including one novel transcript. The frequency of AREs within known genes is much higher than that of AREs in 5′ regions of annotated genes (4 of 21). In the case of transcription factor binding sites on chromosomes 21 and 22, 36% of these regions are situated within known genes or proximal to the 3′ most exon of a gene and the frequency was also higher than that of binding sites within 5′ to known genes (22%) [11]. As for NF-κB binding sites on chromosome 22, 40% of the regions are located in intronic sequences [12]. Indeed, there is evidence that several intronic DNA elements for NF-κB are functionally important in the gene regulation by co-operating with other DNA elements [13,14]. Thus, some of the consensus ARE sequences within annotated genes may be functional in the transcriptional regulation of those genes. Yet, those intronic binding sites are interesting in terms of the potential for distal regulatory elements or promoters for non-coding transcripts or antisense transcripts overlapping the 3′ untranslated regions.

In regard to consensus ARE sequences in unannotated regions, 7 of 21 sites are in regions more than ±20 kb apart from any annotation or novel transcribed regions. Six of 7 sites in unannotated regions are located within regions corresponding to *ab-initio* Genscan transcripts. The remaining one site (X6) is situated at 10-kb downstream to the 3′ end of an *ab-initio* Genscan transcript, yet this ARE may be functional as it has a significant in vivo AR binding ability and the expression levels of the proximal *ab-initio* Genscan transcript (Genscan00000043157) are androgen-inducible. It is interesting to compare our results with NF-κB binding sites on chromosome 22 [12], as 22% of the binding sites for p65 lie in regions more than 50 kb from any annotation. Taken together, our results suggest that there are many unannotated regions that include transcription factor binding sites and have biological functions.

Concerning the differences in binding potency among AREs, several factors such as the nearby chromosomal environment or the chromatin accessibility of specific co-operating factors as well as basal transcriptional factors may be involved. Future comparative studies with different cell context will reveal the variations of chromatin structure and contribute to the identification of novel androgen-responsive genes that are critically involved in the development of prostate cancer.

In summary, the present study demonstrates the usefulness of the genome-wide approach combined with computational analysis and experimental verification in hormone-responsive genes. Our functional analysis reveals that some of the perfect AREs are actual AR binding sites in vivo and may relate to the transcription regulation of proximal genes. Our study will mark the first step toward the elucidation of the entire gene regulatory network mediated by androgen.

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Survival Versus Apoptotic 17β-Estradiol Effect: Role of ERα and ERβ Activated Non-genomic Signaling

FILIPPO ACCONCIA,[1] PIERANGELA TOTTA,[1] SUMITO OGAWA,[2] IRENE CARDILLO,[1] SATOSHI INOUE,[2] STEFANO LEONE,[1] ANNA TRENTALANCE,[1] MASAMI MURAMATSU,[2] AND MARIA MARINO[1]*

[1]*Department of Biology, University "Roma Tre," Rome, Italy*

[2]*Research Center for Genomic Medicine, Saitama Medical School, Saitama, Japan*

The capability of 17β-estradiol (E2) to induce the non-genomic activities of its receptors (ERα and ERβ) and to evoke different signaling pathways committed to the regulation of cell proliferation has been analyzed in different cell cancer lines containing transfected (HeLa) or endogenous (HepG2, DLD1) ERα or ERβ. In these cell lines, E2 induced different effects on cell growth/apoptosis in dependence of ER isoforms present. The E2–ERα complex rapidly activated multiple signal transduction pathways (i.e., ERK/MAPK, PI3K/AKT) committed to both cell cycle progression and apoptotic cascade prevention. On the other hand, the E2–ERβ complex induced the rapid and persistent phosphorylation of p38/MAPK which, in turn, was involved in caspase-3 activation and cleavage of poly(ADP-ribose)polymerase, driving cells into the apoptotic cycle. In addition, the E2–ERβ complex did not activate any of the E2–ERα-activated signal molecules involved in cell growth. Taken together, these results demonstrate the ability of ERβ isoform to activate specific signal transduction pathways starting from plasma membrane that may justify the effect of E2 in inducing cell proliferation or apoptosis in cancer cells. In particular this hormone promotes cell survival through ERα non-genomic signaling and cell death through ERβ non-genomic signaling. J. Cell. Physiol. 203: 193–201, 2005.

Knowledge of the molecular mechanism by which estrogens exert pleiotropic functions in different tissues and organs has evolved rapidly during the past two decades. In particular, the mechanism by which 17β-estradiol (E2) induces cell proliferation has been the object of extensive studies in several tissues (Sutherland et al., 1983; Marino et al., 1998, 2001; Castoria et al., 1999, 2001; Razandi et al., 1999). However, recent reports demonstrated that E2 could even decrease cell growth by significantly increasing apoptosis in breast cancer MCF-7 cell variants, prostate cells, and several other cell types (see Song and Santen, 2003 for review). Whether the E2 apoptotic effects can be explained by the expression of different estrogen receptor (ER) isoforms (i.e., ERα and ERβ) is presently unknown.

It has been assumed that E2 exerts survival proliferative effects mainly by rapid non-genomic mechanisms originating from the hormone binding to ERα (Marino et al., 1998, 2002; Castoria et al., 1999, 2001; Lobenhofer et al., 2000; Fernando and Wimalasena, 2004). In line with this assumption, E2 treatment of MCF-7 cells triggers association of ERα with Src kinase and p85, the regulatory subunit of PI3K, leading to DNA synthesis (Castoria et al., 2001). Moreover, E2 induces rapid non-genomic pathways and DNA synthesis even in ERα transiently transfected cell lines (e.g., Chinese hamster ovary, CHO; cervix epitheloid carcinoma cell line, HeLa) (Razandi et al., 1999; Marino et al., 2002). In addition, multiple and parallel signal transduction pathways are rapidly activated by the E2–ERα complex in hepatoma, HepG2, cells (e.g., ERK/MAPK, PI3K/AKT) (Marino et al., 2003). The disruption of such membrane starting pathways completely prevents the E2-induced DNA synthesis and the cyclin D_1 expression at the specific response elements, activator protein-1 (AP-1) and stimulating protein-1 (SP-1) (Marino et al., 2002, 2003). All these results point to the concept that ERα is the primary endogenous mediator of rapid E2 actions committed to cell proliferation.

Less information is available on the role played by ERβ in E2 proliferative effects. Data from cell cultures, gene expression, and knockout mice clearly indicate that E2-activated ERβ may function as a tumor suppressor by modulating the proliferative effects of ERα (Couse and Korach, 1999; Weihua et al., 2003; Cheng et al., 2004; Paruthiyil et al., 2004, Strom et al., 2004). These studies support a functional antagonism between ERα and ERβ with respect to the E2-induced cell proliferation, but do not clarify either the putative role of ERβ in E2-induced apoptosis or the signal transduction pathways involved. However, the ability of E2–ERβ complex to activate rapid non-genomic mechanisms has been reported. A subpopulation of ERβ transfected into CHO cells is membrane bound and capable of activating IP_3 production, ERK/MAPK and c-Jun kinase phosphorylation (Razandi et al., 1999). Recently, Geraldes and coworkers (Geraldes et al., 2003) reported that E2 reduces ERK/MAPK activity through ERβ stimulation in porcine smooth muscle cells. Moreover, conflicting

Abbreviations: E2, 17β-estradiol; E2-BSA, β-estradiol 6-(o-carboxy-methyl)oxime:BSA; ER, estrogen receptor; ERE, estrogen responsive element; ERK, extracellular regulated kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide-3-kinase; PKC, protein kinase C; PARP, poly(ADP-ribose) polymerase.

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*Correspondence to: Maria Marino, Department of Biology, University "Roma Tre," Viale G. Marconi, 446, I-00146 Rome, Italy. E-mail: m.marino@uniroma3.it

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evidences on the ability of ERβ to activate or inactivate Src and p38 kinases (Castoria et al., 2001; Kousteni et al., 2001; Geraldes et al., 2003; Mori-Abe et al., 2003) has been also reported. In particular, the existence of non-genomic mechanism(s) underling the antiproliferative effects of E2–ERβ complex is to date completely unknown.

Here, the ability of E2 to induce ERs activities has been studied in the HeLa cells devoid of any ERs and rendered E2-sensitive by transient transfection with human ERα or ERβ expression vectors. We report that E2 induced different effects on cell growth/apoptosis decision in the presence of the two different isoforms of receptor. The E2–ERα complex activated multiple signal transduction pathways (i.e., ERK/MAPK, PI3K/AKT, p38/MAPK) involved in cell cycle progression, whereas the E2–ERβ complex activated only p38/MAPK, which in turn, drives cells to apoptosis. A role of E2-induced ERK/MAPK activation in regulating some steps of the pro-apoptotic pathways is also demonstrated. These results were confirmed also in cancer cell lines expressing endogenous level of ERα or ERβ. Altogether our findings indicate a new action mechanism for the E2–ERβ complex pointing to the role of E2-induced rapid non-genomic signals in driving cell proliferation or apoptosis in cancer cells.

MATERIALS AND METHODS

Reagents

17β-estradiol, 17α-estradiol, L-glutamine, gentamicin, penicillin, RPMI-1640 and DMEM (without phenol red), charcoal-stripped fetal calf serum, and estradiol-BSA conjugate (β-estradiol 6-(o-carboxy-methyl)oxime:BSA, E2-BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). The estrogen receptor inhibitor ICI 182,780 was obtained from Tocris (Ballwin, MO). The ERK/MAPK cascade inhibitor, U 0126, the PI3K inhibitor, Ly 294002, and the p38/MAPK inhibitor, SB 203580, were obtained from Calbiochem (San Diego, CA). Lipofectamine reagent was obtained from GIBCO-BRL Life-technology (Gaithersburg, MD). The luciferase kit was obtained from Promega (Madison, WI). GenElute plasmid maxiprep kit was obtained from Sigma Chemical Co. Bradford Protein Assay was obtained from BIO-RAD Laboratories (Hercules, CA). The policlonal anti-phospho-AKT, anti-phospho-p38, and anti-p38 antibodies were obtained by New England Biolabs (Beverly, MA); the policlonal anti-ERα, anti-ERβ, and anti-ERK and the monoclonal anti-phospho-ERK, anti-AKT, anti-Bcl-2, anti-caspase-3, anti-poly(ADP-ribose) polymerase (PARP), and anti-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). CDP-Star, chemiluminescence reagent for Western blot was obtained from NEN (Boston, MA).

All the other products were from Sigma Chemical Co. Analytical or reagent grade products, without further purification, were used.

Cell culture

The ER devoid human cervix epitheloid carcinoma cell line (HeLa) (Marino et al., 2002), the ERα containing hepatoma cell line (HepG2) (Marino et al., 2002, 2003; Moon et al., 2004), and the ERβ containing human colon adenocarcinoma cells (DLD1) (Fiorelli et al., 1999; Di Leo et al., 2001) were used as experimental models. Cells were routinely grown in air containing 5% CO_2 in modified, phenol red-free, DMEM (HeLa cells) or RPMI-1640 (HepG2 and DLD1 cells) media, containing 10% (v/v) charcoal-stripped fetal calf serum, L-glutamine (2 mM), gentamicin (0.1 mg/ml), and penicillin (100 U/ml). Cells were passaged every 2 days (HeLa and DLD1 cells) or every 4 days (HepG2 cells) and media changed every 2 days.

Plasmids and transfection procedures

The expression vectors for pCR3.1-β-galactosidase, human ERα (pSG5-HE0) (Marino et al., 2003), and human ERβ (pCNX2-ERβ) (Ogawa et al., 1998) have been used. Furthermore an empty vector, pCMV5, was used as control (Marino et al., 2001). Plasmids were purified for transfection using a plasmid preparation kit according to manufacturer's instructions. A luciferase dose response curve showed that the maximum effect was present when 1 μg of DNA was transfected in HeLa cells together with 1 μg of pCR3.1-β-galactosidase to normalize transfection efficiency (~55–65%). HeLa cells were grown to ~70% confluence, then transfected using Lipofectamine Reagent according to the manufacturer's instructions. Six hours after transfection the medium was changed and 24 h thereafter cells were stimulated with 10 nM E2.

Cell viability and cell cycle

HeLa cells were grown to ~70% confluence in 6-well plates, then transfected and, after 24 h, stimulated. At different times after treatment cells were harvested with trypsin and centrifuged. Cells were stained with trypan blue solution and counted in a hemocytometer (improved Neubauer chamber) in quadruplicate. For the cell cycle analysis, 10^6 cells were fixed with 1 ml ice-cold 70% ethanol and subsequently stained with 2 μg/ml DAPI/PBS solution. The fluorescence of DNA was measured with a DAKO Galaxy flow cytometer equipped with HBO mercury lamp and the percentage of cells present in sub-G1, G1, S, and G2/M phases was calculated using a FloMax© Software.

Electrophoresis and immunoblotting

Stimulated and un-stimulated cells were lysed as described (Marino et al., 1998). When indicated 1 μM ICI 182,780 or 10 μM U 0126 or 10 μM Ly 294002 or 5 μM SB 203580 were added to the medium 15 or 30 min, respectively, before agonist stimulation. Cells were solubilized in 0.125 M Tris-HCl (pH 6.8) containing 10% SDS (w/v), 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml leupeptin and boiled for 2 min. Proteins were quantified using the Bradford Protein Assay (Bradford, 1976). Twenty microgram solubilized proteins were resolved using SDS–PAGE at 100 V for 1 h. The proteins were then electrophoretically transferred to nitrocellulose for 45 min at 100 V at 4°C. The nitrocellulose was treated with 3% bovine serum albumin in 138 mM NaCl, 26.8 mM KCl, 25 mM Tris-HCl (pH 8.0), 0.05% Tween-20, 0.1% BSA, and then probed at 4°C overnight with either one of anti-ERα, anti-ERβ, anti-phospho-ERK, anti-phospho-AKT, anti-phospho-p38, anti-caspase-3, anti-Bcl-2, or anti-PARP antibodies. The nitrocellulose was stripped by Restore Western Blot Stripping Buffer (Pierce Chemical Company, Rockford, IL) for 10 min at room temperature and then probed with either anti-ERK, anti-AKT, or anti-p38 antibodies (1 μg/ml). Anti-actin antibody (1 μg/ml) was used to normalize the sample loading. Antibody reaction was visualized with chemiluminescence reagent for Western blot.

RESULTS

Divergent effects of E2 in inducing cell growth in the presence of ERα or ERβ

The level of exogenous ERα or ERβ was assessed in HeLa cells untrasfected (none) or transfected with either empty, ERα, or ERβ expression vectors. The Western blot analysis (Fig. 1a) confirmed the absence of ERs in both un-transfected and empty vector-transfected HeLa cells, whereas a unique band at 67 kDa (ERα-containing HeLa cells) or at 57 kDa (ERβ-containing HeLa cells) was detected. The time course of growth of HeLa cells transfected with empty plasmid or ERα or ERβ expression vectors was examined in the presence of E2 and in the presence of the ER inhibitor ICI 182,780. Figure 1b shows that the growth of empty plasmid-transfected HeLa cells was not affected by E2 or ICI 182,780 suggesting that the presence of ER is necessary for the hormone effects. On the other hand, E2 was mitogen for ERα-transiently transfected HeLa cells (Fig. 1c), whereas a decrease in growth was detected after E2 stimulation in ERβ-transfected HeLa cells with respect to unstimu-

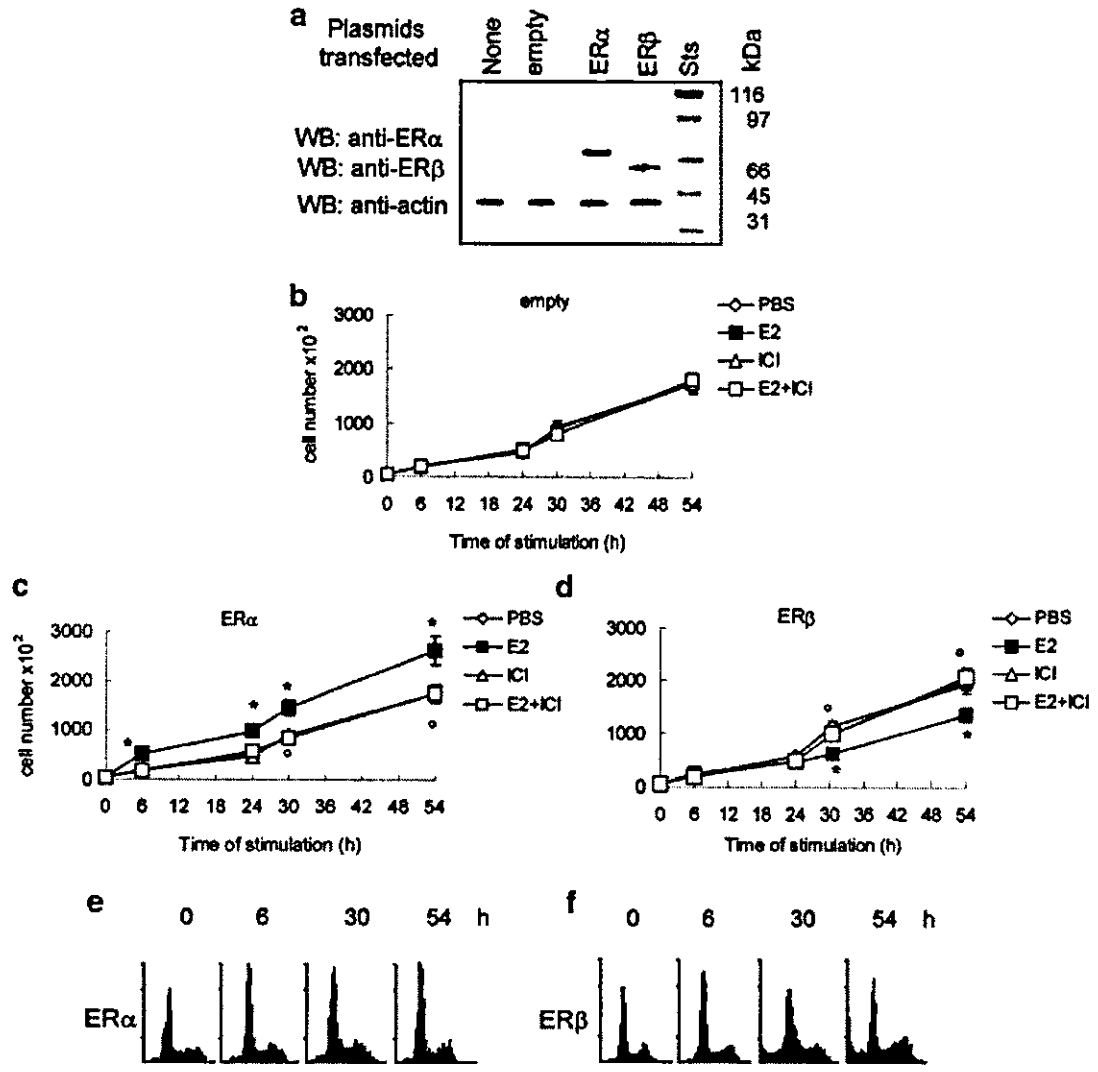

Fig. 1. Level of estrogen receptors (ERs) in transfected and un-transfected and time course of HeLa cell growth in the presence of 17β-estradiol (E2). Western blot analysis of ERα and ERβ levels were performed in un-transfected (none) or transfected HeLa cells with either empty, human ERα or human ERβ expression vectors (**part a**). HeLa cells transfected with empty (**part b**) or human ERα (**part c**) or human ERβ (**part d**) expression vectors were grown in DMEM in the presence of E2 (10 nM) and/or ICI 182,780 (ICI, 1 μM) counted at the indicated times. The data are the mean values ± SD of five independent experiments carried out in duplicate. $P < 0.001$, calculated with Student's t-test, compared with respective un-stimulated values (PBS) (*) or with E2-stimulated values (°). Flow cytometric analysis of the HeLa cells transfected with human ERα (**part e**) or human ERβ (**part f**) vectors after different time of E2-treatment compared with un-stimulated cells (0). The plots indicate cell cycle distribution present in sub-G1, G1, S, and G2/M phases, respectively. For details see the text.

lated ones (Fig. 1d). The cell pre-treatment with the ER inhibitor ICI 182,780 completely blocked the E2 effects both in ERα- and in ERβ-embedded HeLa cells. Further, we analyzed, by flow cytometry, the HeLa cell cycle distribution at different time after treatment. The typical plot of plasmid transfected-HeLa cell population is illustrated in Figure 1e and f (0 h). The first peak indicates the cell number in G1 phase of the cell cycle (50.0 ± 5.0%) followed by S phase (16.3 ± 3.2%), and by the peak of G2/M phase (19.8 ± 2.8%). Increasing the time of E2-stimulation (Fig. 1e; 6, 30, and 54 h), the number of cells in G1 phase of cell cycle increased reaching 65.4 ± 3.8% 54 h after the hormone administration to HeLa cells expressing ERα. On the contrary, when HeLa cells were endowed with ERβ (Fig. 1f; 6, 30, and 54 h), the number of cells in sub-G1 region increased reaching 9.5 ± 1.0% 54 h after the E2 stimulation thus suggesting the presence of DNA fragmentation.

Divergent effects of E2 in inducing an apoptotic cascade in the presence of ERα or ERβ

To determine whether the reported increase of cell population in the sub-G1 phase was truthfully related to the induction of an apoptotic cascade, we analyzed the cleavage of the caspase-3 proform (32-kDa band) which results in the production of the active subunit of the protease (17-kDa band). Caspase-3 proform was expressed in HeLa cells transfected with empty or ERα or ERβ expression vectors (Fig. 2a). No cleavage of caspase-3 was induced by E2 in empty or ERα-containing HeLa cells whereas E2 induced the production of the active subunit in the presence of ERβ.

To confirm that the appearance in the 17-kDa band was associated with an increase in caspase-3 activity, we analyzed one of the known substrates of caspase-3, PARP. This 116-kDa, DNA repair enzyme, is cleaved by

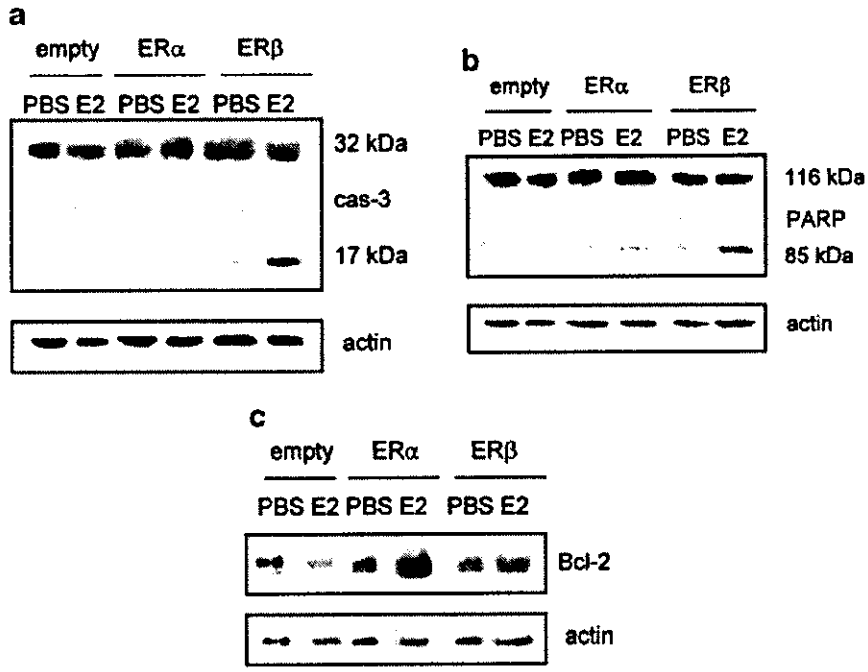

Fig. 2. Effect of E2 in the induction of pro-apoptotic proteins. Western blot analysis of caspase-3 (**part a**), PARP (**part b**) activation, and Bcl-2 (**part c**) levels were performed, as described in "Materials and Methods," on un-stimulated (PBS) and 24 h E2-treated (10 nM) HeLa cells transfected with human ERα, human ERβ, or empty expression vectors. The amounts of protein levels were normalized by comparison with actin expression. Typical blot of three independent experiments. For details see the text.

the caspase-3 producing the inactive 85-kDa fragment (Fig. 2b). By Western blot analysis, treatment of empty- and ERα-containing HeLa cells with E2 did not induce any conversion of PARP in the inactive form. On the contrary, the treatment of ERβ-transfected HeLa cells with E2 resulted in the conversion of PARP into the inactive 85-kDa fragment. These results were consistent with the idea that, in the presence of ERβ, E2 specifically induced an apoptotic cascade involving the caspase-3 activation and a downstream substrate like PARP. This was further confirmed by the expression of Bcl-2 level, the survival factor that can block both necrotic and apoptotic cell death (Dubal et al., 1999). Only the treatment of ERα-transfected HeLa cells with E2 markedly increased the amount of Bcl-2 (Fig. 2c).

Signal transduction pathways involved in the E2-induced apoptotic cascade

We previously reported that the rapid E2-induced activation of ERK/MAPK and PI3K/AKT pathways is sufficient and necessary for E2-induced cell cycle progression (i.e., DNA synthesis and the transcription of cyclin D_1 gene) (Marino et al., 2002, 2003). Then we asked if the inhibition of these rapid signals was involved in the E2–ERβ-induced apoptotic cascade.

No activation of signal transduction proteins was detected in cells transfected with empty vector and stimulated with E2 (data not shown). However, E2 increased ERK and AKT phosphorylation in HeLa cells transiently transfected with ERα (Fig. 3a). After reprobing the membranes using total ERK or AKT antibodies, to recognize the non-phosphorylated form of these proteins, the specific alteration of signaling proteins by E2 was confirmed to occur in the absence of changes in their expression levels (Fig. 3a). On the other hand, E2 failed to elicit any changes in the phosphorylation or expression level of ERK and AKT in cell expressing ERβ (Fig. 3a). Interestingly, a similar activation was observed in cancer cell lines which express endogenous ERα (HepG2) or ERβ (DLD1). In fact, E2 induced the rapid increase of ERK and AKT phosphorylation only in HepG2 cells (Fig. 3b) whereas it was ineffective in DLD1 cells (Fig. 3c). The level of endogenous ERα or ERβ was assessed in HepG2 and DLD1 cells. The Western blot analysis (Fig. 3d) confirmed the presence of a unique band at 67 kDa (HepG2 cells) or at 57 kDa (DLD1 cells) corresponding to ERα or ERβ, respectively.

Generally, the activation of PI3K/AKT and ERK/MAPK pathways causes cell survival in response to many mitogens and growth factors, whereas the activation of p38/MAPK has been associated with the regulation of apoptosis and differentiation processes (Ambrosino and Nebreda, 2001; Harper and LoGrasso, 2001; Talapatra and Thompson, 2001; Shimada et al., 2003; Porras et al., 2004). To verify this possibility, the effect of E2 on p38/MAPK activation was evaluated. A time course of E2-induced p38/MAPK phosphorylation in HeLa cells transfected with ERα or ERβ is shown in Figure 4a. A rapid and transient increase of p38/MAPK phosphorylation was detected from 15 to 30 min after E2 stimulation in ERα-transfected HeLa cells; whereas E2 induced a rapid (15 min) and persistent (24 h) increase of p38/MAPK phosphorylation in ERβ expressing HeLa cells. In the same way, E2 evoked a rapid (15 min) and transient activation of p38/MAPK in ERα-encoding HepG2 cells (Fig. 4b, upper part) and a rapid and persistent (24 h) phosphorylation of p38/MAPK in ERβ-containing DLD1 cells (Fig. 4b, lower part). Note that, the E2-induced p38/MAPK activation was prevented by the pure anti-ER ICI 182,780 in either cell lines (Fig. 4b). The same inhibitor completely

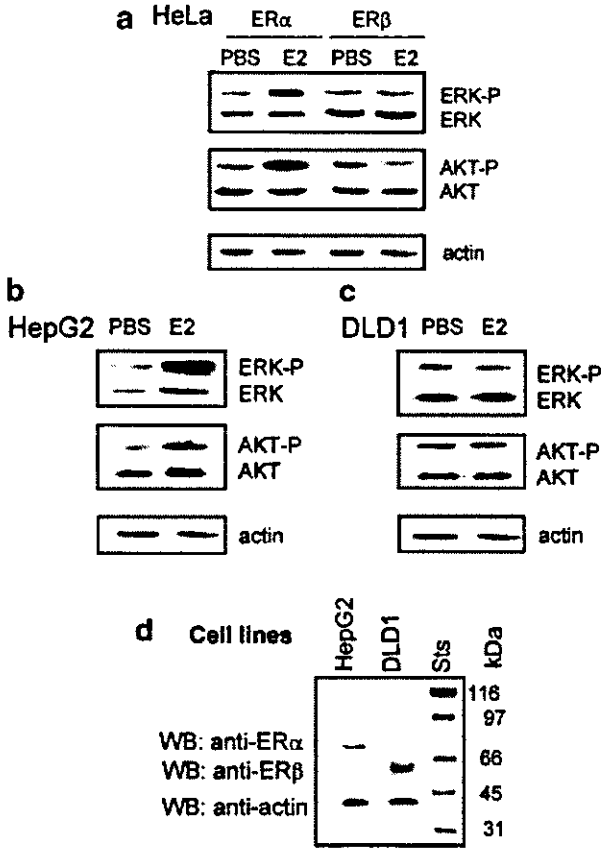

Fig. 3. Signal transduction pathways activated by E2. Western blot analysis of phosphorylated and un-phosphorylated ERK and AKT were performed, as described in "Materials and Methods," on unstimulated (PBS) and 15 min E2-treated (10 nM) HeLa cells transfected with human ERα or human ERβ expression vectors (**part a**) or HepG2 cells (**part b**) or DLD1 cells (**part c**). Western blot analysis of ERα and ERβ levels were performed in HepG2 cells and DLD1 cells (**part d**). The amount of protein levels were normalized by comparison with actin expression. Typical blot of three independent experiments. For details see the text.

prevented the rapid (15 min) E2-induced p38/MAPK phosphorylation also in ER-transfected HeLa cells (Fig. 4c). Furthermore, the E2 inactive stereoisomer, 17α-estradiol, failed to induce p38/MAPK phosphorylation (Fig. 4c), whereas the E2 cell membrane impermeable, E2-BSA (Zheng et al., 1996; Marino et al., 2003), affected the p38/MAPK activation comparably to E2 (Fig. 4c). Altogether these data imply a membrane ER in the rapid and specific E2-induced activation of p38/MAPK signaling.

Cross-talk between proliferative and apoptotic signal transduction pathways and role of E2-induced p38/MAPK

The ability of E2 to induce p38/MAPK phosphorylation even in the presence of ERα was surprising and did not clarify the putative involvement of this kinase in the E2-induced apoptosis. Thus, we asked whether ERK/MAPK and PI3K/AKT cross-talk with p38/MAPK. None of the specific pathway inhibitors used (i.e., Ly 294002 and U 0126) prevented the E2-induced p38/MAPK phosphorylation in ERα-transfected HeLa cells (Fig. 5a) suggesting that the activation of this pathway was parallel and independent on ERK and AKT activation. On the contrary, the cell pre-treatment with the same inhibitors rescued the activation of the pro-apoptotic caspase-3 (Fig. 5b) as well as completely prevented E2-induced, anti-apoptotic, Bcl-2 accumulation (Fig. 5c). The same results were obtained in HepG2 cells (Fig. 5 d, e, and f) further indicating that E2–ERα-induced ERK and AKT activation negatively modulates the apoptotic signals. To direct evaluate the role of p38 in these effects in some experiments cells were pre-treated with the specific p38/MAPK inhibitor, SB 203580 (5 μM), before E2 stimulation. A block of p38/MAPK phosphorylation was evidenced while no effect was present on Bcl-2 levels in both cell lines considered. Note that, caspase-3 cleavage induced by E2 in the presence of U0126 was prevented by the pre-treatment of HepG2 cells with p38/MAPK inhibitor, SB 203580 (30 min) (Fig. 5g). However, the cell pretreatment with the signaling pathways inhibitors alone did not modify the p38/MAPK phosphorylation or caspase-3 and PARP cleavage.

Finally, the pre-treatment of ERβ-transfected HeLa cells with the specific p38/MAPK inhibitor, SB 203580, completely prevented the formation of the caspase-3 active fragment (Fig. 6a) and the cleavage of PARP (Fig. 6b) linking the p38/MAPK activation directly to the apoptosis. As expected, E2 induced p38/MAPK-dependent caspase-3 activation in ERβ-containing DLD1 cells (Fig. 6c) sustaining a pivotal role of the signaling activated by E2–ERβ complex (i.e., prolonged p38/MAPK phosphorylation) in inducing the apoptotic cascade.

DISCUSSION

E2 is known to support cell survival or induce cell death/apoptosis depending on the cell context (Song et al., 2001; Song and Santen, 2003). The mechanism(s) underling these opposite E2 effects could involve the classical/transcriptional mechanism of ER isoforms which, as ligand-dependent transcription factors, modulate the transcription of E2-induced target genes. In addition to this accepted model for the E2 action mechanism, emerging evidences indicated that rapid/non-genomic signaling molecules originating from the cell membrane are involved at least in E2–ERα-induced cell proliferation/survival (Castoria et al., 1999; Razandi et al., 1999, 2000a; Kousteni et al., 2001; Marino et al., 2001, 2002, 2003). These evidences prompted us to examine the non-genomic signaling mechanism(s) generated by the E2–ERβ complex and to compare the role(s) played by these rapid signals with those generated after E2–ERα binding. Although the different functions of ERα versus ERβ on cell proliferation/apoptosis balance has been suggested (Matthews and Gustafsson, 2003; Weihua et al., 2003), the contribution of signal transduction pathways generated by each isoform on these E2-induced cellular functions has not been yet clarified. Therefore, we chose the ER-devoid HeLa cells as experimental model. The transiently transfected HeLa cells allow us to discriminate the effect of each ER isoforms, without the mutual interference, in a E2-induced proliferation model (Marino et al., 2001, 2002). Furthermore, to avoid any dilemma due to the receptors over-expression, some experiments were performed in parallel in two different cancer cell lines which express endogenous ERα (HepG2) (Marino et al., 2001, 2002, 2003; Moon et al., 2004) or ERβ (DLD1) (Fiorelli et al., 1999; Di Leo et al., 2001).

In these experimental conditions, E2 induced different effects on cell growth or apoptosis in dependence on ER isoform present. While the E2–ERα complex activated multiple signal transduction pathways committed to

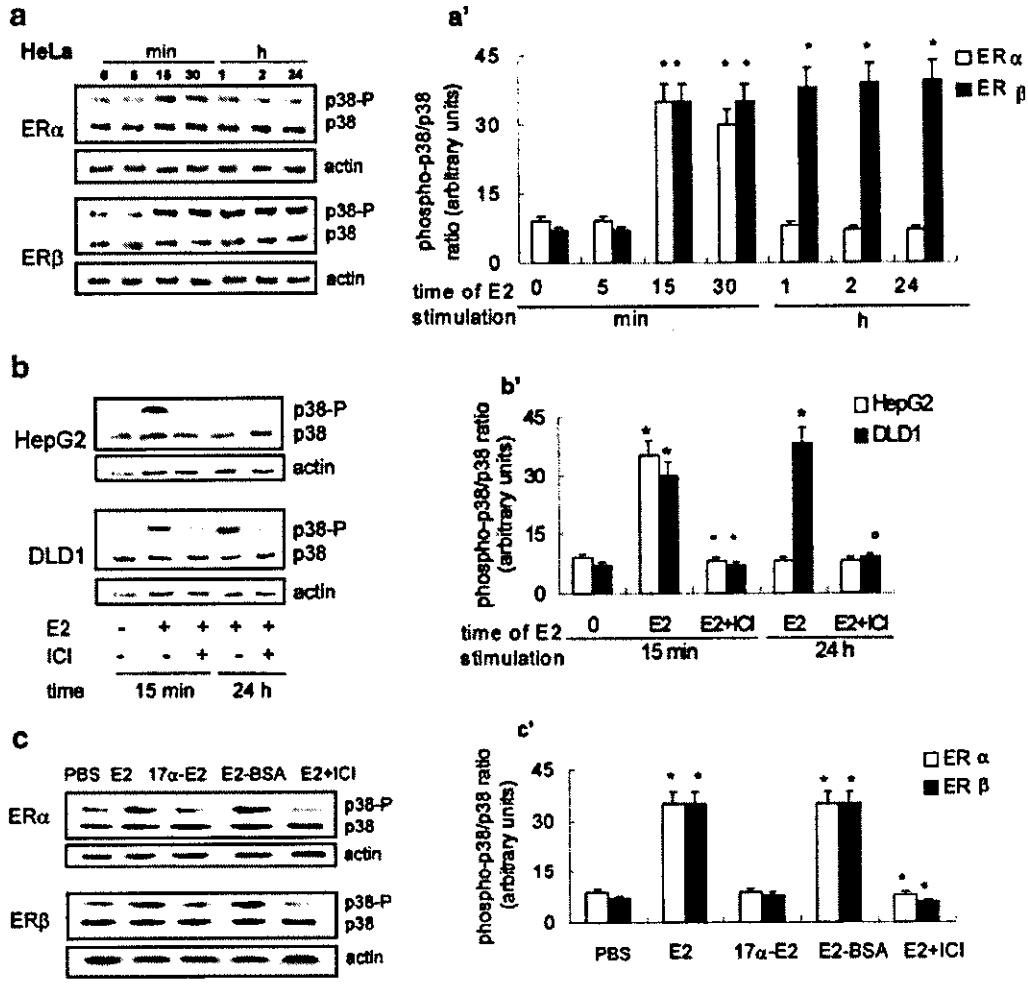

Fig. 4. Effect of E2 on p38/MAPK activation. Time course analysis of p38/MAPK phosphorylation was performed, as described in "Materials and Methods," on untreated (0, –) and E2-treated (10 nM) HeLa cells transfected with human ERα or human ERβ expression vectors (**parts a** and **a'**) or HepG2 or DLD1 cells (**parts b** and **b'**) at the indicated times. The amount of protein levels were normalized by comparison with actin expression. Parts (a) and (b) show the typical blot of three independent experiments; parts (a') and (b') show the data obtained by densitometric analysis, mean values ± SD. $P < 0.001$, calculated with Student's t-test, compared with respective un-stimulated (0, –) values (*) or with E2-stimulated values (°). For details see the text. Western blot analysis of p38/MAPK phosphorylation was performed, as described in "Materials and Methods," in un-stimulated (PBS) or 15 min E2- (10 nM) or 17α-estradiol- (17α-E2; 10 nM) or 17β-estradiol-BSA-(E2-BSA; 10 nM) treated HeLa cells transfected with human ERα or human ERβ expression vectors. In some experiments cells were pretreated with ICI 182,780 (ICI) (1 μM) before E2 stimulation. The amount of protein levels were normalized by comparison with actin expression. **Part (c)** shows the typical blot of three independent experiments; **part (c')** shows the data obtained by densitometric analysis, mean values ± SD. $P < 0.001$, calculated with Student's t-test, compared with respective un-stimulated control (PBS) values (*) or with E2-stimulated values (°). For details see the text.

both cell cycle progression and apoptotic cascade prevention, the E2–ERβ complex induced the rapid and persistent phosphorylation of p38/MAPK, which in turn, drove cells into the apoptotic cycle.

In the E2-stimulated ERα-containing HepG2 cells, we previously demonstrated that E2 enacted the rapid, non-genomic, and membrane starting signal transduction pathways which, in turn, worked cooperatively to achieve cell proliferation. In particular, E2-induced PKC-α was strongly related to DNA synthesis, but was not involved in cyclin D_1 transcription. On the contrary, E2-induced ERK/MAPK and PI3K/AKT pathways were strongly involved in both DNA synthesis and cyclin D_1 transcription (Marino et al., 2002, 2003). Present results clearly indicate, in well accordance with the literature (Razandi et al., 2000b; Kousteni et al., 2001), that these latter pathways have also a critical role in E2 action as a survival agent. While this work was in progress, Fernando and Wimalasena (2004) demonstrated that the prolonged activity of AKT was required to maintain the BAD phosphorylation decreasing its pro-apoptotic effect. In addition, we demonstrate that the E2-induced rapid activation of PI3K/AKT pathway is necessary to increase the level of the anti-apoptotic protein Bcl-2 and to avoid the cleavage of caspase-3 and the induction of apoptotic cascade. Beside AKT-mediated signaling, E2 can also signal through ERK/MAPK pathway. This pathway precedes and modulates AKT phosphorylation (Marino et al., 2003). In fact, the pre-treatment of HepG2 cells with U 0126 (ERK/MAPK inhibitor) rapidly increased the levels of the tumor-suppressor, PTEN, impairing the E2-induced AKT phosphorylation (Marino et al., 2003).

Work of the last years has established that expression and function of component of death machinery are under control of signaling pathways (see Rapp et al., 2004 and literature therein). ERK/MAPK as well as PI3K/AKT cascades cooperate in cellular protection. ERK/MAPK

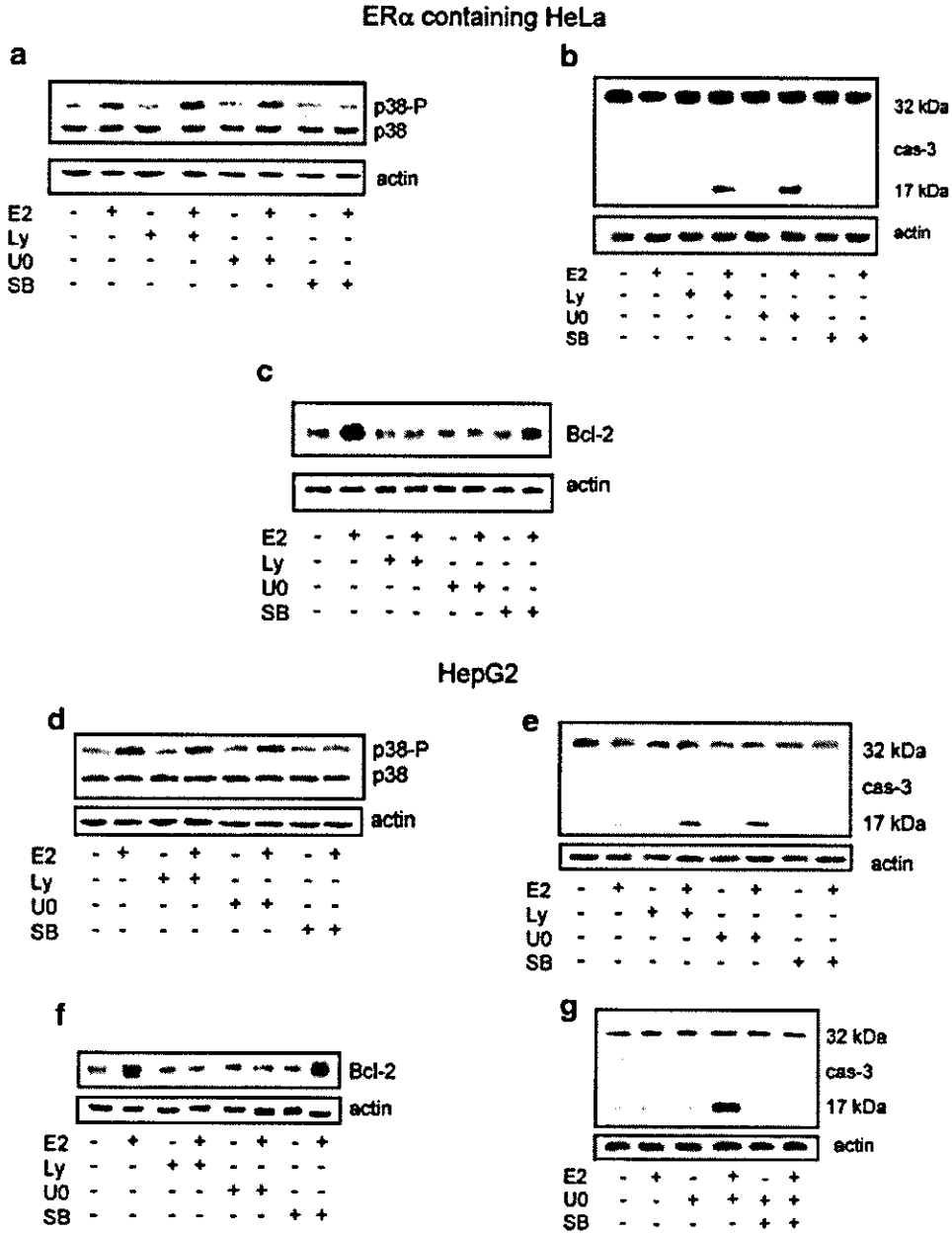

Fig. 5. Cross-talk among E2-induced ERK/MAPK, PI3K/AKT and p38/MAPK activation. Western blot analysis of p38/MAPK (**parts a** and **d**), caspase-3 (**parts b**, **e**, and **g**), and Bcl-2 (**parts c** and **f**) were performed, as described in "Materials and Methods," on un-stimulated (−) or E2-treated (10 nM) (15 min for p38 phosphorylation, 24 h for caspase-3 and Bcl-2 detection) HeLa cells transfected with human ERα expression vector or HepG2 cells. When indicated 10 μM U 0126, Ly 294002 (15 min) or 5 μM SB 203580 (30 min) (ERK/MAPK, PI3K/AKT, and p38/MAPK pathway inhibitors, respectively) were added before E2 administration. The amount of protein levels were normalized by comparison with actin expression. Typical blot of three independent experiments. For details see the text.

control cell survival by targeting Bcl-2 to the mitochondria membranes (Tamura et al., 2004) and together with PI3K/AKT may up-regulate the expression of Bcl-2 (Rapp et al., 2004). Furthermore a direct role of PI3K/AKT in caspase-3 inhibition has been recently reported after polyamine depletion (Zhang et al., 2004). Bcl-2 overexpression, in turn, decreases intracellular Ca^{++} level which can activate p38/MAPK and caspase cascades (Song et al., 2004). Our results, for the first time, show that steroid hormones may regulate this pathway. In fact, the ERK/MAPK and the PI3K/AKT pathways, rapidly activated by the E2–ERα complex, cooperatively enhance the expression of the anti-apoptotic protein (Bcl-2), block the parallel activation of the p38/MAPK, reduce the pro-apoptotic caspase-3 activation, and promote the G1/S transition via the enhancement of cyclin D_1 expression (Marino et al., 2002, 2003; present data).

One of the main findings in this study is the different signal generated by the E2–ERβ complex. There is 96% amino acid identity between the DNA-binding region (C domain) of ERα and ERβ, but in the ligand-binding region (E domain) the homology is only 53% (Kuiper et al., 1998; Matthews and Gustafsson, 2003). As the E domain of ERα is sufficient to elicit non-genomic actions (Marino et al., 2002; Razandi et al., 2002; Acconcia et al.,

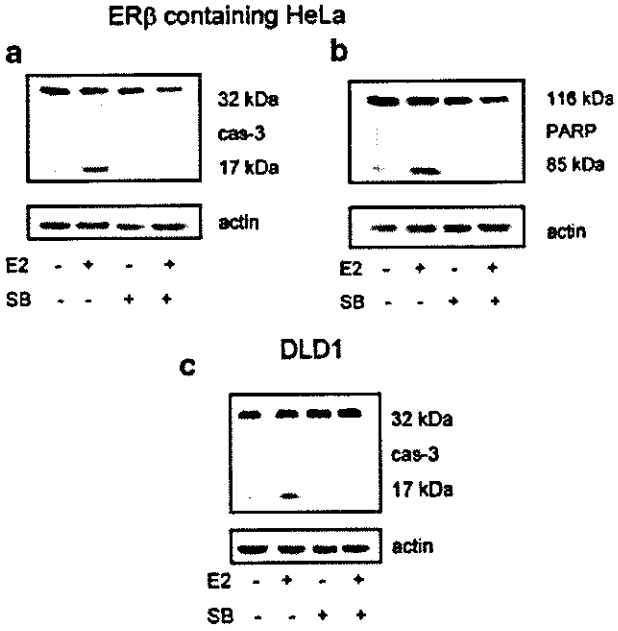

Fig. 6. Involvement of p38/MAPK in pro-apoptotic cascade activation. Western blot analysis of caspase-3 (**parts a** and **c**) and PARP (**part b**) activation were performed, as described in "Materials and Methods," on un-stimulated or 24 h 17β-estradiol-treated (E2; 10 nM) HeLa cells transfected with human ERβ expression vector or in DLD1 cells. When indicated 5 μM SB 203580 (p38/MAPK cascade inhibitor) was added 30 min before E2 administration. The amount of protein levels were normalized by comparison with actin expression. Typical blot of three independent experiments. For details see the text.

2004), it is most likely that different sets of signal transduction proteins may be activated by ERα and ERβ upon E2 binding. Besides these differences the two receptors display a different tissue localization and a different role in proliferation. For example, E2–ERα is a proliferative factor in the uterus, and the uterus of ERβ null mice is hypersensitive to the proliferative action of E2; the co-expression of both ER isoforms is rare during the proliferative phase of mammary gland cells typical of pregnancy, whereas more than 90% of ERβ-expressing mammary gland cells do not proliferate (Weihua et al., 2003); ERβ is abundantly expressed in normal colonic mucosa, but declines in colon adenocarcinoma paralleling the tumor's dedifferentiation (Konstantinopoulos et al., 2003); a progressive decline of ERβ expression has been found in multistage mammary carcinogenesis (Roger et al., 2001) and prostate cancer (Horvath et al., 2001). Very recently it has been reported that the induction of ERβ expression reduces the growth of exponentially proliferating breast cancer cells with a parallel decrease in components of the cell cycle associated with proliferation, namely cyclin D_1, cyclin E, Cdc25A, $p45^{Kip2}$ and an increase in the Cdk inhibitor $p27^{Kip1}$ (Matthews and Gustafsson, 2003; Paruthiyil et al., 2004; Strom et al., 2004). Our data amplify these evidences by adding the ability of ERβ isoform to rapidly induce the persistent membrane starting activation of p38/MAPK without any interference on the survival proliferative pathways, thus impairing the cell cycle components activation.

However, we were surprised to find that the E2–ERα complex increased p38/MAPK phosphorylation. Recently, Lee and Bai (2002) reported that in ERα-expressing endometrial cells, E2 activates the p38/MAPK pathway, which in turn mediates the ERα phosphorylation on threonine-311, promoting the receptor nuclear localization and interaction with specific receptor coactivators. In line with this result the E2-induced p38/MAPK phosphorylation plays a multifunctional role in cellular E2-induced effects. As discussed above, the contemporary increase of Bcl-2 levels, mediated by ERK/MAPK and PI3K/AKT pathways, may decrease the Ca^{++} levels impairing the prolonged p38/MAPK activation (Song et al., 2004).

Ample evidence indicates that the p38/MAPK pathway serves an important role in stress and immune response (Han et al., 1994). Furthermore, p38/MAPK pathway has been associated with a significant slowing in cell proliferation (Han et al., 1994; Badger et al., 1996) and with the regulation of the apoptosis (Kang et al., 2003). In particular p38/MAPK can sensitize cells to apoptosis through the positive regulation of Fas/CD-95 and Bax expression which, in turn, activate caspase cascades (Porras et al., 2004). The E2 capacity in activating p38/MAPK has been reported in a few articles and linked to the preservation of form of ERα- and ERβ-containing vascular endothelial cells (Razandi et al., 2000a), or their migration and proliferation (Geraldes et al., 2003), or even their apoptosis (Mori-Abe et al., 2003). Zhang and Shapiro (2000) reported the ability of E2 to induce p38/MAPK phosphorylation and cell apoptosis in a clone of ERα stably transfected HeLa cells (HeLa-ER5), unresponsive to the E2 proliferative stimuli. The reason for these disparities is not clear but could be related to the divergences in the experimental models, colture condition, E2 treatment period, proliferative capacity, or cell line variability. The E2 stimulation of two cell lines containing endogenous (DLD1) or transfected (HeLa) ERβ demonstrates the ability of human ERβ to drive cancer cells to apoptosis via p38/MAPK-cascade.

In conclusion, besides its role as negative modulator of ERα activities, our findings indicate that ERβ directs the anti-proliferative effects of E2 sustaining the tumor suppressor functions of ERβ. Therefore, the expression of ERs could account for the E2-dependent modulation of cell proliferation. In particular, E2 promotes cell survival through ERα-non-genomic signaling and cell death through ERβ-non-genomic signaling. Thus, the E2 opposite effects in cells co-expressing ERα and ERβ could depend on the balance between the signals originated by each isoform. However, the appearance of new and different signals in the presence of either receptors can not be excluded and it is currently under active investigation in our laboratory.

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Estrogen receptors and their downstream targets in cancer

Kazuhiro Ikeda[1] and Satoshi Inoue[1,2]

[1]Division of Gene Regulation and Signal Transduction, Research Center for Genomic Medicine, Saitama Medical School, Hidaka; and [2]Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

Summary. Estrogen has crucial roles in the proliferation of cancer cells in reproductive organs such as the breast and uterus. Estrogen-stimulated growth requires two estrogen receptors (ERα and ERβ) which are ligand-dependent transcription factors. High expression of ERs is observed in a large population of breast tumors. In addition, the positive expression of ERs correlates with well-differentiated tumors, a favorable prognosis, and responsiveness to an endocrine therapy with anti-estrogen drugs in patients with breast cancer. Transcription activities of ERs can be regulated by interacting proteins such as coactivators and kinases as well as ligand-binding. Moreover, ER isoforms lacking an ability to transactivate are involved in breast cancer. Downstream target genes of ERs have important roles in mediating the estrogen action in breast cancer. We have isolated and characterized several novel estrogen-responsive genes to clarify the molecular mechanism of the estrogen action in target cells. Among these genes, the estrogen-responsive finger protein (Efp) was found to be highly expressed in breast cancer. Efp as a ubiquitin ligase (E3) is involved in the proteasome-dependent degradation of the 14-3-3σ protein, one of cell cycle brakes, this degradation resulting in the promotion of breast cancer growth. A full understanding of the expression and function of ERs and their target genes could shed light on how estrogen stimulates the initiation and promotion of cancer, providing a new approach to diagnose and treat cancer.

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Address for correspondence: Dr. Satoshi Inoue, Division of Gene Regulation and Signal Transduction, Research Center for Genomic Medicine, Saitama Medical School, 1397-1 Yamane, Hidaka-shi, Saitama 350-1241, Japan
Phone: +81-42-985-7206, Fax: +81-42-985-7209
E-mail: s_inoue@saitama-med.ac.jp

Background

Estrogen, a sex steroid hormone, exhibits important biological functions in the target tissues such as reproductive organs. Among these tissues, the growth of the mammary gland and uterine endometrium during pregnancy and the menstrual cycle is dependent on estrogen. In addition to proliferative effects on normal cells, estrogen is considered as a stimulant for the initiation and promotion of tumors in these organs. Epidemiological studies show that prolonged exposure to estrogen, i.e. early menarche, late menopause, and estrogen replacement therapy, can be a risk factor in breast and uterine cancers (Rose, 1996; Clemons and Goss, 2001). In vitro experiments indicate that cells derived from breast and uterine tumors are capable of growing in response to estrogen administration (Holinka *et al.*, 1986; Foster *et al.*, 2001). It is reasonable to assume that the stimulatory effects of estrogen on cell proliferation also contribute to malignant tumor growth. Following prolonged exposure to estrogen, an increase in cell proliferation would be expected to cause an increase in spontaneous DNA replication errors. When mutated in target cells of estrogen, it would enhance the replication of clones of cells carrying such genetic errors. It is, therefore, important to understand mechanisms by which estrogen increases cell proliferation in estrogen-associated cancer.

The estrogen-stimulated growth in tumor cells as well as in normal cells requires the estrogen receptor (ER). It has been shown that about two-thirds of human breast tumors express higher concentrations of ERs than normal breast tissues (Early Breast Cancer Trialists' Collaborative Group, 1998). The ER expression status is related to a variety of histologic characteristics of breast cancer. Most tumors with low grades are ER-positive but, in contrast, tumors demonstrating histologic evidence of poor tumor differentiation are frequently ER-negative (Millis, 1980; Fisher *et al.*, 1981). Breast tumors which lack any ER expression often reveal more aggressive phenotypes (Clarke *et al.*,

1994).

Clinically, endocrine therapy with anti-estrogen drugs or aromatase inhibitors is utilized to treat hormone-related cancer (Howell, 2000; Ali and Combos, 2002). It is expected that tamoxifen, an anti-estrogen drug, binds to ER, making it nonfunctional, while aromatase inhibitors reduce estrogen levels. As discussed below, most breast tumors expressing ER are primarily able to respond to tamoxifen. Aromatase inhibitors such as anastrozole and letrozole are especially useful in patients who are or become resistant to tamoxifen. However, a substantial portion of patients with breast cancer eventually acquire resistance against these treatments. In addition, most of the ER-negative breast tumors can not respond to the anti-estrogen drug. Furthermore, several side effects by treatment with tamoxifen and aromatase inhibitors to ER positive cancer such as breast and cancer, have been reported (Wiseman and Adkins, 1998; Buzdar and Hortobagyi, 2000; Howell, 2000; The ATAC Trialists' Group, 2002).

It is thus important to uncover the precise mechanism of the estrogen action in breast cancer. In particular, the elucidation of regulatory mechanisms for the expression and function of ERs could provide useful information to predict the responsiveness to endocrine therapy and the prognosis. Moreover, it is important to reveal roles of downstream target genes for ERs, which mediate the effects of estrogen on the proliferation of cancer cells while these genes can be targeted to treat and diagnose estrogen-associated tumors.

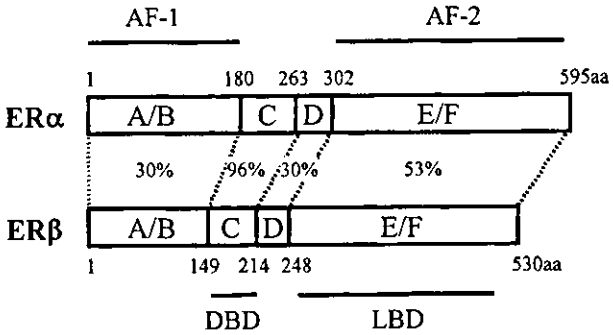

Fig. 1. Schematic representation of human ERα and ERβ. The ERα and ERβ are transcription factors whose activities are regulated by their ligand binding. ERs are members of a nuclear receptor superfamily comprised of six regions (A–F). The ligand-binding domain (LBD) in region E also contains an estrogen-inducible transcription-activating function called AF-2. A constitutively active transcription-activating function (AF-1) is located in the A/B region. Percentages of amino acid identities between the corresponding regions are represented.

Estrogen receptors

As stated above, ER has two subtypes, ERα and ERβ. They belong to the superfamily of nuclear receptors that share similar structures and modes of action (Nuclear Receptors Committee, 1999) (Fig. 1). Namely, estrogen-bound ERs bind as a homodimer or as a heterodimer to an estrogen-responsive element (ERE) with their DNA-binding domain and regulate the transcription of the target genes. ERs contain two independent transcriptional activation functions (AF): the N-terminal A/B domain possesses an autonomous

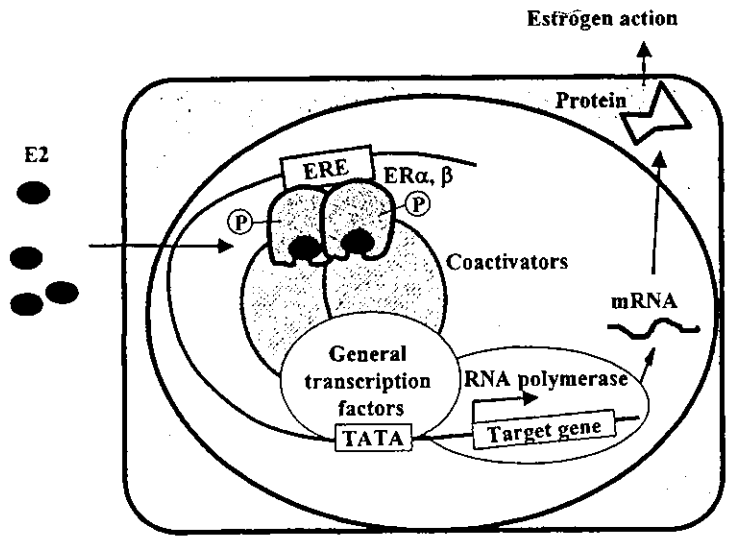

Fig. 2. A model for the regulation of estrogen receptor (ER)-mediated transcription of estrogen-responsive genes. Liganded ERα and ERβ bind as a homodimer or as a heterodimer with an estrogen-responsive element and regulate the target gene transcription. Coactivators are required to mediate ligand-activated transcription by enhancing nuclear receptor transactivation through contacts with the basal transcriptional machinery. Phosphorylation of the ER also modulates the transcription activity.

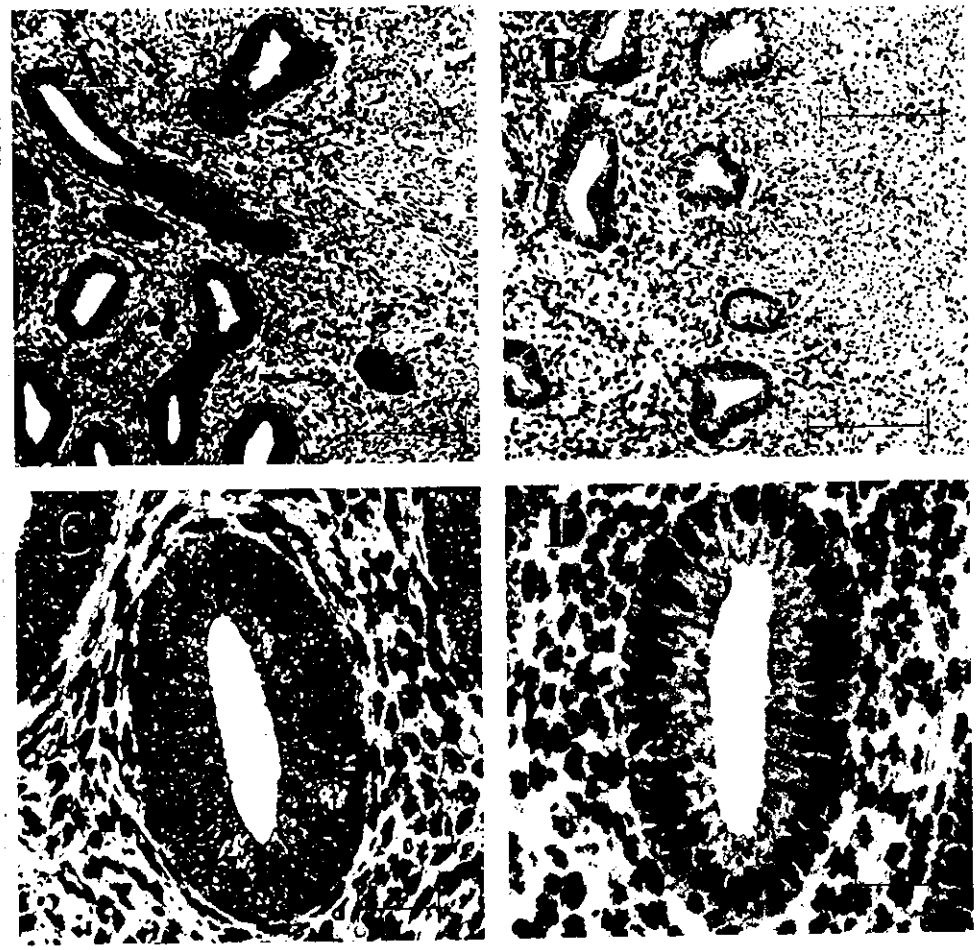

Fig. 3. Immunohistochemical staining of COX7RP (**A** and **C**) and ERα (**B** and **D**) in human endometrium in the proliferative phase of the menstrual cycle. ERα and COX7RP immunoreactivities were detected in the nucleus and cytoplasm, respectively. Strong immunoreactivities of ERα and COX7RP were detected in the glandular epithelia. Scale bar=100 μm (A, B); 10 μm (C, D).

AF-1, while the E-domain possesses a ligand-dependent AF-2. Biological activities of ERs could be controlled by a number of interacting proteins. The ligand-dependent transactivation of ERs requires the recruitment of coactivators such as TIF2 and SRC-1 (Glass and Rosenfeld, 2000). Transcription activities of ERs are also regulated by phosphorylation. In particular, the serine residue at 118 within the A/B domain of human ERα is a major target site of phosphorylation by MAPK in the presence of growth factors (Kato *et al.*, 1995) and by Cdk7 in a ligand-dependent manner (Chen *et al.*, 2000). Recently, we demonstrated that this serine residue is opposingly dephosphorylated by protein phophatase 5 (Ikeda *et al.*, 2004) (Fig. 2). The protein level of ERα is regulated by the ubiquitin-mediated proteasomal degradation (Nawaz *et al.*, 1999; Tateishi *et al.*, 2004). In addition, some elements in the promoter region have been shown to be responsible for a high expression of ER α in breast cancer cells (Hayashi *et al.*, 1997; Tanimoto *et al.*, 1999). Collectively, it is reasonable to assume that these regulatory mechanisms of ERs are closely associated with oncogenesis and tumor growth. Moreover, it is also indispensable for the diagnosis and treatment of estrogen-associated cancer to reveal the regulatory mechanisms for expression levels of the ER mRNA and protein.

The expression of ERβ has been detected in various tumors including breast cancer (Omoto *et al.*, 2002), uterus

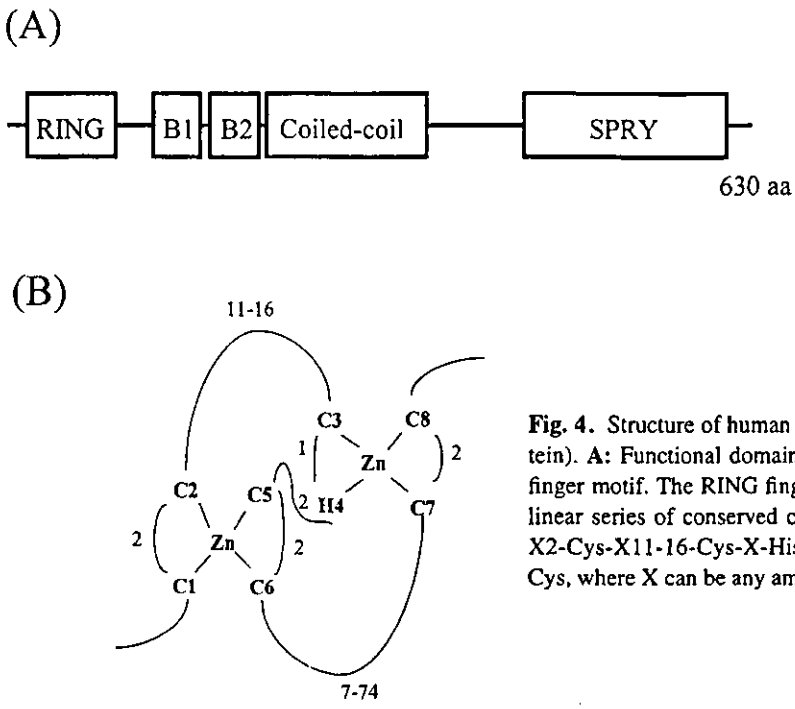

Fig. 4. Structure of human Efp (estrogen-responsive finger protein). **A:** Functional domains of the Efp. **B:** Structure of RING finger motif. The RING finger motif can be defined as a unique linear series of conserved cysteine and histidine residues: Cys-X2-Cys-X11-16-Cys-X-His-X2-Cys -X2-Cys-X7-74-Cys-X2-Cys, where X can be any amino acid.

cancer (Sasano *et al.*, 1999), and prostate cancer (Fujimura *et al.*, 2001). In breast cancer, ERβ shows a tendency to be expressed in ERα-positive carcinomas, while ERα and ERβ double positive cells are also detected. ERβ, as well as ERα, serves as an indicator of a good prognosis in breast cancer (Omoto *et al.*, 2002). It has been found that several variants of ERβ are expressed in breast cancer cells (Leygue *et al.*, 1999). We originally isolated an ERβ isoform, ERβcx (Ogawa *et al.*, 1998), which lacks the last 61 C-terminal amino acids and has an alternative 26 unique amino acids. The ERβcx isoform shows no ligand binding ability and has no capacity to activate transcription in response to estrogen (Ogawa *et al.*, 1998). Moreover, ERβcx shows preferential heterodimerization with ERα rather than with ERβ, inhibiting ERα DNA binding and transactivation. In ERα positive breast cancer, the presence of ERβcx is significantly correlated with the absence of a progesterone receptor (PR) which is a downstream target of activated ER, indicating that ERβcx is a dominant repressor of the ER function in breast cancer. (Saji *et al.*, 2002). These lines of evidence suggest that ERβisoforms are important functional modulators of estrogen-signaling pathways in breast cancer cells and may affect the clinical outcome of patients with breast cancer.

Estrogen-responsive genes in cancer

Estrogen modulates transcription of downstream target genes through ERs. It is thus fundamentally important to identify genes whose expression is regulated by estrogen and to reveal the functions of their protein products. Although a list of ER-target genes has been accumulating, the entire mechanism by which ER enhances the proliferation and progression of tumors remains unknown. In particular, only a few genes are known to be directly regulated by ER through EREs. In order to isolate estrogen-responsive genes having EREs in their transcription regulatory region, we have developed the genomic binding-site cloning (GBSC) method (Inoue *et al.*, 1991, 1999). Using this method, several genomic sequences containing EREs were successfully isolated. Subsequently, novel estrogen-responsive genes were identified nearby the EREs (Inoue *et al.*, 1993; Watanabe *et al.*, 1998). Protein products of these genes include the estrogen-responsive finger protein (Efp), the cytochrome c oxidase subunit VIIa-related polypeptide (COX7RP), and the estrogen receptor-binding fragment-associated antigen 9 (EBAG9).

COX7RP has a well conserved region with cytochrome c oxydase subunit VIIa (Watanabe *et al.*, 1998). Expression of the COX7RP mRNA was up-regulated by estrogen in

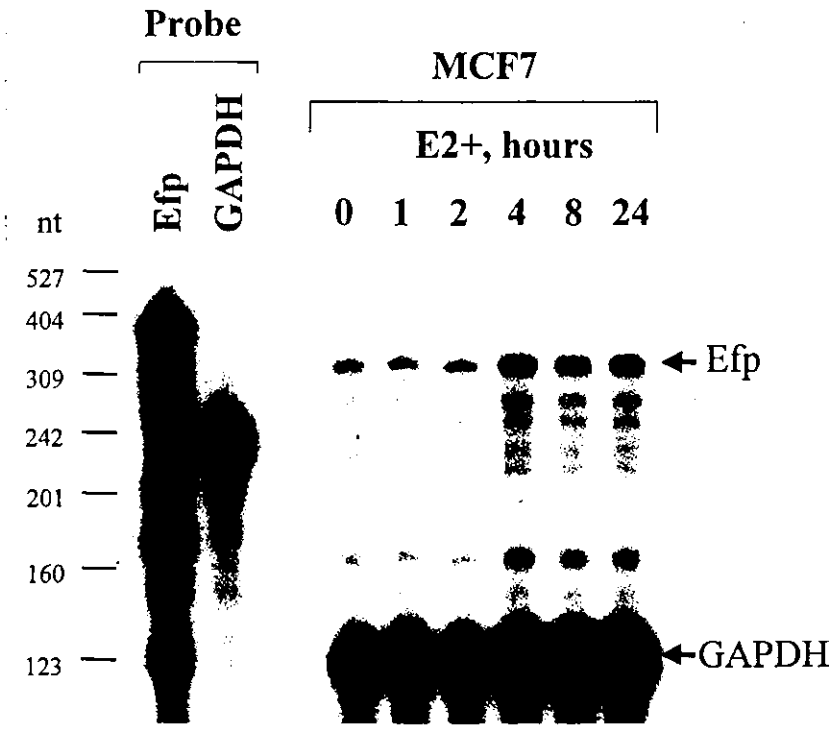

Fig. 5. Estrogen-induced expression of Efp mRNA in MCF7 cells. Total RNAs were isolated from MCF7 cells treated with 10^{-9} M 17β-estradiol at the indicated times. Twenty μg of total RNA was examined by a RNase protection assay using Efp and GAPDH probes. The full-length protected fragment for each probe is indicated.

MCF7 cells. The perfect palindromic ERE found in the first intron possesses an estrogen-dependent enhancer activity in these cells. In addition, an immunohistochemical study demonstrated that the COX7RP protein is co-expressed with the ERα protein in the endometrial glandular epithelium of the human uterus (Fig. 3). We speculate that COX7RP is involved in the regulation of energy production in target cells by estrogen.

Molecular mechanism of Efp function in breast cancer

Among ER-downstream molecules isolated by the GBSC method, we have clarified the molecular mechanism of Efp, which possesses a RING finger motif, two B-boxes, α-helical coiled-coil domains, and a C-terminal SPRY domain (Inoue *et al.*, 1993) (Fig. 4). The RING finger motif is comprised of a unique linear series of conserved cysteine and histidine residues that features a 'cross-brace' arrangement with two zinc ions (Pickart, 2001). Members of the RING finger family grow enormously; some of them have been shown to be responsible for malignant tumors. For instance, PML is responsible for acute premyelocytic leukemia when it forms a fusion protein with the retinoic acid receptor (RAR)α by chromosomal translocation (Jensen *et al.*, 2001). Loss of the tumor suppressor BRCA1 results in chromosomal instability leading to the development of familial breast and ovarian tumors (Ruffner *et al.*, 2001). Efp is predominantly expressed in estrogen target tissues and cells including the mammary gland and uterine epithelial cells (Orimo *et al.*, 1995). Efp is also highly expressed in breast tumors (Ikeda *et al.*, 2000). Expression of the Efp mRNA was shown to be elevated after estrogen treatment in MCF7 cells (Fig. 5). Thus, Efp could function as an estrogen-responsive gene that mediates the estrogen action in cancer. The estrogen-responsive proliferation of the uterine endometrium which expresses abundant ERα was

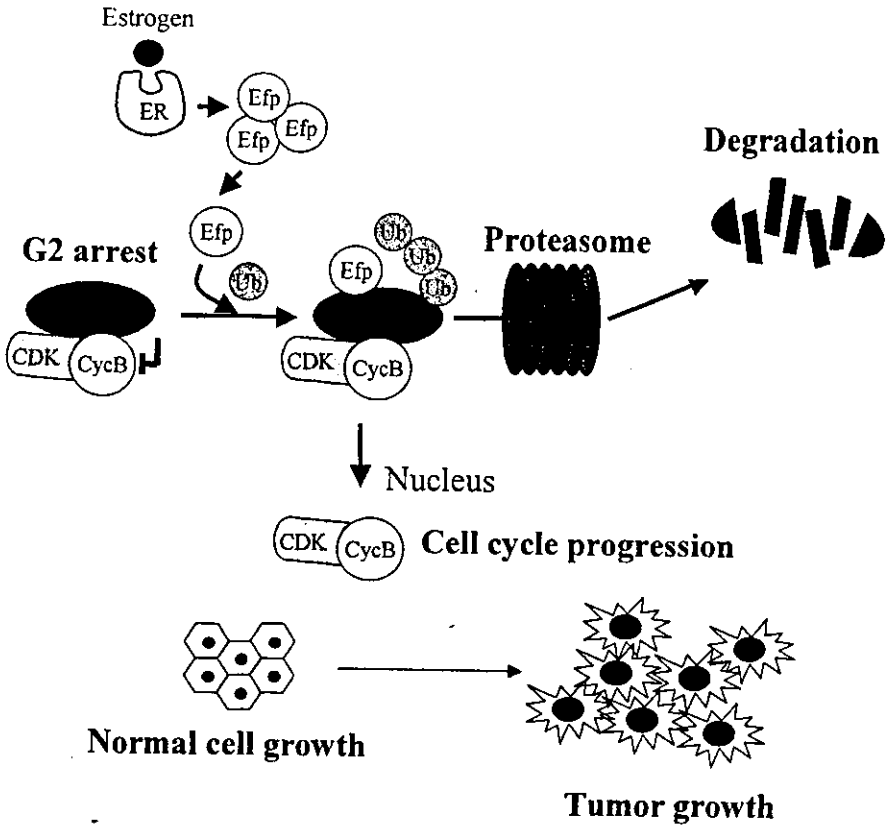

Fig. 6. A simulation model for tumor growth controlled by Efp. The Efp targets 14-3-3σ for proteolysis as a ubiquitin ligase and stimulates tumor growth.

shown to be impaired in Efp knockout mice, suggesting that Efp is a mediator of estrogen-dependent cell growth (Orimo *et al.*, 1999).

To investigate the role of Efp in breast tumor growth, we examined the effects of Efp antisense oligonucleotides on tumor formation in female nude mice inoculated with MCF7 cells (Urano *et al.*, 2002). These mice were ovariectomized or administrated with antisense/sense Efp oligonucleotides. We revealed that the Efp antisense oligonucleotide effectively inhibits the tumor growth generated by MCF7 cells in the recipient mice. MCF7 cells stably expressing Efp (Efp-MCF7) could proliferate even in estrogen-deprived ovariectomized mice. The Efp-MCF7 cells have lower concentrations of the 14-3-3σ protein, which is a negative regulator of the cell cycle progression. The 14-3-3σ protein is important for maintaining G2 arrest by sequestering phosphorylated Cdc2-cyclin B1 from the nucleus into the cytosol (Chan *et al.*, 1999). Interestingly, the expression level of this protein is significantly low in breast tumors (Vercoutter-Edouart *et al.*, 2001; Umbrich *et al.*, 2001). We found that Efp associates with the 14-3-3σ protein. We then demonstrated that Efp functions as a ubiquitin ligase, E3, that ubiquitinates the 14-3-3σ protein, this ubiquitination resulting in the cell cycle progression via the proteasome-dependent degradation of the 14-3-3σ protein (Urano *et al.*, 2002) (Fig. 6).

Perspective

A better understanding of the molecular mechanisms by which estrogen stimulates cell growth can provide new insights into diagnosis, treatment and prevention in estrogen-associated tumors. For this reason, it is indispensable to reveal the expressional and functional regulation of ERs and their target genes. Especially, the identification of estrogen-responsive genes which are closely related to the cancer biology could provide us new approaches for these fields.

Efp, an estrogen-responsive gene, would contribute to the disregulated proliferation of breast cancer cells by the accelerated destruction of a cell cycle regulator, 14-3-3σ. We speculate that Efp could promote tumor growth even in the absence of estrogen and, therefore, the high expression of Efp might be one of reason for acquiring the ability to proliferate independently of estrogen. The future investigation of the relationship between Efp expression and clinical or pathological features could indicate its usefulness as a potential prognostic factor. These trials may lead to the utilization of Efp as a prognostic marker and a therapeutic target in breast cancer. Thus, the accumulation of experimental evidence concerning the estrogen-responsive genes such as Efp can allow us to develop novel cancer treatments separately targeted for each downstream molecule that directly mediates the estrogen action.

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J Bone Miner Metab (in press)

Association of a single nucleotide polymorphism in the lipoxygenase *ALOX15* 5'-flanking region (-5229G/A) with bone mineral density

Tomohiko Urano[1)], Masataka Shiraki[2)], Masayo Fujita[1, 3)], Takayuki Hosoi[4)], Hajime Orimo[5)], Yasuyoshi Ouchi[1)], Satoshi Inoue[1, 3)]

1) Department of Geriatric Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan, 2) Research Institute and Practice for Involutional Diseases, 1609 Meisei, Misato-mura, Minami-Azumi-gun, Nagano, 399-8101, Japan, 3) Research Center for Genomic Medicine, Saitama Medical School, 1397-1, Yamane, Hidaka-shi, Saitama, 350-1241 Japan, 4) Tokyo Metropolitan Geriatric Hospital, 35-2 Sakae-cho, Itabashi-ku, Tokyo, 173-0015, Japan, 5) Health Science University, 7187 Kodachi, Kawaguchiko-machi, Minami-Tsuru-gun, Yamanashi, 401-0380, Japan

Offprint requests to: S. Inoue (Email: INOUE-GER@h.u-tokyo.ac.jp)
Short title: Association of an SNP in *ALOX15* gene with BMD

Abstract

The 12/15-lipoxygenase gene *Alox15* has been identified as a susceptibility gene for bone mineral density in mice through combined genetic and genomic analyses. Here, we studied the association between bone mineral density and an *ALOX15* gene single nucleotide polymorphism to assess the potential involvement of the human *ALOX15* gene in the postmenopausal osteoporosis. Specifically we examined the association between a single nucleotide polymorphism at –5299G/A in the *ALOX15* 5'-flanking region with BMD in 319 postmenopausal Japanese women (66.7 ± 8.9 years, mean ± SD). We found that subjects bearing at least one variant A allele (GA + AA; n=273) had significantly lower Z scores for lumbar spine and total body bone mineral density than did subjects with no A allele (GG; n=46) (lumbar spine, -0.25 ± 1.34 versus 0.48 ± 1.70; p = 0.0014; total body, 0.25 ± 1.01 versus 0.62 ± 1.11; p = 0.048). These findings suggest that the *ALOX15* gene is one of the genetic determinants of BMD in postmenopausal women. Accordingly, this polymorphism could be useful as a genetic marker for predicting the risk of osteoporosis.

Key words adipogenesis, *ALOX15*, PPARγ, osteoporosis, bone mineral density, polymorphism

Introduction

Osteoporosis is characterized by low bone mineral density (BMD), increased bone fragility, and consequently increased risk of fracture [1]. Studies of twins and siblings have shown that BMD is under genetic control, with estimates of heritability ranging from 50% to 90% [2,3]. BMD is regulated by interaction of multiple environmental and genetic factors, each having modest effects on bone mass and bone turnover [4,5]. Polymorphisms of several genes have been investigated to clarify the determinants of BMD [6,7]. These genes of which polymorphisms were associated with BMD include those implicated in bone formation by regulation of osteoblast growth and function, such as *vitamin D receptor* gene [8], *transforming growth factor beta1* (*TGF*β1) gene, *collagen type Iα1* (*COLIA1*) gene [9], *peroxisome proliferator activated receptor-γ* (*PPARγ*) gene [10] and *low-density lipoprotein receptor-related protein 5* (*LRP5*) gene [11]. Identification of candidate genes that affect bone mass will be useful for early detection of individuals who are at risk of osteoporosis and early institution of preventive measures.

The decrease in bone volume associated with osteoporosis is accompanied by an increase in marrow adipose tissues [12, 13]. Indeed, an increase in marrow adipocytes is observed in several conditions that lead to bone loss, such as ovariectomy [14], immobilization [15], and treatment with glucocorticoids [16]. Recent studies have identified rodent quantitative trait locus associated with increased BMD in the mouse gene encoding 12/15-lipoxygenase [17], the enzyme that converts linoleic acid and arachidonic acid into endogenous ligands for the PPARγ [18-20]. Activation of this pathway in marrow-derived mesenchymal progenitors stimulates adipogenesis and inhibits osteoblastogenesis [21, 22]. Mice that are deficient in this gene or have been treated with 12/15-lipoxygenase inhibitors demonstrate increased bone mass as compared with controls [17]. These findings suggest that genetic variants of the 12/15-lipoxygenase encoding gene may affect the BMD in humans as well as mice. The mouse 12/15-lipoxygenase enzyme corresponds to at least three lipoxygenases in humans. 15-lipoxygenase has two isoenzymes: type 1 (human *ALOX15,* encoded by a gene at chromosome 17p13.3) and type 2 (human *ALOX15B,* encoded by a separate gene at 17p13.1). 12-lipoxygenase (human *ALOX12,* encoded by a gene at chromosome 17p13.1) is predominantly expressed in platelets and macrophages and is distinct from 15-lipoxygenase [23]. In the present study, we examined the possibility that there is an association between a polymorphism in the human *ALOX15* gene and BMD in Japanese women to investigate possible contribution of the lipoxygenase in the bone metabolism.