

Tyrosine kinase/p21^{ras}/MAP-kinase pathway activation by estradiol–receptor complex in MCF-7 cells

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The mechanism by which estradiol acts on cell multiplication is still unclear. Under conditions of estradiol-dependent growth, estradiol treatment of human mammary cancer MCF-7 cells triggers rapid and transient activation of the mitogen-activated (MAP) kinases, erk-1 and erk-2, increases the active form of p21^{ras}, tyrosine phosphorylation of Shc and p190 protein and induces association of p190 to p21^{ras}-GAP. Both Shc and p190 are substrates of activated *src* and once phosphorylated, they interact with other proteins and upregulate p21^{ras}. Estradiol activates the tyrosine kinase/p21^{ras}/MAP-kinase pathway in MCF-7 cells with kinetics which are similar to those of peptide mitogens. It is only after introduction of the human wild-type 67 kDa estradiol receptor cDNA that Cos cells become estradiol-responsive in terms of erk-2 activity. This finding, together with the inhibition by the pure anti-estrogen ICI 182 780 of the stimulatory effect of estradiol on each step of the pathway in MCF-7 cells proves that the classic estradiol receptor is responsible for the transduction pathway activation. Transfection experiments of Cos cells with the estradiol receptor cDNA and *in vitro* experiments with *c-src* show that the estradiol receptor activates *c-src* and this activation requires occupancy of the receptor by hormone. Our experiments suggest that *c-src* is an initial and integral part of the signaling events mediated by the estradiol receptor.

Keywords: *c-src*/estradiol/estradiol receptor/MAP-kinases/p21^{ras}

Introduction

Steroid hormones regulate differentiation and proliferation of different cell types. Their receptors act as ligand-inducible transcriptional enhancer factors (Evans, 1988; Green and Chambon, 1988; Beato, 1989; Carson-Jurica *et al.*, 1990; Jensen, 1991). In addition, steroids produce rapid, non-transcriptional responses which in some cases are similar to those evoked by peptide growth factors. Vitamin D rapidly activates phospholipase C of intact enterocytes (Lieberherr *et al.*, 1989) and stimulates MAP-kinase activity of hepatic Ito cells (Benito *et al.*, 1995). Progesterone directly causes an increase of oxytocin receptor binding in the pos-

terior ventromedial hypothalamus (Schumaker *et al.*, 1990); it also rapidly stimulates calcium influx (Blackmore *et al.*, 1990) and induces tyrosine phosphorylation of a protein in sperm (Mendoza *et al.*, 1995). Estrogen and progesterone immediately affect calcium uptake by the myometrium (Batra, 1986). Estrogen induces a rapid release of intracellular calcium in granuloma cells (Morley *et al.*, 1992) and stimulation in different cells of adenylate cyclase and c-AMP-regulated gene transcription (Aronica *et al.*, 1994). These and other non-genomic short-term effects suggest interaction of estradiol and other steroids with cell-surface receptors. The nature of this receptor is debated. Some evidence supports the existence of a unique membrane receptor, other evidence favors the identity or the strong similarity between the receptor which stimulates transcriptional activity and those responsible for the non-genomic action (Pietras and Szego, 1979; Bression *et al.*, 1986; Nemere *et al.*, 1994; Pappas *et al.*, 1995).

MAP-kinases belong to a serine–threonine kinase cascade which is activated by different mitogens and phosphorylate key intracellular enzymes and transcription factors (Charest *et al.*, 1993; Davis, 1993). p21^{ras} regulates the MAP-kinase pathway. An increase of the active form of p21^{ras} invariably leads to an increase in the activity of the serine–threonine kinase cascade. p21^{ras} is an important molecular switch activated by receptor and non-receptor tyrosine kinases (Bogunski and McCormick, 1993; Schlessinger, 1993) through phosphorylation of proteins such as Shc (Egan *et al.*, 1993) and p21^{ras}-GAP associated p190 (Chang *et al.*, 1993). We now observe that estradiol rapidly and transiently activates MAP-kinases erk-1 and erk-2 and increases the level of GTP-bound p21^{ras}, the active form of this small protein, in MCF-7 cells. It also immediately induces tyrosine phosphorylation of Shc and p190 protein and association of p190 to p21^{ras}-GAP. Evidence based on experiments with Cos cells transfected with the estradiol receptor cDNA (HEGO) is presented, showing that the classic receptor is required for MAP-kinase activation. The role of this receptor is independently proved by the finding that each step of the transduction signal pathway stimulated by estradiol in MCF-7 cells is inhibited by the pure anti-estrogen ICI 182 780. Estradiol, in complex with its receptor, immediately activates *c-src* in Cos-cell transfected with receptor cDNA, stimulates *c-src* immunoprecipitated from MCF-7 cell lysate and interacts *in vitro* with cytosol estradiol receptor. *c-src* could be the initial target of the estradiol–receptor complex in the process leading to activation of tyrosine phosphorylation/p21^{ras}/MAP-kinase pathway.

Results

Effect of estradiol on MAP-kinase (erk-1 and erk-2) activity of MCF-7 cells

MCF-7 cells were maintained for a week in the absence of phenol-red, a substance with a weak estrogenic activity

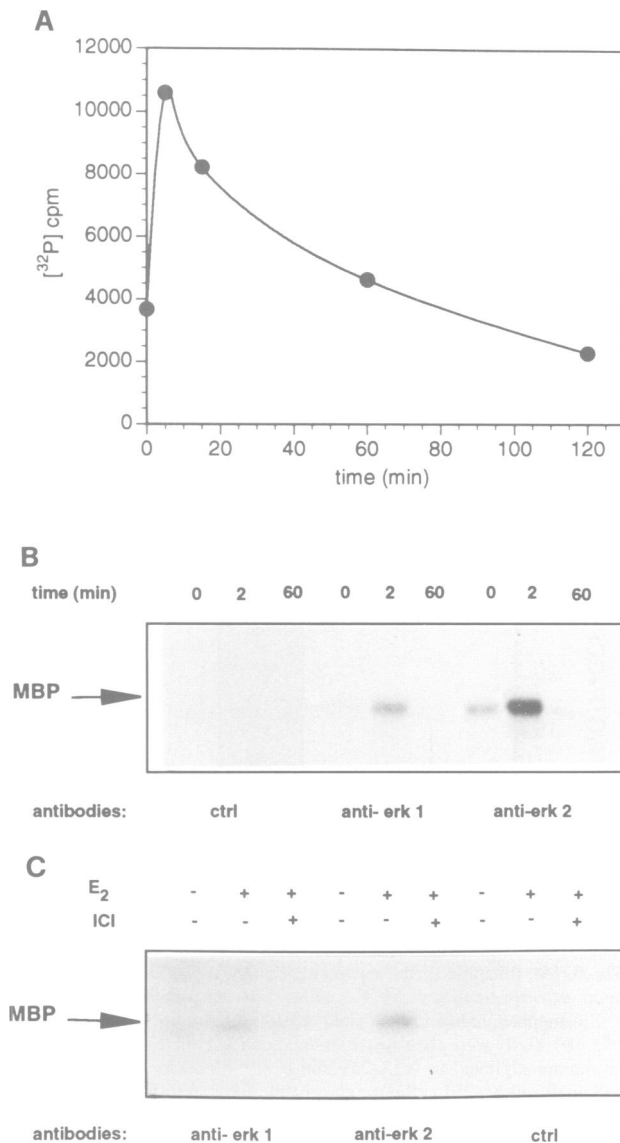


Fig. 1. Activation of MAP-kinase(s) in estrogen-treated MCF-7 cells. (A) MCF-7 cells were untreated or treated for indicated times with 10 nM 17 β -estradiol. The cells were lysed and lysate aliquots were used to assay MAPK activity on MBP. ³²P counts incorporated in MBP were assayed on glass filters. The values are expressed as a difference between samples \pm MBP. (B) MCF-7 cells were untreated or treated for indicated times with 10 nM 17 β -estradiol. The cells were lysed and lysates immunoprecipitated using either control antibodies (ctrl) or anti-erk-1 or anti-erk-2 antibodies. (C) MCF-7 cells were untreated or treated for 2 min with 10 nM 17 β -estradiol either in the absence (E₂) or in the presence of 10 μ M anti-estrogen ICI 182 780 (ICI). Lysates were immunoprecipitated by using anti-erk-1, anti-erk-2 or control antibodies (ctrl). The immunoprecipitates (B and C) were assayed for MAP-kinase activity using MBP. Phosphorylated proteins were resolved on 15% SDS-PAGE and revealed by autoradiography. The arrows indicate the position of MBP.

(Berthois *et al.*, 1986a), and in presence of charcoal-treated serum. Under these conditions cell growth is estradiol dependent. The cells were left untreated or treated with 17 β -estradiol for 2, 15, 60 and 120 min, then lysed. Aliquots of cell lysates were incubated in the presence of [γ -³²P]ATP with or without MBP as described in Materials and methods (Figure 1A). A ~3-fold increase of MBP phosphorylation was observed in the samples containing lysates from cells treated with estradiol for 2 min. The

kinase activity was decreased after 15 min of estradiol treatment and declined towards the basal level after 60 min.

The MAP-kinase or extra-cellular signaling regulated kinase (ERK) family includes different members with a high degree of homology endowed with kinase activity on common substrates (Rossomando *et al.*, 1989; Thomas *et al.*, 1992). Therefore, it was of interest to establish which one of these kinases was responsive to estradiol. Lysates from MCF-7 cells untreated or treated with estradiol \pm ICI 182 780 anti-estrogen were immunoprecipitated using two different monoclonal antibodies specifically raised against either erk-1 or erk-2 kinases. Figure 1B and C shows that both immunoprecipitates from 2 min estradiol-treated cells were more active in phosphorylating MBP than the corresponding precipitates from untreated cells. The hormonal stimulation was far more evident for erk-2 than for erk-1. No MBP phosphorylation was observed by control antibody immunoprecipitates. The effect of estradiol on both erk-1 and erk-2 was rapid and transient. The kinase activity of erk-1 as well as that of erk-2 was clearly stimulated at 2 min and declined to basal level at 60 min (Figure 1B). The 2 min estradiol stimulation of erk-1 and erk-2 was inhibited by ICI 182 780 (Figure 1C). In the experiment presented in Figure 1C about a third of the cells utilized in the experiment in B were employed.

Estradiol-dependent activation of erk-2 in Cos-7 cells overexpressing estradiol receptor

Cos-7 cells lack estrogen receptor. They were transfected with pSG1-HEG0 plasmid encoding the whole human estrogen receptor open reading frame (Tora *et al.*, 1989). In parallel, cells were also transfected with pTR-B0 control plasmid. After 72 h, cells were treated with estradiol for 2 min, in the absence or presence of one of two different non-steroidal anti-estrogens: the pure anti-estrogen ICI 182 780 and the partially agonist hydroxy-tamoxifen, in two different experiments. Erk-2 was immunoprecipitated from cell lysates by anti-erk-2 antibodies and its MAP-kinase activity measured as MBP phosphorylation. Figure 2 shows that erk-2 kinase activity is very low or absent in Cos-7 cells which do not express estrogen receptor and the level of this activity is unaffected by estradiol treatment. In contrast, when the cells were transfected with estrogen receptor-encoding plasmid, the kinase activity was increased upon estradiol treatment and was reduced to the basal levels when the hormone was added in the presence of ICI 182 780 (Figure 2A) or OH-tamoxifen (Figure 2B). The stimulation by estradiol of erk-2 kinase activity was confirmed when control Cos cells were transfected with pSG1 plasmid instead of pTR-B0 (not shown). These experiments show that occupancy of classic estradiol receptor by the hormone is required to confer estradiol responsiveness of erk-2 to Cos cells.

Increase by estradiol of GTP-bound p21^{ras}

We have analyzed the guanine nucleotides bound to p21^{ras} in [³²P]orthophosphate-labeled MCF-7 cells either untreated or treated for 2 min with 10 nM estradiol, \pm the pure anti-estrogen, ICI 182 780. Figure 3 shows that the amount of GTP bound to p21^{ras} is increased upon estradiol treatment of cells and this increase is abolished by addition of ICI 182 780 (Figure 3A). The amount of

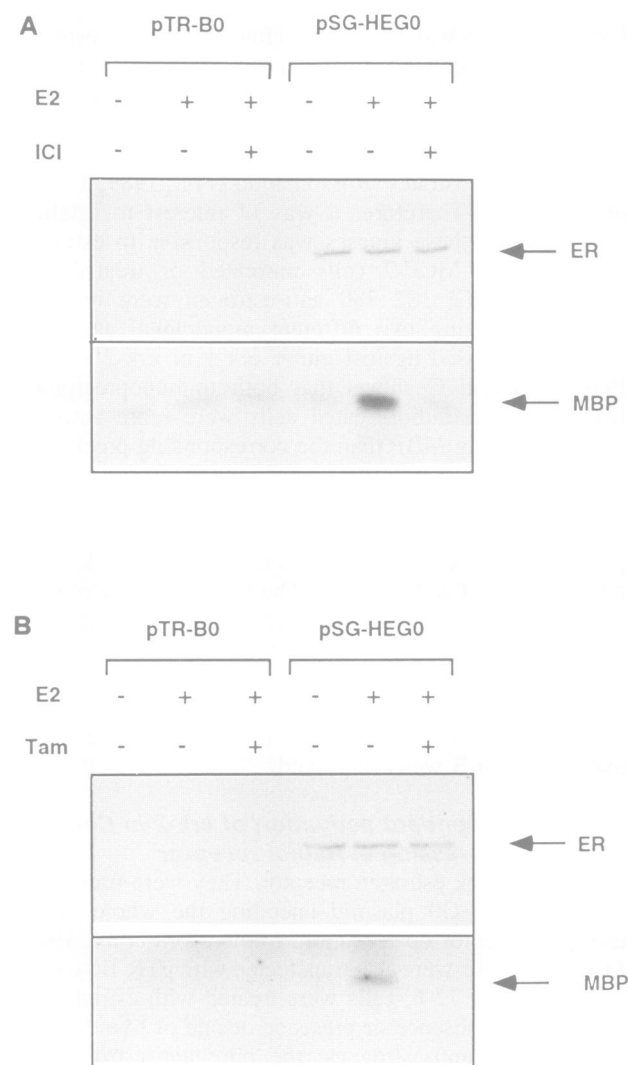


Fig. 2. Activation of erk-2-kinase upon estrogen treatment in Cos-7 cells transfected with human estradiol receptor cDNA. (A) Cos-7 cells were transfected with pTR-B0 control plasmid or with pSG1-HEG0 plasmid encoding the wild-type estrogen receptor ORF. Cells, expressing or not expressing the estrogen receptor, were untreated or treated for 2 min with 10 nM 17 β -estradiol \pm 10 μ M ICI 182 780 (ICI). The cells were lysed and lysates immunoprecipitated using anti-erk-2 antibodies. (B) Cells expressing or not expressing the estrogen receptor were untreated or treated for 2 min with 10 nM 17 β -estradiol (E2) \pm 1 μ M OH-tamoxifen (Tam). Lysates were immunoprecipitated by anti-erk-2 antibodies. The immunoprecipitates were assayed for MAP-kinase activity by using MBP. Phosphorylated proteins were resolved on 15% SDS-PAGE and revealed by autoradiography. Western blots with H222 anti-estrogen receptor antibodies of each lysate submitted to immunoprecipitation are shown in each panel. The arrows indicate estrogen receptor and MBP positions.

bound nucleotides as well as the GTP/GTP + GDP ratio was also measured using a PhosphorImager. The GTP/GTP + GDP ratio averaged from three different experiments was 11.7% in untreated cells, it increased ~2.5-fold (29%) upon estradiol treatment and was reduced to the control value (10.5%) when estradiol was added in the presence of ICI 182 780 (Figure 3B). The estradiol-induced increase over the basal level (control) was significant ($P \leq 0.001$); also significant was the effect of ICI 182 780 on estradiol treated cells ($P \leq 0.005$), whereas the difference between control cells and cells treated with both estradiol and anti-estrogen was not significant.

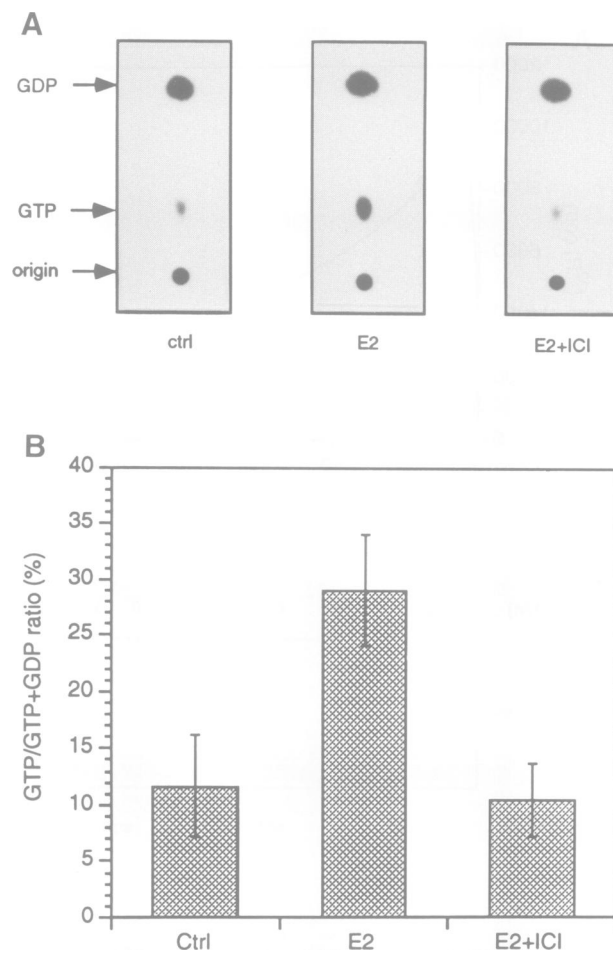


Fig. 3. Identification of the nucleotide bound to p21^{ras} in MCF-7 cells upon estrogen treatment. MCF-7 cells were radiolabeled with [³²P]orthophosphate and incubated \pm 10 nM estradiol \pm 10 μ M ICI 182 780. Cells were then lysed and nucleotide-bound to p21^{ras}, immunoprecipitated by Y13-259 anti-p21^{ras} monoclonal antibodies, and analyzed by PEI cellulose chromatography (A). (B) presents the GTP/GTP + GDP ratio of three experiments, including the experiment shown in the upper panel, calculated by measuring ³²P counts on the nucleotide spots using a PhosphorImager Analyzer. The average values and the standard errors are shown.

Phosphorylation of Shc by estradiol

In our previous experiments, maximal stimulation of protein tyrosine phosphorylation in MCF-7 cells has been observed after 10 s of estradiol treatment (Migliaccio *et al.*, 1993). Therefore, to study tyrosine phosphorylation of proteins involved in p21^{ras} activation by estradiol we treated MCF-7 cells with hormone for 10 s.

Cells were left untreated or treated for 10 s with 17 β -estradiol \pm ICI 182 780. Cells were lysed, lysates incubated with either control or anti-Shc antibodies and the immunoprecipitates analyzed by Western blotting with either anti-P-tyrosine antibodies or anti-Shc antibodies. Figure 4 shows that anti-Shc antibody precipitated two proteins. One had a mol. wt of 46 kDa and the other, 52 kDa. Both were detected by anti-shc antibody blotting. Immunoblotting with anti-P-tyrosine antibodies revealed no tyrosine phosphorylation of these proteins in untreated cells whereas a phosphorylated 46 kDa band appeared only after estradiol treatment, together with a slightly tyrosine phosphorylated 180 kDa protein which could be mSos. This phosphorylation was abolished by ICI 182 780.

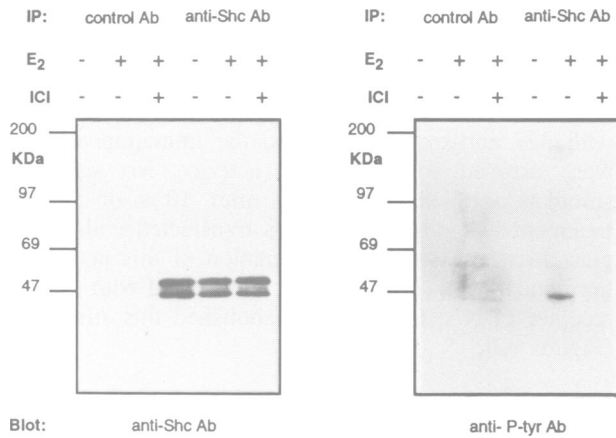


Fig. 4. Effect of estradiol on tyrosine phosphorylation of Shc proteins in MCF-7 cells. Subconfluent MCF-7 cells were used, untreated or treated for 10 s with 10 nM 17 β -estradiol (E2) \pm 10 μ M ICI 182 780 (ICI). Cell lysates containing \sim 2 mg/ml of protein were submitted to immunoprecipitation by rabbit IgG (control Ab) or by anti-Shc polyclonal antibodies (anti-Shc Ab) as described under Materials and methods. The immunoprecipitated samples were run on SDS-PAGE, blotted on nitrocellulose filters and probed with anti-Shc (anti-Shc) or anti-P-tyrosine (anti-P-tyr) antibodies.

No protein interacting with either anti-P-tyrosine or anti-Shc antibodies was precipitated by control antibodies.

These results suggest that, through phosphorylation of Shc, estradiol induces formation of the Shc/GRB2/Sos complex which plays an important role in p21^{ras} activation.

Estradiol effect on tyrosine phosphorylated proteins associated with p21^{ras}-GAP

MCF-7 cells were lysed after 10 s and 60 min of estradiol treatment and the p120 GTPase activating protein (p21^{ras}-GAP) immunoprecipitated. Using anti-P-tyrosine antibodies for Western blotting of proteins electrophoretically separated did not reveal any significant phosphorylation of p120 p21^{ras}-GAP in either untreated or estradiol-treated cells, but identified two polypeptides barely detectable in untreated cells with mobilities corresponding to a mol. wt of \sim 190 kDa and 62 kDa (Figure 5A). The interaction with the anti-P-tyrosine antibodies of these two proteins appeared largely enhanced by 10 s of estradiol treatment and declined after 60 min. Blots of the proteins immunoprecipitated by anti-GAP antibody with the same antibody revealed similar amounts of the 120 kDa GAP in cells untreated or treated with estradiol for 10 s or 60 min (Figure 5A). These findings show that estradiol immediately and reversibly affects tyrosine phosphorylation of proteins associated with GAP. This effect is more evident for p190 than for p62. Parallel samples of cell lysate were incubated with control antibody. Western blotting of the immunoprecipitates neither revealed tyrosine phosphorylated proteins associated with GAP, nor GAP protein (Figure 5A). ICI 182 780 reduced the level of tyrosine phosphorylation of the GAP-associated p190 after 10 s of estradiol treatment (Figure 5B). When proteins immunoprecipitated by anti-GAP-antibodies were blotted with anti-p190 antibodies, it became evident that 10 s of estradiol treatment increased the association of p190 with GAP and ICI 182 780 reduced this increase (Figure 5B). Therefore, estradiol stimulates both tyrosine phosphorylation and association of p190 with GAP.

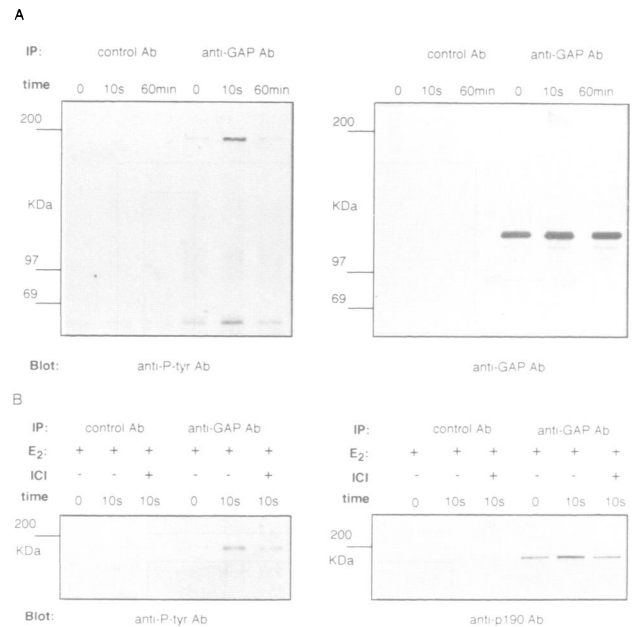


Fig. 5. Effect of estradiol on tyrosine phosphorylation of p21^{ras}-GAP associated proteins in MCF-7 cells. (A) MCF-7 cells untreated (0) or treated with 10 nM 17 β -estradiol for 10 s (10s) or 60 min, were lysed. (B) MCF-7 cells untreated (0) or treated for 10 s (10s) with 10 nM 17 β -estradiol \pm 10 μ M ICI 182 780 (ICI), were lysed. Cell lysates containing \sim 2 mg/ml of protein were submitted to immunoprecipitation by rabbit IgG antibodies (control Ab) or by anti-p21^{ras}-GAP polyclonal antibodies (anti-GAP Ab) as described under Materials and methods. The immunoprecipitated samples were run on SDS-PAGE, blotted on nitrocellulose filters and probed with anti-ras-GAP (anti-GAP Ab), anti-P-tyrosine (anti-P-tyr Ab) or anti-p190 (anti-p190 Ab) antibodies.

Estradiol-receptor complex in vitro interacts with c-src and stimulates c-src kinase activity in vitro and in vivo

Experiments were set up in the attempt to identify the initial events promoting the signal transduction. We verified whether estradiol-receptor complex interacts with c-src and activates its kinase activity. Bovine calf uterus cytosol was incubated with c-src purified from human platelets and covalently bound to Sepharose. Figure 6A shows that estradiol-receptor complex interacts with c-src-Sepharose since it is eluted as a 67 kDa protein together with proteolytic products of the receptor from c-src-Sepharose by 0.4 M KCl. No receptor was found in the eluate from soy bean trypsin inhibitor coupled to Sepharose. In addition, an excess of cold estradiol liganded receptor decreased the amount of [³H]estradiol-receptor complex eluted from c-src-Sepharose from 106 to 10 fmoles (Figure 6A). These findings show that the estradiol receptor-c-src-Sepharose interaction is specific and saturable. Figure 6B shows the *in vitro* effect of estradiol-pure receptor complex on src-kinase activity. MCF-7 cell lysates were incubated with 327 anti-src antibodies and the src-kinase activity of immunoprecipitates was measured using acid-treated enolase as substrate in the absence and in the presence of pure calf estradiol receptor, preincubated or not with 17- β estradiol. A drastic stimulation of the activity was observed when the immunoprecipitate was incubated with estradiol receptor pre-loaded with hormone. No significant stimulation was observed in immunoprecipi-

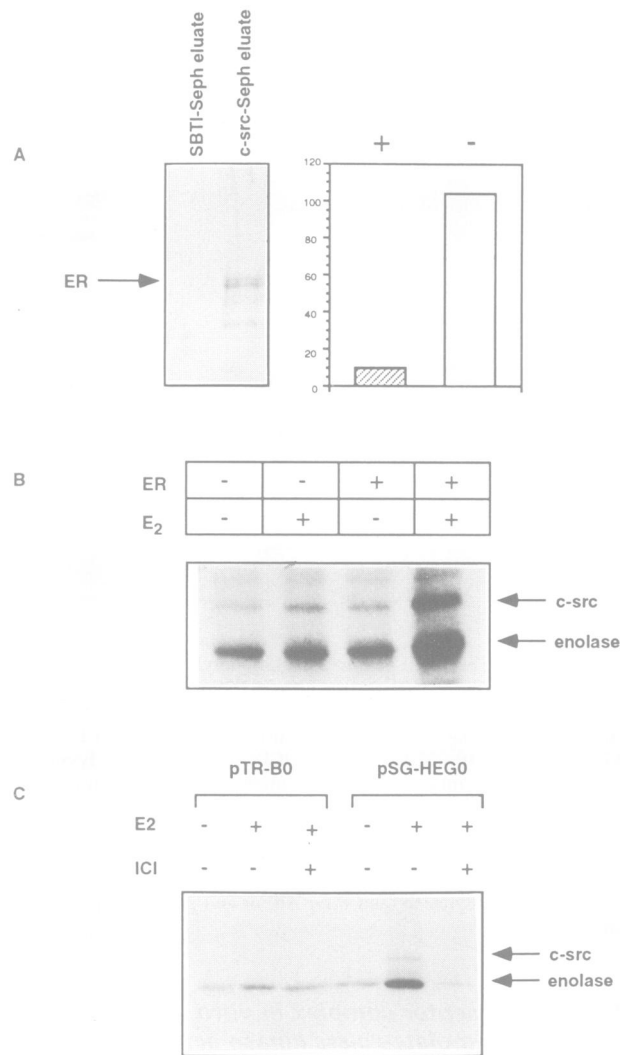


Fig. 6. *In vitro* interaction of estradiol receptor with c-src-Sepharose and *in vitro* and *in vivo* stimulation of src-kinase activity by estradiol receptor. (A) Bovine uterus cytosol in TGDK-buffer was incubated either with a c-src-Sepharose column or with a soy bean trypsin inhibitor-Sepharose column (SBT1-Sepharose). The columns were extensively washed and finally eluted by 0.4 M KCl. Eluates were submitted to electrophoresis and Western blot with H222 monoclonal antibodies. The bars in the right portion of A show the [³H]estradiol-calf uterus receptor complex in fmole eluted from c-src-Sepharose column in the presence (+) or in the absence (-) of an excess of cold estradiol-receptor complex. (B) MCF-7 cell lysates were incubated with 327 anti-src antibodies. The immunoprecipitate was incubated ± estradiol or + pure calf uterus estradiol receptor preincubated with or without 17 β -estradiol. All the samples were then incubated with acid-treated enolase and [γ -³²P]ATP. The reaction was stopped by adding Laemmli SDS-sample buffer and samples submitted to gel electrophoresis followed by autoradiography. Arrows indicate enolase and c-src position. (C) Cos-7 cells were transfected with pTR-B0 control plasmid or with pSG1-HEG0 plasmid encoding the wild-type estrogen receptor ORF. Both cells, expressing or not expressing the estrogen receptor, were untreated or treated for 2 min with 10 nM 17 β -estradiol either in the absence (E₂) or in the presence of 10 μ M anti-estrogen ICI 182 780 (ICI). The cells were lysed and lysates incubated with 327 anti-src antibodies. The immunoprecipitates were assayed for src-kinase activity using acid-treated enolase. Arrows indicate enolase and c-src position.

tates incubated with either estradiol alone or estradiol-free receptor.

To assess the *in vivo* effect of estradiol-receptor complex

on src-kinase activity, Cos-7 cells were transfected with pSG1-HEG0 plasmid encoding human estrogen receptor or control pTR-B0 plasmid and untreated or treated with estradiol ± ICI 182 780. Cell lysates were then incubated with 327 anti-src antibody and the immunoprecipitates were assayed for src-kinase activity. No significant stimulation of kinase activity after 10 s of estradiol treatment was observed in cells transfected with control plasmid whereas a sharp stimulation of this activity by hormone was observed in cells transfected with estrogen receptor cDNA. ICI 182 780 abolished this stimulation (Figure 6C).

Discussion

The mechanism by which estradiol promotes cell proliferation is unknown. The steroid might act through interaction of its own receptor with specific DNA sequences regulating the expression of genes required for cell multiplication (Loose-Mitchell *et al.*, 1988; Weisz and Bresciani, 1993), or through production of growth factors by the target cells (Dickson and Lippman, 1987) or other as yet unidentified mechanisms. The present findings show that in MCF-7 cells maintained under conditions of estradiol-dependent cell growth, estradiol activates enzymes of a pathway generally shared by peptide mitogens as well as oncogene products. In this pathway Raf-1 phosphorylates and activates MAP kinase (MEK) which in turn phosphorylates and activates MAP-kinases (Crews *et al.*, 1992; Macdonald *et al.*, 1993).

We now observe that estradiol activates MAP-kinases in MCF-7 cells. The serine-threonine kinase cascade which includes MAP-kinases is regulated upstream by p21^{ras}. In fact, in our experiments the MAP-kinase activity stimulation by estradiol is associated with increased p21^{ras}-GTP. The relevance of this estradiol-activated process with mitogenesis is substantiated by kinetics of the enzyme activation which mimic activation kinetics by other mitogens (Blenis, 1993).

Tyrosine kinases enhance the active form of p21^{ras}. Activated src phosphorylates Shc which then associates to the GRB2/mSos complex with consequent release of GDP from inactive p21^{ras} and switch of p21^{ras} to the active GTP-bound state (Egan and Weinberg, 1993). src also phosphorylates the p21^{ras}-GAP associated p190 protein. Thereafter, this protein interacts with p21^{ras}-GAP, reducing the GTPase stimulatory activity of p21^{ras}-GAP and increasing p21^{ras} GTP (Bouton *et al.*, 1991; Moran *et al.*, 1991; Chang *et al.*, 1993). It has been recently reported that estradiol induces immediate and transient c-src activation in MCF-7 cells (Migliaccio *et al.*, 1993). In this paper we show that estradiol triggers, in the same cells, phosphorylation of two src substrates, Shc and p190. These findings extend our previous observation and support the possibility that c-src plays a pivotal role in the signaling transduction pathway triggered by this hormone. In addition the interest in estradiol action on p190 phosphorylation is reinforced by the knowledge that p190 functions *in vitro* as a GAP for Rho and Rac proteins (Settleman *et al.*, 1992) so that, through this phosphorylation leading to association of p190 to p21^{ras}-GAP, estradiol may couple mitogenic pathways signaling

through p21^{ras} with pathways regulating cytoskeletal organization and cell adhesion.

Our findings raise a very intriguing question: how can estradiol trigger *c-src* activation, increase active p21^{ras} and stimulate MAP-kinases? Estradiol could interact with either Neu/erb-B2 as recently reported (Matsuda *et al.*, 1993) or alternatively with its own receptor. According to the first hypothesis, upon ligand binding, the tyrosine kinase activity in the cytoplasmic domain of this membrane protein should induce erb-B2 autophosphorylation and activation. The constitutively active, transforming erb-B2 is coupled with the p21^{ras}/MAP kinase pathway (Ben-Levy *et al.*, 1994). Furthermore, in human breast carcinoma cell lines, Neu/erb-B2 interacts with *c-src*, suggesting that *c-src* is an integral part of the signaling events mediated by this receptor (Luttrell *et al.*, 1994). On the basis of these findings, a direct interaction of estradiol with erb-B2 in MCF-7 cells could induce its tyrosine phosphorylation and activation of the *src*/p21^{ras}/MAP-kinase pathway. However, under our experimental conditions we were unable to observe estradiol-induced erb-B2 tyrosine phosphorylation in MCF-7 cells (data not shown). It is possible that estradiol acts through Neu/erb-B2 only in cells lacking estradiol receptor like the cells employed in the reported experiments (Matsuda *et al.*, 1993). Therefore, we explored the second hypothesis with transient transfection experiments. Introduction of human receptor estradiol cDNA (HEG0) in Cos cells was required for estradiol activation of erk-2 and *c-src*. This shows that the receptor mediating the hormonal activation of these enzymes is the classic receptor universally known for its gene stimulatory activity. This has also been proven by the inhibition by a pure anti-estrogen of each step of the pathway activated by estradiol in MCF-7 cells.

Localization of this receptor on cell membranes would allow its interaction with active *c-src* and activation of the signal transduction pathway. Although it is generally accepted that estradiol receptor is mostly nuclear, Berthois *et al.* (1986b) demonstrated saturable specific binding of estradiol on the surface of MCF-7 cells. Furthermore, the recent report on a membrane-associated receptor, whose immunoreactivity was tested with different antibodies, each directed against a different epitope of the 'nuclear' estradiol receptor (Pappas *et al.*, 1995), indicates that a subpopulation of the classic receptor is associated with the cell membrane. We observe that 10–20% occupancy by estradiol of the total binding sites present in MCF-7 cells is sufficient to trigger the maximal *c-src* activation by this hormone (unpublished data). This finding shows that only a subpopulation of the receptor is involved in the estradiol activated process and supports the possibility that this subpopulation could correspond to the membrane-associated receptors.

Use of transfection experiments with deleted and point-mutated estradiol receptor and *c-src* DNA should allow identification of the receptor and *c-src* domains involved in the estradiol-induced activation of *c-src* activity. The findings of transfection experiments as well as estradiol dependent activation of kinase activity in anti-*src* immunoprecipitates call for further investigations. It remains to be verified whether the hormonal activation of *c-src* needs other proteins in addition to the receptor. The specific and saturable interaction of *c-src*–Sepharose with cytosol

estradiol receptor could also require additional protein(s). Such a requirement is suggested by the recent report that mutation of a chaperone protein functionally associated with the 90 kDa heat shock protein (Hsp 90) affects the activity of two Hsp 90 substrates, *v-src* and estradiol receptor (Kimura *et al.*, 1995).

In conclusion, the experiments presented in this paper show that estradiol, like peptide growth factors, activates the tyrosine-kinase/p21^{ras}/MAP-kinase pathway. Interestingly, this activation requires ligand occupancy of the classic estradiol receptor which is universally known as a transcriptional activator. Either alone or in association with other protein(s), *c-src* could be an initial target of the estradiol–receptor complex. Suppression of estradiol dependence of cell growth by transfection of MCF-7 cells with *src* and p21^{ras} mutants could provide additional support to the conclusion that this transduction pathway is responsible for estradiol mitogenic activity.

Materials and methods

Materials

Monoclonal Ig 2bk anti-P-tyrosine antibodies (clone 4G10), polyclonal rabbit anti-p21^{ras}-GAP antibodies were from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Anti-p21^{ras} rat monoclonal antibodies, (clone Y13–259), 327 mouse anti-*src* monoclonal antibodies, anti-mouse IgG and anti-rat IgG goat antibodies and protein A–Sepharose beads were from Oncogene Science Inc. (Manhasset, NY, USA). Anti-erk-1 and anti-erk-2 rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA, USA). Anti-Shc and anti-p190 rabbit polyclonal and mouse monoclonal antibodies were from Transduction Laboratories (Lexington, KY, USA). Acrylamide, BIS, TEMED, ammonium persulfate, SDS, Tween-20, pre-stained mol. wt markers for protein electrophoresis and protein assay kit were from Bio-Rad (Richmond, CA, USA). BA-85 nitrocellulose was from Schleicher & Schuell (Dassel, Germany). Gelatine and Norit A were from Serva (Heidelberg, Germany). Anti-mouse and anti-rat IgG alkaline phosphatase (AP) conjugates antibodies were from Promega (Madison, WI, USA). Anti-mouse and anti-rabbit IgG HRP-conjugates and ECL reagents were from Amersham (Bucks, UK). Anti-rat IgG (fractionated anti-serum), protein G–Sepharose, estradiol, BSA (fraction V), sodium orthovanadate, enolase, myelin basic protein (MBP), HEPES, PIPES, Tris, glycine, EDTA, Triton X-100, PMSF, poly(ethylene)imine(PEI)-cellulose plates, guanosine-nucleotide phosphates, leupeptin, pepstatin, antipain, soy bean trypsin inhibitor were from Sigma (St Louis, MO, USA). OH-tamoxifen and ICI 182 780 were a gift from ICI (Macclesfield, UK). Reagents for cell culture media, including fetal calf serum, were from GIBCO (USA). All other reagents used were of analytical grade.

Cell culture

Human breast cancer MCF-7 cells were routinely grown in 10% CO₂ in air atmosphere using plastic Petri dishes (100 mm) in Dulbecco's modified Eagle's medium (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. Before experiments subconfluent cells were maintained for 1 week in phenol red-free DMEM with 5% fetal calf serum treated twice with charcoal-coated dextran (CSS) prepared using a previously described method (Migliaccio *et al.*, 1993). Cells were passaged every 7 days and media changed every 2–3 days.

Immunoprecipitation of erk-1 and erk-2 kinases

MCF-7 cells either untreated or treated with 10 nM 17β-estradiol ± 10 µM ICI 182 780 were washed three times with ice-cold PBS, pH 7.4 and scraped. Cells were then added with 2 ml of ice-cold lysis buffer: 50 mM Tris–HCl, EDTA 4 mM, 25 mM KCl, 1 mM PMSF, 1 µg/ml each of antipain, leupeptin and pepstatin, 10 µg/ml of soy bean trypsin inhibitor, 1 mM sodium orthovanadate and 1% Triton X-100, pH 7.4. Lysate was left at 4°C under shaking, centrifuged at 15 000 g for 30 min and protein concentration of the clear supernatant was assayed. Cell lysates were diluted at a protein concentration of about 1 mg/ml, incubated with ~1 µg/ml of either rabbit immunoglobulins or rabbit

polyclonal anti erk-1 or anti-erk-2 antibodies for 90 min at 4°C, then added with 40 µl of a 50% suspension of protein G-Sepharose and incubated for an additional 30 min. The samples were centrifuged and pellets washed with 1 ml of lysis buffer four times and used for the MAP kinase assay.

MAP kinase assay

MAP kinase assay was performed as previously described (Sontag *et al.*, 1993) at 30°C for 20 min in a final volume of 50 µl containing 50 mM HEPES (pH 8.0), 10 mM MnCl₂, 1 mM DTT, 1 mM benzamide, 0.3 mg/ml MBP, 50 µM [γ -³²P]ATP (10 µCi) and cell lysate (10 µl). Reaction was stopped by addition of 10% TCA. The samples were spotted onto GFC filters. Each filter was exhaustively washed with TCA, dried and counted by liquid scintillation. ³²P incorporated into MBP was measured as the difference between samples incubated with and without MBP. Alternatively, cell lysates were immunoprecipitated with anti-erk antibodies as described above and pellets were assayed for MBP phosphorylation in a final volume of 50 µl of the above described mixture. Reactions were stopped with 2× SDS sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (13.5% acrylamide gel) followed by autoradiography.

p21^{ras}.bound nucleotide analysis

MCF-7 cells in Petri dishes were maintained in phosphate-free DMEM containing 5% CSS for 16 h, then incubated with 0.5 mCi/dish of [³²P]orthophosphate (as orthophosphoric acid) for 3 h. Cells from three dishes were either untreated or treated with 10 nM 17 β -estradiol \pm 10 µM ICI 182 780, washed three times with ice-cold PBS, pH 7.4, and scraped. Cells were then added to 0.7 ml of ice-cold lysis buffer and lysates centrifuged as described above. Cleared supernatants (0.5 ml) were incubated with protein A-Sepharose beads (40 µl) for 60 min to remove non-specific complexes and centrifuged. The supernatants were incubated overnight at 4°C with 20 µl of protein A-Agarose beads previously coupled to goat anti-rat IgG plus anti-p21^{ras} antibody Y13-259. After centrifugation, pellets were washed three times with lysis buffer, each immunoprecipitate was resuspended in 15 µl of 1 M KH₂PO₄, pH 4.0, incubated for 10 min at 4°C, for 5 min at 80°C and centrifuged in an Eppendorf centrifuge for 2 min. Seven microlitre aliquots of the supernatants were immediately spotted on PEI-cellulose plates. The developing solvent was 1 M KH₂PO₄, pH 4.0. This solvent quantitatively eluted nucleotides from the origin. Autoradiography was performed with an intensifying screen at -70°C using an Amersham MP film. Nucleotide standards were visualised by UV light on PEI-cellulose plates containing a fluorescent indicator. Autoradiographic intensities were quantitated using a PhosphorImager (Molecular Biodynamics, USA).

Immunoprecipitation of Shc

Cell lysates were prepared as described above and incubated with ~5 µg/ml of either rabbit immunoglobulins or rabbit polyclonal anti-Shc antibodies for 2 h at 4°C, then added to 40 µl of a 50% suspension of protein G-Sepharose and incubated for an additional 30 min. The samples were centrifuged and pellets washed four times with 1 ml of lysis buffer, eluted in Laemmli sample buffer and submitted to SDS-PAGE and immunoblotting with either anti-Shc antibodies or anti-P-tyrosine antibodies, as described below.

Immunoprecipitation of p21^{ras}-GAP

MCF-7 cells either untreated or treated with 10 nM 17 β -estradiol \pm 10 µM ICI 182 780 were washed three times with ice-cold PBS, pH 7.4 and scraped. Cells were lysed in 2 ml of ice-cold lysis buffer and centrifuged as described above. The supernatant was diluted to a final concentration of 1 mg/ml of proteins with the lysis buffer (final volume 2.2 ml) and incubated with 80 µl of a 50% suspension of protein G-Sepharose for 90 min at 4°C. At the end of incubation each sample was divided in two aliquots and incubated overnight in the cold with either 1 µg of control rabbit immunoglobulins or anti p21^{ras}-GAP rabbit polyclonal antibodies. The following day, each sample was added to 80 µl of a 50% suspension of protein G-Sepharose and incubation was continued for an additional h. The samples were centrifuged and pellets washed four times with 1 ml of lysis buffer, eluted by boiling in 100 µl of Laemmli sample buffer and submitted to SDS-PAGE and immunoblotting with anti-p21^{ras}-GAP, anti-P-tyrosine or anti-p190 antibodies as described below.

Immunoprecipitation and purification of c-src

Cell lysates were prepared as described above, incubated with ~1 µg/ml mouse monoclonal anti-c-src (clone 327) antibodies for 60 min 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 the c-src-kinase assay.

pp60^{c-src} was purified from human blood platelets as reported (Fukami and Lipmann, 1985). The final fractions were concentrated by ultrafiltration, using a PM 10 membrane (Amicon) and dialyzed against the stock solution [50 mM Tris-HCl, pH 7.5, containing 1 mM dithiothreitol, 20% glycerol (v/v) and 0.1% Triton X-100]. The purified src-kinase and soy bean trypsin inhibitor (Sigma) were coupled to CNBr-Sepharose (Pharmacia) according to manufacturer's instructions.

Interaction of estradiol receptor with c-src-Sepharose

Calf uterus cytosol was prepared as previously described (Castoria *et al.*, 1993) and equilibrated at 2°C for 2 h with high specific activity 10 nM [³H]estradiol. Parallel samples were incubated in the presence of a 100-fold excess of cold estradiol. KCl was added to the samples to reach a final concentration of 0.15 M and specific hormone binding activity was determined in duplicate by the dextran-coated charcoal method. Cytosol samples (0.8 ml) containing 787 fmoles of crude estradiol receptor (ER)/mg protein were applied to 0.15 ml of src- or soy bean trypsin inhibitor-Sepharose columns and cycled for 3 h at 2°C. The flow-through was discarded and columns were washed with 20 vol of Tris-HCl buffer, pH 7.5, containing EGTA 0.2 mM, DTT 1 mM, KCl 0.15 M (TGDK-buffer) and a protease inhibitors cocktail (leupeptin, antipain, pepstatin, each at 20 µg/ml, plus PMSF 2 mM). The columns were finally eluted with 0.4 ml of the same buffer containing 0.4 M KCl. Proteins were precipitated by TCA from eluates and washed twice with ether-ethanol (1:1 v/v), then solubilized in Laemmli sample buffer (40 µl). Electrophoretically separated proteins were transferred to nitrocellulose filter and blotted with anti-estradiol receptor H222 antibodies as described (Auricchio *et al.*, 1995).

Competition of [³H]ER binding to src-Sepharose by unlabeled ER complex was measured as follows: 150 µl samples containing 367 fmoles of crude ER incubated for 2 h at 2°C with 10 nM [³H]estradiol \pm 100-fold excess of cold hormone were added to 1.25 ml of TGDK-buffer \pm 3058 fmol of cold estradiol-crude receptor complex pretreated with charcoal. Samples were incubated batchwise for 2 h at 2°C with 0.15 ml of src-Sepharose pre-equilibrated with TGDK-buffer. Suspensions were centrifuged and the pellets were washed with TGDK-buffer until no radioactivity was detected in the supernatant. Pellets were suspended in 0.4 ml of TGD-buffer containing 0.4 M KCl and specific binding sites in the supernatants assayed.

Purification of estradiol receptor

Pure ER was prepared from bovine uterus as described (Abbondanza *et al.*, 1993). In the final step, the preparation was applied to AER 311 Mab-Pr G-Agarose DMP-linked and eluted as hormone-free receptor at pH 12. Pure ER was then equilibrated at 2°C for 2 h with 10 nM cold estradiol and used. AER 311 Mab are antibodies raised against calf uterus ER (Abbondanza *et al.*, 1993).

c-src-kinase assay

Activity of src-kinase was assayed using acidified enolase as a substrate. The incubation was performed in 100 mM PIPES-NaOH containing 20 mM MnCl₂ by mixing 20 µl of anti-c-src immunoprecipitates, 2.5 µl of pure ER (pre-equilibrated or not with 10 nM cold estradiol) and 2.5 µl of acid-treated enolase. Control samples containing 2.5 ml of mock-purified ER (pre-equilibrated or not with estradiol) were run in parallel. The reaction was started by addition of 10 µM [γ -³²P]ATP, continued at 30°C for 10 min and stopped with 2 mM ATP. The samples were reduced with an equal volume of 2× SDS Laemmli sample-buffer and aliquots of them (25 µl) were submitted to SDS-PAGE (acrylamide 10%). The dried gel was exposed to Hyperfilm film (Amersham) for 12 h.

Electrophoresis and immunoblotting

Samples were submitted to SDS-PAGE (7.5–10% acrylamide, acrylamide/BIS ratio 37.5:1). At the end of the run, proteins from gel slabs were electrophoretically blotted 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 Tris-HCl, 150 mM NaCl, 0.05% Tween-20 pH 8.0 (TBST buffer) containing 3% BSA to block non-specific binding sites

(blocking solution) and incubated for ~2 h. They were then incubated for 2–3 h with mouse anti-P-tyrosine monoclonal antibodies or with rabbit anti-p21^{ras}-GAP polyclonal antibodies (1 µg/ml in blocking solution), thereafter washed at least three times for 10 min with TBST buffer. After washing, the filters probed with anti-P-tyrosine antibodies were incubated with AP-linked anti-mouse IgG antibodies (1:4000 dilution in TBST) and the filters treated with anti-p21^{ras}-GAP antibodies were incubated with AP-linked anti-rabbit IgG antibodies (1:7500 dilution in TBST) for 45 min at room temperature. Finally, filters were washed again after the procedure described above and protein-antibody complexes revealed according to the manufacturer's instructions. The filters treated with anti-p190 antibodies were incubated with horseradish peroxidase-linked anti-rabbit IgG antibodies (1:15000 dilution in TBST containing +5% non-fat dry milk) for 45 min at room temperature and immunocomplexes revealed by ECL. For the blots of anti-Shc immunoprecipitates the filters were blocked in 3% non-fat dry milk in TBST, incubated with mouse monoclonal anti-Shc (1:250 in TBST containing +5% non-fat dry milk) or anti-P-tyrosine antibodies (1:1000 in TBST containing +5% non-fat dry milk) and washed as described above. The immunocomplexes on filters were detected by ECL according to the instructions of the manufacturer using horseradish peroxidase-linked anti-mouse IgG antibodies (1:15 000 dilution in TBST containing +1% non-fat dry milk).

Transfection of Cos-7 cells with pSG1-HEG0 plasmid encoding estrogen receptor ORF

The Cos-7 cells were routinely cultured in 75 cm² plastic flasks in DMEM medium supplemented with 5% fetal calf serum. Ten micrograms of the pSG1-HEG0 (Tora *et al.*, 1989) expression vector encoding the human ERcDNA, or alternatively 10 µg of the control plasmid pTR-B0 (Burglin and De Robertis, 1987) or the control plasmid pSG1, were transiently transfected into Cos-7 cells by electroporation (220 V, 250 µF) using the Gene Pulser apparatus provided with a capacitance extender (Bio-Rad). After 24 h, medium was changed to DMEM fenol red-free supplemented with 5% charcoal-stripped fetal calf serum for 3 days.

Protein assay

Proteins were assayed using Bio-Rad kit for protein assay.

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