Src antibody and are marked with arrows (left panels). The same cells did not incorporate BrdU when visualized with fluorescein-conjugated mouse monoclonal anti-BrdU antibodies (middle panels). The nuclear staining is also shown (right panels).

To exclude the possibility that microinjection by itself was responsible for the observed reduction of BrdU incorporation, we injected into cell nuclei the pSG5 plasmid together with an expressing green fluorescent protein (GFP) vector (pEGFP), as injection marker, since the empty plasmid cannot be revealed. The results are presented in the legend of Figure 1. Microinjection of the pSG5 empty plasmid did not affect the steroid-stimulated S-phase entry of either MCF-7 or T47D cells. In addition, whereas microinjection of either pEGFP alone or pEGFP together with pSG5 did not modify the steroid-elicited DNA synthesis, co-microinjection of Src K⁻ with pEGFP inhibited the steroid-stimulated S-phase entry, with an efficiency similar to that observed in cells microinjected with Src K⁻ alone (not shown).

We also considered the possibility that overexpression of an SH2 domain-containing protein was responsible for the observed inhibition of BrdU incorporation. Therefore, we microinjected a plasmid expressing the kinase-active form of Src (Src wt) at a DNA concentration (50 ng/ μ l) that did not stimulate cells to enter the S-phase in the absence of ligand (not shown). Microinjection of this amount of wild-type Src (Src wt) did not affect the steroid-induced S-phase entry (third bars in Figure 1A and B).

In conclusion, the data show that Src is required for steroid-stimulated cell cycle progression.

Microinjection of anti-Ras antibody inhibits the steroid-stimulated DNA synthesis of target cells

To assess whether target cells require Ras signalling for DNA synthesis induced by steroids, we used the anti-Ras monoclonal antibody (mAb) Y259, which recognizes antigenic determinants common to H-, K- and N-Ras, and blocks binding of Ras to Raf (Mulcahy *et al.*, 1985). It was injected into the cytoplasm of quiescent MCF-7 and T47D cells at two different concentrations. Purified IgGs from non-immunized rabbits were co-injected with anti-Ras mAb to identify the injected cells. The specificity of this approach was assessed by rabbit IgG injection, as a control. Immediately after microinjection, 10 nM oestradiol or the progesterin R5020 were added to the medium of MCF-7 and T47D cells, respectively, together with BrdU, and after 24 h the cells were fixed and stained. Several experiments were analysed statistically (see the legend of

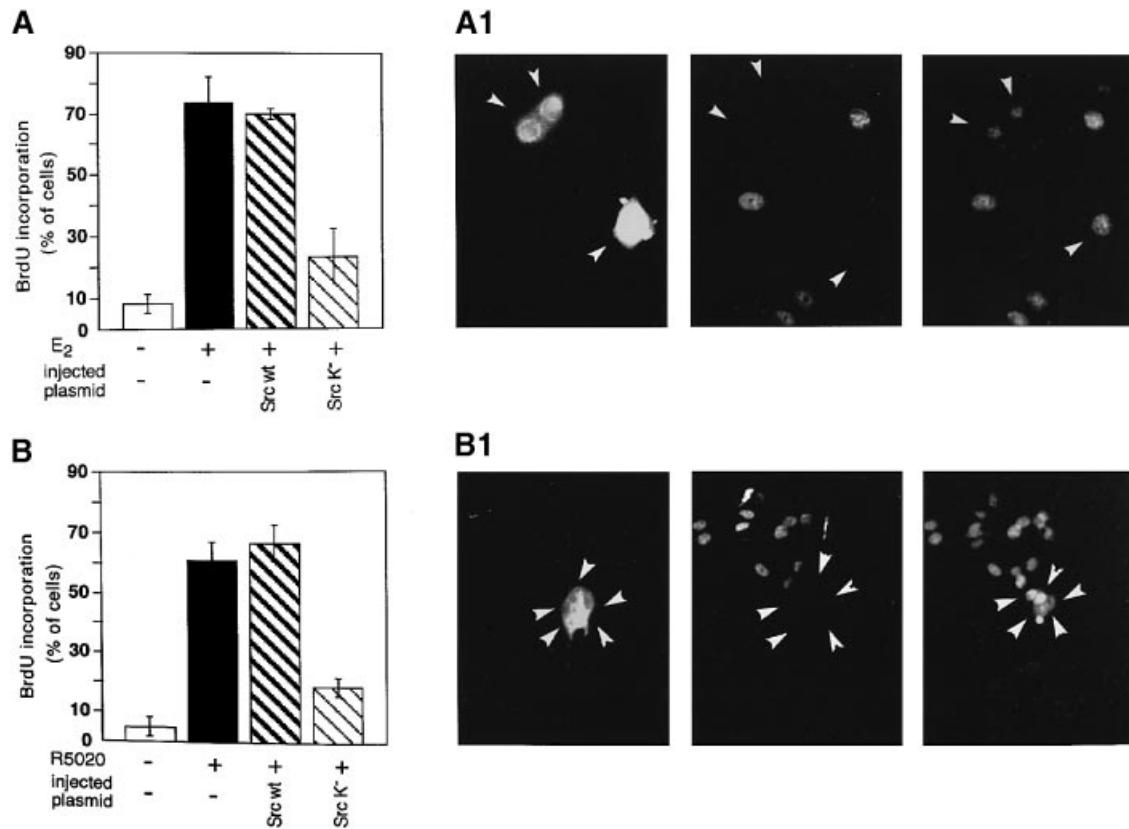


Fig. 1. Specific inhibition of steroid-dependent S-phase entry of MCF-7 and T47D cells by the dominant-negative Src. Constructs expressing Src K⁻ (Src K⁻) or Src wild-type (Src wt) were microinjected into nuclei of quiescent MCF-7 (A and A₁) or T47D (B and B₁) cells. Either 10 nM oestradiol (A and A₁) or 10 nM progesterin R5020 (B and B₁) was added to the cells together with BrdU, and 24 h later they were fixed and stained. Coverslips were analysed for DNA synthesis. (A and B) For each cell line, several coverslips were analysed and DNA synthesis in injected cells was calculated by the formula: percentage of BrdU-positive cells = (No. of injected BrdU-positive cells/No. of injected cells) × 100. For each plasmid, data are derived from at least 150 microinjected cells. Results of more than three independent experiments have been averaged; the mean and SEM are shown. The statistical significance of these results was also evaluated by paired *t*-test. *P*-values were <0.001 for both MCF-7 and T47D cells microinjected with Src K⁻. The difference in BrdU incorporation between the cells microinjected with Src K⁻ and those microinjected with Src wt was also significant (*P* < 0.005). The difference in BrdU incorporation between the cells microinjected with Src wt and uninjected cells stimulated with both oestradiol and progesterin was not significant. (A₁ and B₁) Representative fields are presented. Cytoplasmic fluorescence in the left panels is from reactivity with the Src antibody of Src K⁻-expressing MCF-7 or T47D cells. The white arrowheads mark the injected cells. The middle panels show the staining with fluorescein-conjugated anti-BrdU antibody. The control Hoechst 33258 staining of nuclei is presented in the right panels. A construct expressing the pSG5 empty plasmid was also microinjected into nuclei of quiescent MCF-7 and T47D cells together with an injection marker, the pEGFP-expressing plasmid. The cells were left unstimulated or stimulated with either oestradiol or the progesterin, R5020. BrdU was added, and after 24 h the coverslips were analysed for DNA synthesis. A total of 58 and 50% of the EGFP-expressing MCF-7 and T47D cells, respectively, entered S-phase. The BrdU incorporation of unstimulated cells was, in each case, <7%, whereas the steroid-stimulated BrdU incorporation was 58 and 57% for MCF-7 and T47D cells, respectively.

Figure 2). BrdU incorporated into newly synthesized DNA was evaluated as described in the previous section. The data are presented in Figure 2. At a lower concentration of anti-Ras mAb, the inhibition of BrdU incorporation in oestradiol- (Figure 2A) and progesterin- (Figure 2B) stimulated cells, although strong, was only partial (52 and 54% for MCF-7 and T47D cells, respectively). At a higher concentration of antibody (~3-fold higher), the inhibition of BrdU incorporation was almost total for both MCF-7 and T47D cells (Figure 2A₁ and B₁). The steroid-stimulated S-phase entry of cells was unaffected by injection of purified IgGs from non-immunized rabbits (compare the second and third bars in Figure 2A and A₁, and B and B₁). Representative fields of MCF-7 and T47D microinjected cells were captured. Figure 2A₂ and B₂ shows the cytoplasmic fluorescence derived from the staining of IgG co-injected with anti-Ras mAb (left panels) and the nuclear fluorescence of the BrdU staining (middle panels). The nuclear staining is also shown (right panels).

Taken together, the data demonstrate that targeting of Ras interferes with steroid-induced progression of target cells into S-phase. The efficiency of this interference depends on the concentration of the injected antibody.

Effect of geldanamycin on steroid-induced Ras-Raf-1 association and DNA synthesis

MCF-7 cells, which respond to oestradiol treatment with Ras and Erk_s activation (Migliaccio *et al.*, 1996), were stimulated for 2 min with 10 nM oestradiol and cell lysates were immunoprecipitated with anti-Raf-1 antibodies (Figure 3). The upper panel in Figure 3A shows that immunoprecipitation of Raf-1 is specific and its efficiency in the different samples is similar. Association of Ras with Raf-1 was followed by immunoblotting using anti-Ras mAb (Figure 3A, middle panel) and Raf-1 phosphorylating activity was assayed using H1 histone as a substrate (Figure 3A, lower panel). The data show that oestradiol increases the Ras-Raf-1 association and

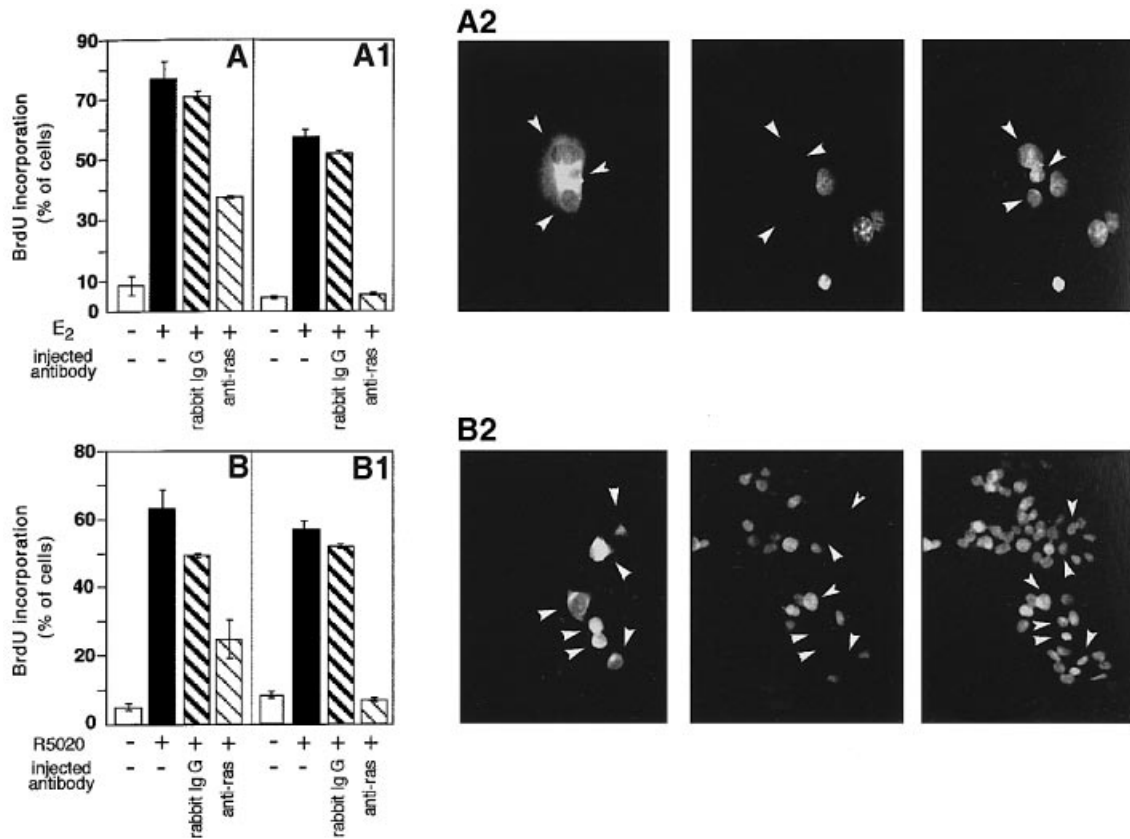


Fig. 2. Specific inhibition of steroid-activated S-phase entry of MCF-7 and T47D cells by different concentrations of anti-Ras mAb. Quiescent MCF-7 or T47D cells were microinjected with either 1.3 (A, A2, B and B2) or 3.3 $\mu\text{g}/\mu\text{l}$ (A1 and B1) of anti-Ras mAb. Purified rabbit IgGs, either 1.3 (A and B) or 0.7 $\mu\text{g}/\mu\text{l}$ (A1 and B1), were included as a marker for successfully injected cells. Cells were injected with rabbit IgGs alone, as a control. Either 10 nM oestradiol (A, A1 and A2) or the progestin R5020 (B, B1 and B2) was added to the cells together with BrdU. After 24 h, cells were fixed and stained. (A, A1, B and B1) For each cell line, several coverslips were analysed and the extent of DNA synthesis in injected cells was calculated as reported in the legend to Figure 1. For each antibody, the data are derived from at least 100 injected cells. Results were pooled; the mean and SEM are shown. The statistical significance of these results was also evaluated by paired *t*-test. *P*-values were <0.001 for both MCF-7 and T47D cells injected with either lower or higher concentration of anti-Ras mAb. The difference in BrdU incorporation between the stimulated cells microinjected with rabbit IgG alone and those microinjected with anti-Ras mAb were also significant ($P < 0.001$). The difference in BrdU incorporation between the cells microinjected with rabbit IgGs alone and uninjected cells stimulated with both oestradiol and progestin was not significant. (A2 and B2) Representative fields of cells microinjected with a lower anti-Ras mAb concentration are presented. In the left panels, cells microinjected with anti-Ras mAb are marked with white arrowheads. The middle panels show the staining of the same cells with fluorescein-conjugated anti-BrdU antibody. The control Hoechst 33258 staining of nuclei is presented in the right panels.

stimulates Raf-1 activity. In analogy with other systems, blockade of Ras–Raf-1 association should lead to loss of hormone effects on downstream steps of the signalling transduction cascade, such as Raf-1 and Erk_s activation, without impairing the activity of the upstream Src.

To assess this hypothesis, we used the ansamycin antibiotic geldanamycin (GA). Treatment of MCF-7 cells with GA for 10 min did not affect ER, either in its hormone-binding capacity assayed by the dextran-coated charcoal method, or at the protein level by immunoblot (not shown), but it abolished both Ras–Raf-1 association (Figure 3A, middle panel) and Raf-1 kinase activity after 2 min of oestradiol stimulation (Figure 3A, lower panel). GA also abolished the oestradiol-induced activation of Erk-2 (Figure 3B, left panel) whereas it was without strong effect on Src kinase activity (Figure 3B, right panel). This shows that the target of GA action in the pathway is the Ras–Raf-1 association.

GA also disrupts the progestin-stimulated Ras–Raf-1 association and blocks Erk-2 activation without significant interference with Src activation in T47D cells (not shown). Whereas steroid treatment of either MCF-7 (Figure 3C,

upper panel) or T47D cells (lower panel) strongly stimulates the BrdU incorporation into newly synthesized DNA, the GA treatment abolished it. These data show that GA blocks the steroid-stimulated entry into S-phase and indicate that this block is caused by the disruption of Ras–Raf-1 association and Raf-1 activation.

The role of Erk_s activation in steroid-stimulated S-phase entry of MCF-7 and T47D cells

Quiescent MCF-7 and T47D cells were stimulated with either 10 nM oestradiol or 10 nM progestin R5020 for 24 h. BrdU was included in the cell media together with different concentrations (0–50 μM) of either the MEK inhibitor, PD98059 (Alessi *et al.*, 1995), or the p38/HOG inhibitor, SB203580 (Lee *et al.*, 1994; Cuenda *et al.*, 1995). Quiescent MCF-7 and T47D cells were treated with vehicle alone, as a control. Figure 4 shows that treatment of MCF-7 (Figure 4A) and T47D cells (Figure 4B) with PD98059 strongly reduces the steroid-stimulated BrdU incorporation into newly synthesized DNA. In contrast, SB203580 did not affect the steroid-elicited S-phase entry. The BrdU incorporation of the untreated

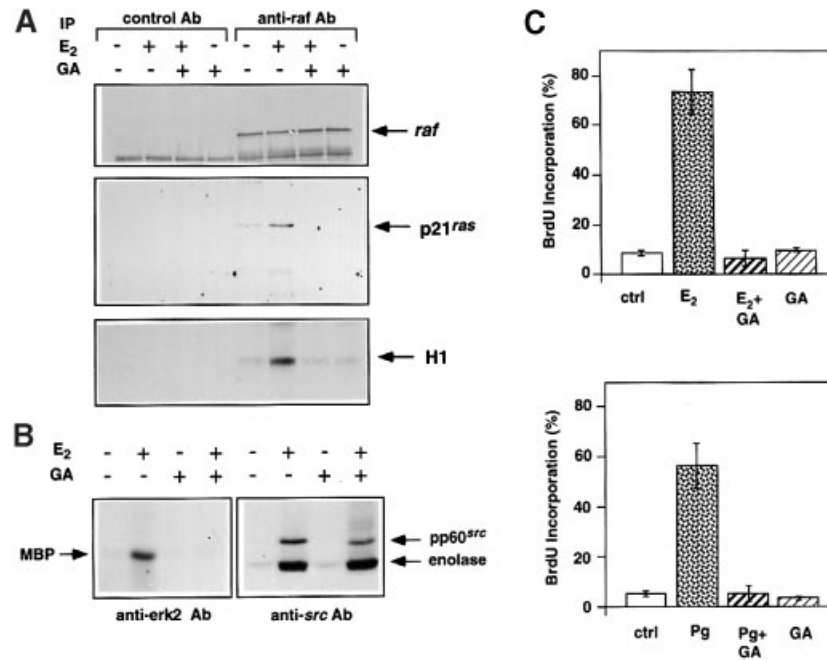


Fig. 3. Steroids induce Ras–Raf-1 association and Raf-1 activation. Geldanamycin abolishes these effects as well as the steroid-dependent S-phase entry. In (A) and (B), MCF-7 cells were untreated or treated with 0.89 μ M geldanamycin (GA) for 10 min, then incubation was pursued in the absence or presence of 10 nM oestradiol for 2 min. (A) Cell lysates were immunoprecipitated with either control or anti-Raf-1 antibodies and the immunoprecipitates blotted with either anti-Raf (upper panel) or anti-Ras antibodies (middle panel) and assayed for Raf-1 kinase activity using H1 histone as a substrate (lower panel). (B) Cell lysates were immunoprecipitated with anti-Erk-2 antibody and the immunoprecipitates assayed for Erk-2 activity using MBP as a substrate (left panel). Lysates were incubated separately with anti-Src antibody. The immunoprecipitates were assayed for Src kinase activity using acid-treated enolase as a substrate (right panel). (C) MCF-7 and T47D cells were untreated or treated with either 10 nM oestradiol (E₂) or the progestin R5020 (Pg), in the absence or presence of 0.89 μ M GA together with BrdU. Cells were treated with GA alone, as a control. After 24 h, cells were fixed and stained. For each cell line, several coverslips were analysed and the BrdU incorporation was calculated by the formula: percentage of BrdU-positive cells = (No. of BrdU-positive cells/No. of total cells) \times 100. Results from different experiments were pooled. In the upper panel, data from MCF-7 cells and in the lower panel data from T47D cells are shown. The mean and SEM are also shown.

cells was <10% and was not affected by treatment of the cells with either PD98059 or SB203580 (not shown). In addition, quiescent MCF-7 and T47D cells were stimulated with either 10 nM oestradiol for 2 min or 10 nM progestin R5020 for 5 min. Figure 4A1 and B1 shows that steroid treatment of target cells stimulated the Erk-2 kinase activity assayed using myelin basic protein (MBP) as a substrate. Addition of 50 μ M PD 98059 to the cell medium 10 min before steroids abolished the stimulatory effect of oestradiol (Figure 4A1) and progestin R5020 (Figure 4B1) on Erk-2 activity, whereas 50 μ M SB 203580 was without effect (Figure 4A1 and B1).

The data indicate that Erk_s activation is involved in the mitogenic response of target cells to steroid hormones.

Steroid stimulation of DNA synthesis of target cells requires ER and PgR

To verify the role of ER and PgR in steroid-elicited DNA synthesis, we treated both the oestradiol-stimulated MCF-7 and the progestin-stimulated T47D cells with either anti-oestrogens, ICI 182,780 and OH-tamoxifen, or the anti-progestin RU486 (Roussel Uclaf, France), respectively. Figure 5A (middle and right panels) shows that the steroid-dependent BrdU incorporation of both MCF-7 and T47D cells is inhibited by treatment with anti-oestrogens or the anti-progestin, respectively. In each case, BrdU incorporation of the untreated cells was <10% and this basal incorporation was unaffected by treatment of the cells with antagonists (not shown). It is noteworthy that antagon-

ists also prevent the oestradiol and progestin activation of the Src/Ras/Erk_s pathway (Migliaccio *et al.*, 1996, 1998).

The requirement for ER in oestradiol-stimulated DNA synthesis was verified further using MDA-MB231 cells, not expressing ER (Davidson *et al.*, 1987). A Western blot with H222 anti-ER monoclonal antibodies of lysates from MDA-MB231 cells confirmed the absence of ER in the hormone-independent cells (not shown). The MDA-MB231 cells were made quiescent by serum starvation, then left unstimulated or stimulated with oestradiol in the absence or presence of ICI 182,780. The BrdU incorporation into newly synthesized DNA was analysed by immunofluorescent staining. Figure 5A (left panel) shows that MDA-MB231 cells fail to enter S-phase when stimulated by oestradiol. In contrast, they actively incorporate BrdU when 10% serum is added to the medium.

Figure 5B shows that oestradiol treatment of MDA-MB231 is without effect on Erk-2 kinase activity. In contrast, 5 min of epidermal growth factor (EGF) treatment of the same cells strongly increases the Erk-2 activity. These data show that cells lacking ER are unable to respond to oestradiol in terms of both Src/Ras/Erk_s pathway activation and DNA synthesis.

The oestradiol-stimulated DNA synthesis of NIH 3T3 fibroblasts requires ER α expression and is not mediated by its transcriptional action

To approach the question of whether ER-mediated transcription effects have a central role in the oestradiol-

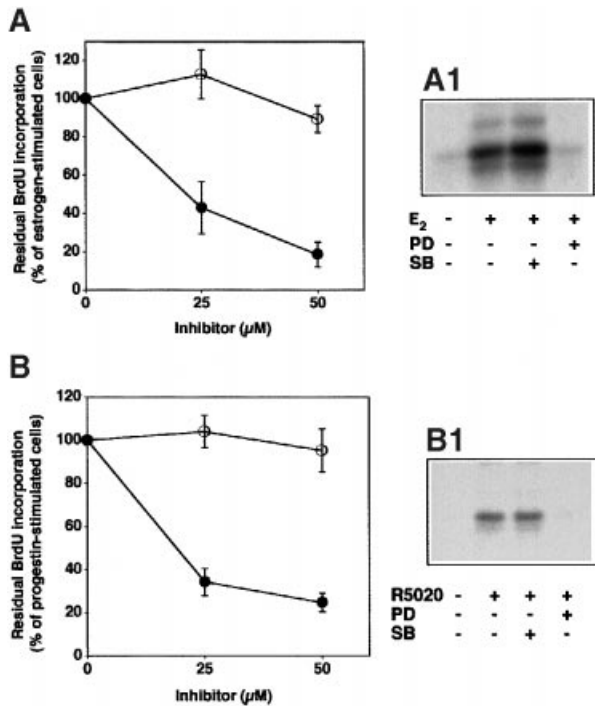


Fig. 4. The MEK inhibitor PD98059, but not the p38/HOG inhibitor, SB203580, inhibits the steroid-stimulated Erk activity and S-phase entry. MCF-7 and T47D cells were made quiescent, then stimulated for 24 h with either 10 nM oestradiol or the progestin R5020 in the absence or presence of either PD98059 (0–50 μM, ●) or SB203580 (0–50 μM; ○). BrdU was included and DNA synthesis monitored. Cells were fixed and stained for BrdU incorporation (A and B). For each cell line, data from several coverslips were pooled and the residual BrdU incorporation was expressed as a percentage of steroid-stimulated cell incorporation, which was 76 and 55% for MCF-7 and T47D, respectively. The mean and SEM are shown. Quiescent MCF-7 and T47D cells were left unstimulated or stimulated with 10 nM oestradiol or the progestin R5020 for 2 or 5 min, respectively, in the absence or presence of 50 μM of either PD98059 or SB203580. The inhibitors were added to the medium 10 min before steroids. Lysates from either MCF-7 (A₁) or T47D cells (B₁) were immunoprecipitated with anti-Erk-2 antibody. The Erk-2 activity was assayed using MBP as a substrate.

elicited progression into S-phase of the cell cycle, NIH 3T3 fibroblasts were used. They were transiently transfected with expression plasmids encoding either the human wild-type ER (pSG5-HEGO) or its mutant, HE241G (pSG5-HEGO/Δ250–303), which does not bind DNA in HeLa cells (Ylikomi *et al.*, 1992). Control cells were transfected with the empty pSG5 plasmid.

We first evaluated the ability of HEGO- or HE241G-expressing plasmids to activate the hormone-regulated transcription. Thus, an ERE-tk-CAT reporter gene was co-transfected into NIH 3T3 cells. The CAT activity was calculated using the β-galactosidase expression vector as an internal control (Figure 6A). Only fibroblasts transfected with HEGO respond to oestradiol with ERE-tk-CAT stimulation, which was completely inhibited by the pure anti-oestrogen ICI 182.780. The transfected NIH 3T3 fibroblasts were stimulated for 24 h with 10 nM oestradiol. BrdU was added to the medium together with oestradiol, and its incorporation into DNA was analysed. Several independent transfections were performed and multiple coverslips analysed. BrdU-positive cells expressing either ER or its mutant were counted (Figure 6B). The non-

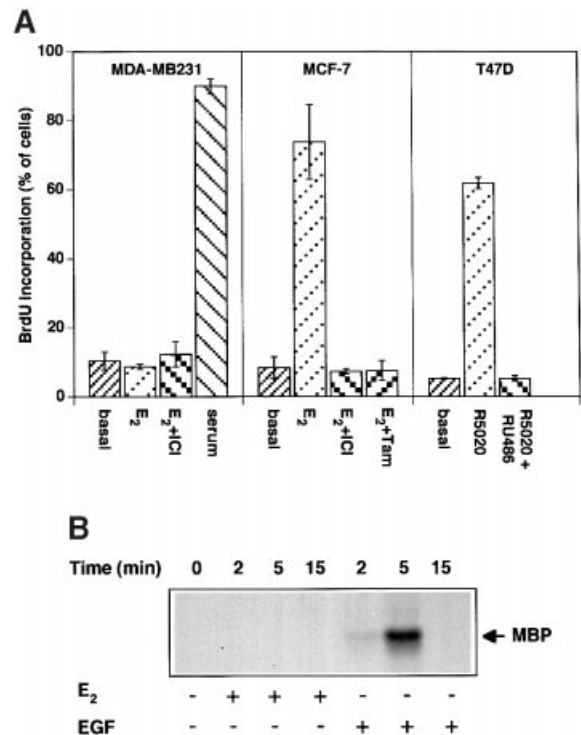


Fig. 5. Steroid-stimulated DNA synthesis requires ER and PgR. (A) ER-negative MDA-MB231 cells (left panel) were made quiescent by serum starvation then left unstimulated or stimulated for 24 h with either 10 nM oestradiol (in the presence or absence of 1 μM ICI 182.780) or 10% serum. Quiescent ER-positive MCF-7 cells (middle panel) were unstimulated or stimulated for 24 h with 10 nM oestradiol, in the absence or presence of either 1 μM ICI 182.780 or 0.1 μM OH-tamoxifen. Quiescent PgR-positive T47D cells (right panel) were unstimulated or stimulated with the progestin R5020 (10 nM) in the absence or presence of 1 μM RU486. BrdU was included and the cells were fixed and stained for BrdU incorporation. For each cell line, several coverslips were analysed and the BrdU incorporation was calculated as in Figure 3. Results from different experiments were pooled and statistical analysis was performed. The mean and SEM are shown. (B) Quiescent ER-negative MDA-MB231 cells were left unstimulated or stimulated for the indicated times with either 10 nM oestradiol or 100 ng/ml EGF (Boehringer Mannheim). Cell lysates were immunoprecipitated with anti-Erk 2 antibody. The Erk-2 activity was assayed using MBP as a substrate.

transfected NIH 3T3 did not respond to oestrogen treatment (<5% BrdU incorporation). Data were pooled and statistically analysed. Figure 6B shows that oestradiol strongly stimulates progression of ER-transfected NIH 3T3 fibroblasts towards S-phase. Interestingly, a similar response in terms of cell cycle progression was obtained in NIH 3T3 cells transfected with the transcriptionally inactive ER mutant. In addition, the oestradiol-induced S-phase entry of NIH 3T3 cells transfected with either HEGO or HE241G cDNA was completely inhibited by the pure anti-oestrogen ICI 182.780. In the absence of oestradiol as well as in oestradiol-treated NIH 3T3 cells transfected with the pSG5 control plasmid, a very low BrdU incorporation was observed. The viability of the cells expressing ER or its mutant was verified. They entered S-phase when the medium was replaced by 10% serum-containing Dulbecco's modified Eagle's medium (DMEM; not shown).

The role of Src in oestradiol-elicited S-phase entry of ER-transfected fibroblasts was also addressed. NIH 3T3

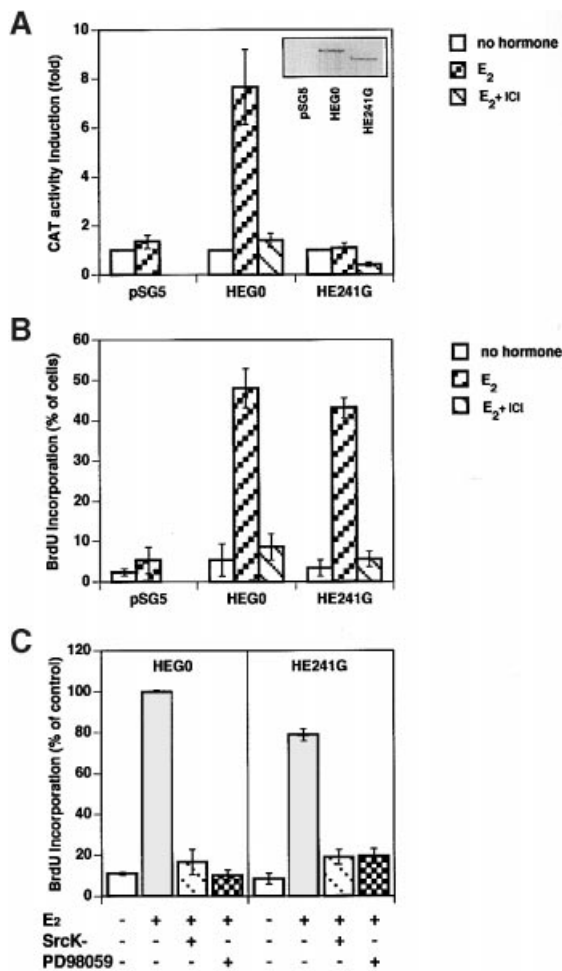


Fig. 6. Oestradiol stimulates S-phase entry of NIH 3T3 fibroblasts transfected with either the wild-type ER or a transcriptionally inactive mutant. (A) NIH 3T3 fibroblasts were transfected with either the control pSG5, or pSG5-HEG0 or pSG5-HE241G plasmids. Expression of ER_s is shown in the insert. In each case, the ERE-tk-CAT reporter gene was co-transfected together with RSV-βGAL as described in Materials and methods. The NIH 3T3 cells were made quiescent then left unstimulated (no hormone) or stimulated by 10 nM oestradiol alone or together with 1 μM ICI 182,780. The CAT activity from cell extracts was assayed and expressed relative to β-GAL activity as reported in Materials and methods. Two different experiments were performed, and the CAT activity was expressed as fold induction over the unstimulated cell level. Mean and SEM are shown. (B) NIH 3T3 fibroblasts transfected with pSG5, pSG5-HEG0 or pSG5-HE241G plasmids were made quiescent and then left unstimulated (no hormone) or stimulated with 10 nM oestradiol. When indicated, 1 μM ICI 182,780 was added. BrdU was included in the cell medium and its incorporation into DNA assessed by immunofluorescent staining. Several coverslips were analysed and data from at least 200 scored cells were represented according to the formula: percentage of BrdU-positive cells = (No. of BrdU-positive transfected cells/No. of transfected cells) × 100. In each case, the BrdU incorporation of non-transfected fibroblasts (<5%) was evaluated and subtracted. Mean and SEM are shown. (C) NIH 3T3 fibroblasts were transfected with pSG5-HEG0 (left panel) or pSG5-HE241G (right panel) alone or together with the Src K⁻-encoding plasmid. They were left unstimulated or stimulated with 10 nM oestradiol. When indicated, PD98059 (25 μM) was added to the cell medium. BrdU incorporation into DNA was analysed by immunofluorescent staining. Several coverslips were analysed and BrdU incorporation was expressed as a percentage of the control, which corresponds to oestradiol-stimulated incorporation of BrdU into HEG0-transfected cells (53%). The BrdU incorporation of non-transfected fibroblasts (<7%) was subtracted in each case. Results from at least 100 scored cells were represented. Mean and SEM are shown.

cells were co-transfected with Src K⁻-encoding plasmids together with the wild-type ER or its mutant. Figure 6C shows that co-expression of Src K⁻ strongly inhibits the oestradiol-elicited S-phase entry of NIH 3T3 cells. In addition, the role of Erk in steroid-stimulated DNA synthesis of NIH 3T3 cells expressing either HEG0 or HE241G was also verified. A significant reduction in the number of cells incorporating BrdU was observed by PD98059 addition to the cell medium. The results demonstrate that ER-expressing NIH 3T3 cells use the same proliferative pathway as MCF-7 and T47D cells when stimulated by oestradiol. Furthermore, lack of ER transcriptional activity does not affect the oestradiol-induced cell cycle progression.

Discussion

Regulation of cell proliferation is one of the main aspects of steroid action not yet elucidated (Gorski, 1997). Identification of the initial targets involved in the proliferative action of steroids is still a matter of debate, although it has been reported that steroids regulate transcription of early genes, cell cycle-controlling genes and cyclin-dependent kinase activity (Musgrove *et al.*, 1993; Weisz and Bresciani, 1993; Planas-Silva and Weinberg, 1997).

Oestradiol, like growth factors (Davis, 1993; Schlessinger, 1993), immediately and reversibly stimulates the Src activity of MCF-7 cells (Migliaccio *et al.*, 1993, 1996). The analogy between oestradiol and growth factors is reinforced by the observation that the ER interacts with and activates Src. Both interaction and activation are dependent on the receptor occupancy by the ligand (Migliaccio *et al.*, 1996, 1998). Experiments with Cos cells transiently transfected with the human ERα cDNA indicate that this receptor activates Src kinase (Migliaccio *et al.*, 1996).

Progestins activate Src in T47D and MCF-7 cells; both cell lines are derived from human mammary cancer. Activation of Src by progestin requires ligand binding to PgR-B pre-associated with ER. Even for progestin, activation of Src requires its association with ER, indicating the existence of an unexpected cross-talk between the two steroid receptors (Migliaccio *et al.*, 1998). Therefore, Src is an initial and central target of both oestradiol and progestin action. It has been shown that Src family kinases are required for PDGF- (and other growth factors) induced DNA synthesis in NIH 3T3 fibroblasts (Twamley-Stein *et al.*, 1993; Roche *et al.*, 1995; Barone and Courtneidge, 1996). A requirement for Src or other tyrosine kinases in steroid-stimulated proliferation of target cells was suggested previously by experiments with tyrosine kinase inhibitors (Di Domenico *et al.*, 1996; Migliaccio *et al.*, 1998). To prove that Src is required for steroid-induced S-phase entry of cells, we performed experiments on cells microinjected with catalytically inactive Src. Conditions of steroid-dependent cell growth have been employed that avoid use of metabolic inhibitors, since these might block proliferative pathways used by steroids. Serum starvation has also been excluded because it strongly interferes with the viability of MCF-7 and T47D cells (not shown). In addition, Erk_s activity of a different breast cancer cell (T5) is drastically reduced under serum-free conditions (Coutts and Murphy, 1998). Therefore, cells were made

quiescent by removing steroids from the medium and avoiding compounds with a weak steroid activity, such as phenol red. Cells were then microinjected with a construct expressing kinase-inactive Src (Src K⁻) and stimulated with hormone. The number of cells in S-phase 24 h after steroid stimulation was greatly reduced. Since the Src mutant associates with the classical ER in transiently transfected cells (not shown), it is possible that, when overexpressed in MCF-7 and T47D cells, this mutant displaces the binding of wild-type Src to activated ER.

Oestradiol-induced Src stimulation leads to tyrosine phosphorylation of Shc and consequent Ras activation in MCF-7 cells (Migliaccio *et al.*, 1996). Such an activation was also observed after progestin stimulation of T47D cells (Migliaccio *et al.*, 1998). Extracellular signals can trigger different processes, such as proliferation or differentiation, by activating Ras-dependent signalling pathways (Marshall, 1996). The mitogenic response to a variety of growth factors and oncogenes is blocked by inhibiting Ras (Mulcahy *et al.*, 1985; Smith *et al.*, 1986). Our present finding that a drastic reduction of S-phase entry is observed in MCF-7 and T47D cells microinjected with Ras-neutralizing mAb demonstrates the requirement for Ras for cell cycle progression of cells stimulated by steroids.

The new finding that oestradiol and progestin stimulation of target cells induces both Ras–Raf-1 association and Raf-1 activation corroborates our previous work on steroid activation of Ras and the Ras-dependent phosphorylation cascade. The Ras–Raf association follows Ras activation and is required for recruitment of Raf-1 at the cell membrane where Raf-1 is activated and in turn activates Mek-1 (Avruch *et al.*, 1994). Activation of Ras–Raf signalling induces cyclin D1 overexpression, repression of the cyclin–cdk inhibitor, p27^{kip1}, as well as Myc expression; furthermore, Raf-1 activates the cdc25 phosphatase which in turn activates the cdk_s (for references, see Kerkhoff and Rapp, 1998). The Raf-1/Mek_s/Erk_s cascade acts downstream of Ras (Bogunski and McCormick, 1993; Schlessinger, 1993; Marshall, 1996). Since Ras can trigger multiple signalling pathways in addition to this cascade (Rodriguez-Viciano *et al.*, 1997; Campbell *et al.*, 1998), it was necessary to verify whether Raf-1/Mek_s/Erk_s kinase cascade activation by steroids (Migliaccio *et al.*, 1996, 1998; Di Domenico *et al.*, 1996) is required for S-phase entry of stimulated cells.

Inhibition of steroid-stimulated S-phase entry of target cells by microinjection experiments with the Y259 anti-Ras mAb indicates that Ras–Raf-1 association is required since this antibody blocks such an association (Mulcahy *et al.*, 1985). This indication is supported further by the experiments with GA. This ansamycin antibiotic has antitumour activity (Supko *et al.*, 1995). It specifically binds and inhibits hsp90, a ubiquitously expressed component of the cellular protein folding machinery involved in regulating many proteins such as steroid receptors and signalling proteins (Rutherford and Zuker, 1994; Pratt and Toft, 1997). Raf-1 is a member of a multimolecular complex that includes hsp90 (Schulte *et al.*, 1995, 1996). It has been reported that GA interferes with this complex, causes loss of Ras–Raf-1 association and Raf-1 activity, blocking the downstream MEK/MAP kinases, and also prevents phorbol ester-induced proliferation (Schulte *et al.*,

1995, 1996). We now observe that steroids induce Ras–Raf-1 association and GA disrupts this association and prevents stimulation of Erk-2 activity as well as the cell cycle progression of steroid-stimulated cells.

PD98059 is a synthetic compound acting *in vitro* and *in vivo* as a selective inhibitor of MEK (Alessi *et al.*, 1995; Dudley *et al.*, 1995; Pang *et al.*, 1995). Therefore, it has been used to elucidate the role of the MAP kinase cascade in a variety of biological responses (Khwaya *et al.*, 1998; Yen *et al.*, 1998) including the insulin-like growth factor-1 (IGF-1)-stimulated mitogenic signalling in MCF-7 cells (Dufourny *et al.*, 1997), the heregulin-induced cell cycle progression in T47D cells (Fiddes *et al.*, 1998) and Fos induction by oestradiol in SK-N-SH cells (Watters *et al.*, 1997). PD98059 inhibits activation of Erk-2 and DNA synthesis stimulated by steroids. p38/HOG, like Erk_s, is a member of the MAP kinase family. Its inhibitor, SB203580 (Lee *et al.*, 1994; Cuenda *et al.*, 1995), does not affect Erk-2 activity and the DNA synthesis stimulation by steroids. This indicates that p38/HOG, in contrast to Erk_s, has no role in these processes. Furthermore, the recent observation that PD98059 inhibits cyclooxygenase might suggest that this effect is involved in the inhibitory effect of PD98059 on steroid stimulation of DNA synthesis. This possibility is excluded by the finding that SB203580, which also inhibits the cyclooxygenase activity (Borsch-Haubold *et al.*, 1998), does not modify the steroid-stimulated S-phase entry. The findings observed using anti-Ras mAb Y259, GA and PD98059 indicate that the Ras-dependent Raf/Mek/Erk_s cascade activation by steroids has a critical role in committing the cells to S-phase entry.

The requirement for ER and PgR for S-phase entry is shown by the inhibitory effect of antagonists on steroid-stimulated DNA synthesis of target cells. It is supported further by the lack of oestradiol stimulation of S-phase entry in the ER-negative MDA-MB231 cells. These findings, together with the inhibition by antagonists of the Src/Ras/Erk_s pathway in target cells (Migliaccio *et al.*, 1996, 1998) as well as the lack of oestradiol activation of the same pathway in MDA-MB231 cells, indicate that steroid receptors are needed for S-phase entry through Src/Ras/Erk_s pathway activation.

Such a requirement is reinforced further by the findings that NIH 3T3 fibroblasts become oestradiol responsive, in terms of DNA synthesis, only when engineered to express the wild-type ER α . Interestingly, they enter S-phase, upon oestradiol stimulation, even when engineered to express a transcriptionally inactive form of ER (Δ 250–303). In addition, in the NIH 3T3 fibroblasts, the stimulation of S-phase entry by oestradiol, mediated by either the wild-type ER α or the transcriptionally inactive ER, is inhibited, as in MCF-7 cells, by co-expression of Src K⁻ as well as by PD98059. This observation clearly indicates that the Src/Ras/Erk_s pathway is engaged by oestradiol to transmit its mitogenic signal even in very different cellular contexts.

In contrast to the view that ER-mediated transcription effects play a key role in the proliferative mechanism of oestradiol in target cells (Loose-Mitchell *et al.*, 1988; Weisz and Bresciani, 1993), our findings strongly indicate that the transcriptional activity of the ER is not needed for S-phase entry.

The present report shows that very early, non-nuclear

events are responsible for steroid-induced cell cycle progression, stating a new view of the proliferative mechanism of steroids. Moreover, it offers new hints for alternative strategies in the therapy of human breast cancer, suggesting the use of compounds targeting the Src/Ras/Erk_s pathway.

Materials and methods

Constructs

The cDNAs coding for the wild-type (HEGO) and the mutant (HE241G) form of ER were cloned into the pSG5 expression vector as described (Tora *et al.*, 1989; Ylikomi *et al.*, 1992). ERE-tk-CAT was constructed as described (Truss *et al.*, 1991). cDNAs coding for both the wild-type and kinase-inactive forms of Src were cloned into pSG5 as described (Twamley-Stein *et al.*, 1993; Roche *et al.*, 1995; Barone and Courtneidge, 1996).

Cell culture and microinjection techniques

All reagents for cell culture media were from Gibco (USA). Human breast cancer MCF-7 cells were grown at 37°C in a 5% CO₂ in air atmosphere in DMEM supplemented with phenol red, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), gentamicin (50 µg/ml), insulin (6 ng/ml), hydrocortisone (3.75 ng/ml) and 5% fetal calf serum (FCS). Human breast cancer T47D cells were grown at 37°C in 5% CO₂ in RPMI-1640 medium supplemented with phenol red, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), gentamicin (50 µg/ml), insulin (0.02 U/ml) and 10% FCS. Human breast cancer MDA-MB231 cells were grown at 37°C in DMEM supplemented with phenol red, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), gentamicin (50 µg/ml), insulin (0.02 U/ml) and 10% FCS. For S-phase entry analysis, MDA-MB231 cells were seeded onto gelatine-pre-coated glass coverslips and grown to 40–50% confluence. Prior to the experiments, the medium was changed to DMEM lacking phenol red and serum. Cells were left in this medium for 24 h, then unstimulated or stimulated for 24 h either with 10 nM oestradiol (Sigma) in the absence or presence of ICI 182.780 (1 µM), or with DMEM containing 10% serum. The DNA synthesis was monitored by adding 100 µM (final concentration) BrdU (Sigma) to the medium. The cells were then fixed for immunostaining. For microinjection experiments, MCF-7 and T47D cells were seeded onto gelatine-pre-coated glass coverslips and grown to 40–50% confluence. Prior to the experiments, the cells were passaged in the same media lacking phenol red and containing charcoal-stripped FCS prepared as previously described (Di Domenico *et al.*, 1996). Cells were left in these media for 3–4 days. Purified plasmids (50 ng/µl) were injected into cell nuclei, and purified antibodies (either 1.3 or 3.3 µg/µl) were injected into cell cytoplasm by a semi-automated (Eppendorf) microinjection system as described (Barone *et al.*, 1994; Barone and Courtneidge, 1996). Azide-free rat monoclonal antibodies against v-H-ras (259; Santa Cruz Biotechnology Inc., CA) were diluted in phosphate-buffered saline (PBS), and purified rabbit IgGs (Sigma) were included as injection marker. Before injection, the antibody mixture was centrifuged for 10 min. Purified rabbit IgGs were injected alone, as a control. The empty plasmid pSG5 (25 ng/µl) was injected into cell nuclei together with 25 ng/µl of GFP expression vector (pEGFP from Clontech, CA), to help the identification of microinjected cells. In some experiments, pEGFP (25 ng/µl) was injected together with Src K⁻-expressing plasmid (25 ng/µl). Oestradiol or R5020 (Roussel-Uclaf, France) were added 6 h after injection of cDNA or immediately after injection of antibodies, and DNA synthesis monitored by adding 100 µM (final concentration) BrdU to the medium. The cells were incubated at 37°C for another 24 h, then fixed for immunostaining.

Transfection of NIH 3T3 fibroblasts and CAT activity assay

Cells were cultured as described (Barone and Courtneidge, 1996). For immunofluorescence experiments, they were seeded onto glass coverslips, grown at 60–70% confluence, then transfected using the SuperFect Transfection Reagent (Qiagen, GmbH) according to the manufacturer's instructions. Three µg of pSG5-HEGO or pSG5-HE241G plasmids were transfected together with 1 µg of pEGFP, to help the identification of transfected cells. The pSG5 empty plasmid was used as a control. The ratio of either pEGFP/ER or pEGFP/Δ250–303 ER co-transfected cells was determined by the immunofluorescence staining of ER or its mutant form as reported below. It showed that ~90% of the cells expressing pEGFP were positive for either ER or Δ250–303 ER. When indicated,

3 µg of pSG5-HEGO or pSG5 HE241G plasmids were co-transfected with 6 µg of Src K⁻-encoding plasmid. Quantification from co-transfection experiments was assessed by immunofluorescence staining as reported below. It showed that >90% of Src K⁻-expressing cells were positive for ER or its mutant. Twelve hours after transfection, the medium was changed to phenol red-free DMEM containing 0.5% charcoal-stripped FCS, 0.5 µg/ml transferrin, 5 µg/ml insulin and antibiotics. Fibroblasts were left quiescent for 30 h, then unstimulated or stimulated by 10 nM oestradiol. When indicated, 1 µM anti-estrogen ICI 182.780 (from Zeneca, Italy) was added to the medium and DNA synthesis monitored by addition of 100 µM BrdU. The cells were incubated at 37°C for an additional 24 h and finally fixed for immunostaining. For ERE-tk-CAT activity, NIH 3T3 cells were plated at subconfluence in phenol red-free DMEM containing 10% charcoal-stripped FCS, then transfected by the SuperFect Transfection Reagent with 1.5 µg of HEGO or HE241G, alone or together with 1.5 µg of ERE-tk-CAT. Either the pSG5 empty plasmid or the ERE-tk-CAT (both at 3 µg) were transfected alone, as controls. In each case, 1 µg of the β-galactosidase expression vector (RSV-βGAL) was co-transfected as an internal control. After 12 h, the medium was changed to DMEM containing 0.5% charcoal-stripped FCS, 0.5 µg/ml transferrin, 5 µg/ml insulin and antibiotics. The NIH 3T3 fibroblasts were untreated or treated with 10 nM oestradiol, and 3 h later they were lysed for CAT activity assay. The lysis procedure as well as the CAT activity assay of cell extracts were performed using the CAT ELISA kit (from Boehringer Mannheim) according to the manufacturer's instructions. The CAT activity was calculated relative to β-galactosidase activity as described (Hall *et al.*, 1983).

Immunofluorescence

Cells on coverslips were washed once with PBS, fixed for 20 min with paraformaldehyde (3%, w/v in PBS), permeabilized for 5 min with Triton X-100 (0.2%, v/v in PBS) and incubated for 1 h with PBS containing FCS (1%, v/v). For Src detection, coverslips were stained by incubation with mouse anti-Src mAbs (clone 327 from Oncogene Science Inc., Manahasset, NY) diluted 1:100 in PBS for 45 min followed by three washings with PBS. Coverslips were then incubated in Texas red-conjugated goat anti-mouse antibody (Calbiochem, CA) diluted 1:200 in PBS. DNA synthesis was analysed by coverslip incubation for 10 min with HCl (1.5 M in PBS) followed by three washings in PBS. Coverslips finally were incubated with diluted (1:1 in PBS) fluorescein-conjugated mouse anti-BrdU mAbs (clone BMC 9318 from Boehringer Mannheim Co., IN). For the control plasmid pSG5, coverslips were incubated for 10 min with HCl (1.5 M in PBS) followed by three washings in PBS. Coverslips were then incubated with diluted (1:10 in PBS) mouse anti-BrdU mAbs (clone BMC9318 from Boehringer Mannheim), then washed three times with PBS. Mouse antibody was detected using diluted (1:200 in PBS) Texas red-conjugated goat anti-mouse antibodies (Calbiochem). Injected cells were visualized by green fluorescence from EGFP. For antibody injections, rabbit antibody was detected using a diluted (1:200 in PBS) Texas red-conjugated anti-rabbit antibody (Sigma) followed by acid treatment and BrdU detection, as described above. For the pEGFP/ER or pEGFP/Δ250–303 ER ratio, detection of either ER or Δ250–303 ER overexpressed in NIH 3T3 fibroblasts was performed as follows. Coverslips were incubated for 1 h with PBS containing FCS (1%, v/v), bovine serum albumin (BSA; 0.2% w/v) and sodium azide (0.2% w/v) followed by three washings with PBS. Coverslips were then stained by incubation with rat ER mAb (H222) diluted 1:20 in PBS containing 0.2% BSA and 0.2% sodium azide for 12 h followed by three washings in PBS. Coverslips were then incubated in diluted (1:100 in PBS containing 0.2% BSA) Texas red-conjugated affinity-pure or (for co-transfection experiments with SrcK⁻ in NIH 3T3 fibroblasts) fluorescein-conjugated anti-rat IgG (Jackson ImmunoResearch Laboratories; West Grove, PA) for 45 min followed by three washings in PBS. DNA synthesis was analysed by coverslip incubation for 10 min in HCl (1.5 M in PBS), followed by three washings in PBS. Coverslips finally were incubated with diluted (1/10 in PBS) mouse anti-BrdU mAbs, then washed three times with PBS. Mouse antibody was detected using diluted (1:200 in PBS) Texas red-conjugated goat anti-mouse antibodies (Calbiochem). All coverslips were washed three times in PBS, incubated for 10 min with PBS containing Hoechst 33258 (Sigma) at a final concentration of 1 µg/ml and finally washed three times with PBS. The coverslips were inverted and mounted in Moviol (Calbiochem, CA) on glass slides. Slides were analysed using an Axiophot fluorescent microscope (Zeiss). Significant fields were captured and processed using a KS300 system (Zeiss).

Raf-1 kinase activity assay

Raf-1 kinase activity was assayed using cell lysates prepared in lysis buffer as described (Migliaccio *et al.*, 1998) except using 1% NP-40 instead of 1% Triton X-100. Lysates were immunoprecipitated with anti-Raf-1 antibodies (clone C-12 from Santa Cruz) and immunocomplexes were washed once with lysis buffer, twice with 0.5 M LiCl, 0.1 M Tris-HCl pH 8 and finally with kinase buffer [25 mM HEPES pH 7.4, 25 mM glycerol-2-phosphate, 1 mM dithiothreitol (DTT), 10 mM MgCl₂, 10 mM MnCl₂]. The Raf kinase activity of the immunocomplexes was assayed using 20 µl of kinase buffer, which contained 5 µg of H1 histone (Boehringer Mannheim) dissolved at 1 mg/ml in 0.2 M NaCl, 0.1 mM EDTA, 1 mM DTT, 1 mM leupeptin, antipain and pepstatin, 20 mM Tris-HCl pH 7.4.

Immunoprecipitations and Src or Erk-2 kinase assays

Cells were lysed and lysates immunoprecipitated with either anti-Src or anti-Erk-2 antibodies, as described (Migliaccio *et al.*, 1996, 1998; Di Domenico *et al.*, 1996). Src and Erk-2 kinase activities were assayed using enolase and MBP as substrates, respectively, according to the same reports.

Electrophoresis and immunoblotting

Electrophoresis and immunoblotting were performed as described (Migliaccio *et al.*, 1998). Filters were probed either with anti-Raf-1 or with anti-Ras (v-H-ras, Y13-259 from Oncogene Science) antibodies as reported (Schulte *et al.*, 1996). Immunoreactive proteins were revealed using the ECL detection system (Amersham, UK).

Acknowledgements

We are grateful to Giovanni Santelli and Concetta Pietropaolo who supported this work with useful discussions and by allowing access to essential equipment in their respective laboratories. We also thank P.Chambon for the HEGO- and the HE241G-expressing plasmids, M.Beato for the ERE-tk-CAT reporter plasmid, and J.Johnson of the Developmental Therapeutics Program, National Cancer Institute (Rockville, MD) for the gift of geldanamycin. The H222 anti-ER monoclonal antibodies were kindly provided by Abbott Labs (Abbott Park, IL). The editorial assistance of Gian Michele La Placa as well as the technical assistance of Maria Lombardi, Flavia Vitale and Nicolino Valerio Dorrello are also acknowledged. This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro and Ministero dell'Universita e della Ricerca Scientifica (Fondi 40 and 60%). M.V.B. is the recipient of a Fondazione Italiana per la Ricerca sul Cancro scholarship.

References

Alessi,D.R.,Cuenda,A., Cohen,P., Dudley,D.T. and Saltiel,A.R. (1995) PD098059 is a specific inhibitor of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *J. Biol. Chem.*, **270**, 27489-27494.

Auricchio,F., Di Domenico,M., Migliaccio,A., Castoria,G. and Bilancio,A. (1995) The role of estradiol receptor in the proliferative activity of vanadate in MCF-7 cells. *Cell Growth Differ.*, **6**, 105-113.

Avruch,J., Zhang,X.F. and Kyriakis,J.M. (1994) Raf meets Ras: completing the framework of a signal transduction pathway. *Trends Biochem. Sci.*, **19**, 279-283.

Barone,M.V. and Courtneidge,S.A. (1996) Myc but not Fos rescue of PDGF signalling block caused by kinase-inactive Src. *Nature*, **378**, 509-512.

Barone,M.V., Crozat,A., Tabae,A., Philipson,L. and Ron,D. (1994) CHOP (GADD153) and its oncogenic variant, TLS-CHOP, have opposing effects on the induction of G₁/S arrest. *Genes Dev.*, **8**, 453-464.

Beato,M., Herrlich,P. and Schutz,G. (1995) Steroid-hormone receptors: many actors in search of a plot. *Cell*, **83**, 851-857.

Bogunski,M.S. and McCormick,F. (1993) Protein regulating Ras and its relatives. *Nature*, **366**, 643-654.

Borsch-Haubold,A.G., Pasquet,S. and Watson,S.P. (1998) Direct inhibition of cyclooxygenase-1 and -2 by the kinase inhibitors SB203580 and PD98059. SB203580 also inhibits thromboxane synthase. *J. Biol. Chem.*, **273**, 28766-28772.

Boyer,B., Roche,S., Denoyelle,M. and Thierry,J.P. (1997) Src and Ras are involved in separate pathways in epithelial cell scattering. *EMBO J.*, **16**, 5904-5913.

Campbell,S.L., Khosravi-Far,R., Rossman,K.L., Clark,G.J. and Der,C.J.

(1998) Increasing complexity of Ras signaling. *Oncogene*, **17**, 1395-1413.

Cantley,L.C., Auger,K.R., Carpenter,C., Duckworth,B., Graziani,A., Kapeller,R. and Soltoff,S. (1991) Oncogenes and signal transduction. *Cell*, **64**, 281-302.

Chalbos,D., Vignon,F., Keidar,I. and Rochefort,H. (1982) Estrogens stimulate cell proliferation and induce secretory proteins in a human breast cancer cell line (T47D). *J. Clin. Endocrinol. Metab.*, **55**, 276-283.

Coutts,A.S. and Murphy,L.C. (1998) Elevated mitogen-activated protein kinase activity in estrogen-nonresponsive human breast cancer cells. *Cancer Res.*, **58**, 4071-4074.

Cuenda,A., Rouse,J., Doza,Y.N., Meier,R., Cohen,P., Gallagher,T.F., Young,P.R. and Lee,J.C. (1995) SB203580 is a specific inhibitor of a MAP-kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.*, **364**, 229-233.

Davidson,N.E., Gelmann,E.P., Lippman,M.E. and Dickson,R.B. (1987) Epidermal growth factor receptor gene expression in estrogen receptor-positive and negative human breast cancer cell lines. *Mol. Endocrinol.*, **1**, 216-223.

Davis,R.G. (1993) The mitogen-activated protein kinase signal transduction pathway. *J. Biol. Chem.*, **268**, 14553-14556.

Di Domenico,M., Castoria,G., Bilancio,A., Migliaccio,A. and Auricchio,F. (1996) Estradiol activation of human colon carcinoma-derived Caco-2 cell growth. *Cancer Res.*, **56**, 4516-4521.

Dickson,R. and Lippman,M.E. (1995) Growth factors in breast cancer. *Endocr. Rev.*, **16**, 559-589.

Downward,J. (1997) Cell cycle: routine role for Ras. *Curr. Biol.*, **7**, 258-260.

Dudley,D.T., Pang,L., Decker,S.J., Bridges,A.J. and Saltiel,A.R. (1995) A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl Acad. Sci. USA*, **92**, 7686-7689.

Dufourny,B., Alblas,J., van Teeffelen,H.A.A.M., van Schaik,F.M.A., van der Burg,B., Steenbergh,P.H. and Sussenbach,J.S. (1997) Mitogenic signalling of insulin-like growth factor I in MCF-7 human breast cancer cells requires phosphatidylinositol 3-kinase and is independent of mitogen-activated protein kinase. *J. Biol. Chem.*, **272**, 31163-31171.

Evans,R.M. (1998) The steroid and thyroid hormone receptor superfamily. *Science*, **240**, 889-895.

Fantl,V., Stamp,G., Andrews,A., Rosewell,I. and Dickson,C. (1995) Mice lacking cyclin D1 are small and show defects in eye and mammary gland development. *Genes Dev.*, **9**, 2364-2372.

Fiddes,R.J., Janes,P.W., Sivertsen,S.P., Sutherland,R.L., Musgrove,E.A. and Daly,R.J. (1998) Inhibition of the MAP kinase cascade blocks heregulin-induced cell cycle progression in T-47D human breast cancer cells. *Oncogene*, **16**, 2803-2813.

Filmus,J., Robles,A.I., Shi,W., Wong,M.J., Colombo,L.L. and Conti,C.J. (1994) Induction of cyclin D1 overexpression by activated ras. *Oncogene*, **9**, 3627-3633.

Gorski,J. (1997) Do we need new paradigms? *Endocr. News*, **22**, 4-12.

Guy,C.T., Muthuswamy,S.K., Cardiff,R.D., Soriano,P. and Muller,W.J. (1994) Activation of the c-src kinase is required for the induction of mammary tumors in transgenic mice. *Genes Dev.*, **8**, 23-32.

Hall,C.V., Jacob,P.E., Ringold,G.M. and Lee,F. (1983) Expression and regulation of *Escherichia coli lacZ* gene fusions in mammalian cells. *J. Mol. Appl. Genet.*, **2**, 101-110.

Horwitz,K.B., Mockus,M.B. and Lessey,B.A. (1982) Variant T47D human breast cancer cells with high progesterone-receptor levels despite estrogen and antiestrogen resistance. *Cell*, **28**, 633-642.

Kasid,A. and Lippman,M.E. (1987) Estrogen and oncogene mediated growth regulation of human breast cancer cells. *J. Steroid Biochem.*, **27**, 465-470.

Kerkhoff,E. and Rapp,U.R. (1998) Cell cycle targets of Ras/Raf signalling. *Oncogene*, **17**, 1457-1462.

Khwaya,A., Lehmann,K., Marte,B.M. and Downward,J. (1998) Phosphoinositide 3-kinase induces scattering and tubulogenesis in epithelial cells through a novel pathway. *J. Biol. Chem.*, **273**, 18793-18801.

Korach,K.S. (1994) Insights from the study of animals lacking functional estrogen receptor. *Science*, **266**, 1524-1527.

Lee,J.C. *et al.* (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature*, **372**, 739-746.

Levenson,A.L. and Jordan V.C. (1997) MCF-7: the first hormone-responsive breast cancer cell line. *Cancer Res.*, **57**, 3071-3078.

- Loose-Mitchell,D.S., Chiappetta,C. and Stancel,G.M. (1998) Estrogen regulation of c-fos messenger ribonucleic acid. *Mol. Endocrinol.*, **2**, 946–951.
- Lubahn,D.B., Moyer,J.S., Golding,T.S., Couse,J.F., Korach,K.S. and Smithies,O. (1993) Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc. Natl Acad. Sci. USA*, **90**, 11162–11166.
- Lydon,J.P., De Maio,F.J., Funk,C.R., Mani,S.K., Hughes,A.R., Montgomery,C.A., Jr, Shyamala,G., Conneely,O.M. and O'Malley, B.W. (1995) Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes Dev.*, **8**, 2266–2278.
- Mangelsdorf,D.J. et al. (1995) The nuclear receptor superfamily: the second decade. *Cell*, **83**, 835–839.
- Marshall,C.J. (1994) MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. *Curr. Opin. Genet. Dev.*, **4**, 82–89.
- Marshall,C.J. (1996) Ras effectors. *Curr. Opin. Cell Biol.*, **8**, 197–204.
- Migliaccio,A., Pagano,M. and Auricchio,F. (1993) Immediate and transient stimulation of protein tyrosine phosphorylation by estradiol in MCF-7. *Oncogene*, **8**, 2183–2191.
- Migliaccio,A., Di Domenico,M., Castoria,G., de Falco,A., Bontempo,P., Nola,E. and Auricchio,F. (1996) Tyrosine kinase/p21^{ras}/MAP-kinase pathway activation by estradiol–receptor complex in MCF-7 cells. *EMBO J.*, **15**, 1292–1300.
- Migliaccio,A., Piccolo,D., Castoria,G., Di Domenico,M., Bilancio,A., Lombardi,M., Gong,W., Beato,M. and Auricchio,F. (1998) Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen. *EMBO J.*, **17**, 2008–2018.
- Mulcahy,L.S., Smith,M.R. and Stacey,D.W. (1985) Requirement for *ras* proto-oncogene function during serum-stimulated growth of NIH3T3 cells. *Nature*, **313**, 241–243.
- Musgrove,E.A., Hamilton,J.A., Lee,C.S., Sweeney,K.J., Watts,C.K. and Sutherland,R.L. (1993) Growth factor, steroid and steroid antagonist regulation of cyclin gene expression associated with changes in T-47D human breast cancer cell cycle progression. *Mol. Cell. Biol.*, **13**, 3577–3587.
- Ottenhoff-Kalff,A.E., Rijkse,G., van Beurden,E.A.C.M., Hennipman,A., Michels,A.A. and Staal,G.E.J. (1992) Characterization of protein tyrosine kinases from human breast cancer: involvement of the *c-src* oncogene product. *Cancer Res.*, **52**, 4773–4778.
- Pang,L., Sawada,T., Decker,S.J. and Saltiel,A.R. (1995) Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor. *J. Biol. Chem.*, **270**, 13585–13588.
- Parker,M.G. and White,R. (1996) Nuclear receptors spring into action. *Nature Struct. Biol.*, **3**, 113–115.
- Pietras,R.J. et al. (1995) HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells. *Oncogene*, **10**, 2435–2446.
- Planas-Silva,M.D. and Weinberg,R.A. (1997) Estrogen-dependent cyclinE–cdk2 activation through p21 redistribution. *Mol. Cell. Biol.*, **17**, 4059–4069.
- Pratt,W.B. and Toft,D.O. (1997) Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr. Rev.*, **18**, 306–360.
- Roche,S., Koegl,M., Barone,M.V., Roussel,M.F. and Courtneidge,S.A. (1995) DNA synthesis induced by some but not all growth factors requires Src family protein tyrosine kinases. *Mol. Cell. Biol.*, **15**, 1102–1109.
- Rodriguez-Viciano,P., Warne,P., Khwaja,A., Marte,B.M., Pappin,D., Das,P., Waterfield,M.D., Ridley,A. and Downward,J. (1997) Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. *Cell*, **89**, 457–467.
- Rosen,N., Bolen,J., Schwartz,A.M., Cohen,P., De Seau,V. and Israel,M.A. (1986) Analysis of pp60 c-src protein kinase activity in human tumor cell line and tissues. *J. Biol. Chem.*, **261**, 13754–13759.
- Rutherford,S.L. and Zuker,C.S. (1994) Protein folding and the regulation of signaling pathways. *Cell*, **79**, 1129–1132.
- Schlessinger,J. (1993) How receptor tyrosine kinases activate *ras*. *Trends Biochem. Sci.*, **18**, 273–275.
- Schulte,T.W., Blagosklonny,M.V., Ingui,C. and Neckers,L. (1995) Disruption of the raf-1 hsp90 molecular complex results in destabilization of raf-1 and loss of raf-1-ras association. *J. Biol. Chem.*, **270**, 24585–24588.
- Schulte,T.W., Blagosklonny,M.V., Romanova,L., Mushinski,J.F., Monia,B.P., Johnston,J.F., Nguuyen,P., Trepel,J. and Neckers,L.M. (1996) Destabilization of raf-1 by geldanamycin leads to disruption of the raf-1–MEK–mitogen-activated protein kinase signalling pathway. *Mol. Cell. Biol.*, **16**, 5839–5845.
- Sicinski,P. et al. (1995) Cyclin D1 provides a link between development and oncogenesis in the retina and breast. *Cell*, **82**, 621–630.
- Smith,M.R., DeGubius,S.J. and Stacey,D.W. (1986) Requirement for c-ras proteins during viral oncogene transformation. *Nature*, **320**, 540–543.
- Supko,J.G., Hickman,R.L., Grever,M.R. and Malspeis,L. (1995) Preclinical pharmacologic evaluation of geldanamycin as an antitumor agent. *Cancer Chemother. Pharmacol.*, **36**, 305–315.
- Tora,L., Mullick,A., Metzger,D., Ponglikitmongkol,M., Park,I. and Chambon,P. (1989) The cloned human oestrogen receptor contains a mutation which alters its hormone binding properties. *EMBO J.*, **8**, 1981–1986.
- Truss,M., Chalepakis,G., Slater,E.P., Mader,S. and Beato,M. (1991) Functional interaction of hybrid response elements with wild-type and mutant steroid hormone receptors. *Mol. Cell. Biol.*, **11**, 3247–3258.
- Twamley-Stein,G.M., Pepperkok,R., Ansoorge,A. and Courtneidge,S.A. (1993) The Src family tyrosine kinases are required for platelet-derived growth factor-mediated signal transduction in NIH3T3 cells. *Proc. Natl Acad. Sci. USA*, **90**, 7696–7700.
- Vignon,F., Bouton,M.M. and Rochefort,H. (1987) Antiestrogens inhibit the mitogenic effect of growth factors on breast cancer cells in the total absence of estrogens. *Biochem. Biophys. Res. Commun.*, **146**, 1502–1508.
- Watters,J.J., Campbell,J.S., Cunningham,M.J., Krebs,E.G. and Dorsa,D.M. (1997) Rapid membrane effects of steroids in neuroblastoma cells: effects of estrogen on mitogen activated protein kinase signalling cascade and c-fos immediate early gene transcription. *Endocrinology*, **138**, 4030–4033.
- Weisz,A. and Bresciani,F. (1993) Estrogen regulation of proto-oncogenes coding for nuclear proteins. *Crit. Rev. Oncogen.*, **4**, 361–388.
- Yen,A., Roberson,M.S., Varvayanis,S. and Lee,A.T. (1998) Retinoic acid induced mitogen-activated protein (MAP) extracellular signal-regulated kinase (ERK) kinase-dependent MAP kinase activation needed to elicit HL-60 cell differentiation and growth arrest. *Cancer Res.*, **58**, 3163–3172.
- Ylikomi,T., Bocquel,M.T., Berry,M., Gronemeyer,H. and Chambon,P. (1992) Cooperation of proto-signals for nuclear accumulation of estrogen and progesterone receptors. *EMBO J.*, **11**, 3681–3694.

Received November 25, 1998; revised and accepted March 2, 1999