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Involvement of Nuclear Receptor Coactivator SRC-1 in Estrogen-Dependent Cell Growth of MCF-7 Cells

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Steroid hormones regulate cell growth and function through the transcriptional control of target genes by their cognate nuclear receptors. These receptors bind to ligands and associate with transcriptional cofactors to stimulate transcription. SRC-1, one of the nuclear receptor coactivators, is known to interact with nuclear receptors and enhance transactivation function in a ligand-dependent manner. In this study, to assess the function of SRC-1 in cell growth regulated by nuclear receptor ligands, we established a stable transformant cell line overexpressing human SRC-1 and studied the action of 17β-estradiol (E_2) on cell growth as well as the expression of E_2-responsive genes in MCF-7 cells. We found that SRC-1 overexpression potentiates cell growth stimulated by E_2 in accordance with enhancement of transcriptional activation of exogenous and endogenous E_2-responsive genes. These findings clearly indicate the importance of nuclear receptor coactivators for the activities of steroid/lipophilic vitamins in cell growth and gene expression.

The biological actions of steroid and lipophilic hormones are mediated by the transcriptional control of target genes by their nuclear receptors, which form the steroid/thyroid receptor superfamily. There are two domains that are responsible for the transactivation function of nuclear receptors. One of these functions, designated activation function-1 (AF-1), is localized in the N-terminal A/B domain, and the C-terminal ligand-binding domain encompasses the AF-2 function. The transactivation function is activated by binding of its cognate ligand, then forms a larger complex to stimulate transcription. This complex is considered to contain basic transcription machinery and coactivator complexes. For ligand-induced transactivation of nuclear receptors, two coactivator complexes have been identified to date. One is a recently identified complex, DRIP/TRAP (1, 2), and the other is thought to contain CBP/P300 (3), SRC-1 (4, 5), TIF2/GRIP1 (6, 7), AIB1/RAC3/ACTR/TRAM-1 (8–11) and SRA (12). These factors, called nuclear receptor coactivators, enhance the transactivation function of nuclear receptors by directly associating in a ligand-dependent way. One of the molecular mechanisms underlying the actions of coactivators may be due to its intrinsic histone acetyltransferase activity, by which histones are acetylated to activate the chromatin structure from a transcriptionally repressed state (10, 13, 14).

SRC-1 is a nuclear receptor coactivator sharing sequence homology with TIF2/GRIP1 and AIB1/RAC3/ACTR/TRAM-1 as a member of the p160 coactivator family, and exhibits histone acetylation activity (13). This coactivator is expressed in many tissues and cells (15). SRC-1 is shown to associate many with nuclear receptors through interactions with helix 12 in the C-terminal ligand binding domain, and enhance the transcriptional activation in a ligand-dependent manner (16, 17). As the sequences of helix 12 are well conserved among the nuclear receptors, SRC-1 seems to act as a common coactivator for nuclear receptors, rather than as a specific coactivator for limited receptors (18, 19). Recently, Xu *et al.* reported that the loss of SRC-1 gene in mice results in a lack of estradiol (E_2)-responsiveness in the development of reproductive organs (20), indicating a physiological importance of SRC-1 in E_2 actions in naïve animals. E_2 is well known to regulate cell growth and differentiation of female reproductive organs, and the actions are considered to be mediated by transcriptional control of E_2 target genes by nuclear estrogen receptors (ERα and ERβ; 21, 22). Therefore, in this study, to directly assess the function of SRC-1 in E_2-induced cell growth/differentiation controlled by ERs, we established a sta-

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ble transformant overexpressing human SRC-1 in the E_2-dependent breast cancer cell line MCF-7. We studied the action of E_2 on cell growth and the expression of E_2 target genes. We found that SRC-1 overexpression potentiates cell growth stimulation by E_2, reflecting enhanced E_2-responsiveness to gene expression by SRC-1. These results suggest the importance of SRC-1 in E_2 actions on cell growth as well as transcriptional control by ER.

MATERIALS AND METHODS

DNA. Human SRC-1a cDNA (hSRC-1a; identical to F-SRC-1 [GenBank accession number: HSU59302]; 23) was cloned by RT-PCR from HeLa cells, subcloned into pBluescriptII SK(−) cloning vector (Stratagene) at the *Sma*I–*Xba*I site, and a 4.3-kb *Xho*I–*Xba*I cDNA fragment was finally inserted into the pcDNA3 expression vector (Invitrogen). The cDNAs containing a 1.1-kb fragment of the N-terminal SRC-1a, 0.6-kb fragment of C-terminal TIF2 (6), and 1-kb fragment of C-terminal AIB1(8), respectively, were radiolabeled by random priming (TaKaRa biomedicals, Japan) and employed as probes for Northern blot analysis of the expression of p160 nuclear receptor coactivators. For the analysis of pS2 expression, a 0.3-kb pS2 fragment was synthesized as probe by PCR using a plasmid containing pS2 cDNA (kindly donated by P. Chambon).

Cells. Human MCF-7 breast cancer cells and their transformants were routinely maintained in phenol red-free DMEM (Life Technologies) supplemented with 10% FBS (Hyclone), and subcultured [1:3] every 3 to 5 days. For transformant culture, G418 (Sigma) was added at a concentration of 200–300 μg/ml. Cells were given fresh medium every 2 days. To establish stable transformants, parent MCF-7 cells were transfected with pcDNA3-hSRC-1a by calcium phosphate precipitation and cultured for 10 days in the presence of 500 μg/ml G418 for transformant selection. Then the cells were harvested and replated onto fresh dishes, and further cultured for 2 weeks in 500 μg/ml G418. Individual colonies were selected, and each colony was expanded in 200–300 μg/ml G418 before expression check.

Cell growth assay. Cell growth was estimated by XTT colorimetric assay (24, 25). Quadruplicate cultures [1–3 $\times$ 10^4 cells] of pcDNA3-hSRC-1a-transfected S7 clone or pcDNA3-transfected control clone (C1 clone) were inoculated into the wells of 24-well tissue culture plates, and cultured for the indicated periods in 0.5 ml phenol red-free DMEM supplemented with 2.5% charcoal-stripped FBS (medium A). One day after seeding (day 1), E_2 or 4-hydroxytamoxyfen (OHT) was added at the final concentrations indicated in the figures. Every 2 days, medium was removed and fresh medium containing ligands was given. At the end of culture, cells were treated at 37°C for 45 min with 0.2 mg/ml XTT (Sigma) and 15 μM PMS (Sigma) in medium A. Optical density of each well was measured by a spectrophotometer (Beckmann) at a test wavelength of 450 nm, and background wavelength of 650 nm. Preliminary experiments showed a linear relationship between cell number and optical density in the range of 1–15 $\times$ 10^4 MCF-7 cells/cm^2 (data not shown).

Northern blot. To test pS2 expression, 1–2 $\times$ 10^6 cells were plated on 90-mm-diameter culture dishes in phenol red-free DMEM plus 5% charcoal-stripped FBS (medium B). Cells were given fresh medium every 2 days. After 1 week culture, the cells were washed with phenol red-free DMEM and treated with medium A containing E_2 for 12 or 24 h. Then crude total RNA was extracted by acidic phenol/chloroform (26). Total RNA (10 μg) was fractionated in 1.5% agarose gel and transferred onto BA-S85 nitrocellulose membranes (Schleicher & Schull). For Northern blot of p160 coactivators, poly(A)+ RNA was extracted from cells using a commercial kit (Pharmacia biotech). Poly(A)+ RNA (1 μg) was electrophoresed and transferred onto membranes. The membranes were first probed with ^{32}P-labeled cDNA fragments for 16 h, then washed three times with 2× SSC–0.1% SDS at room temperature, and twice with 0.5× SSC–0.1% SDS at 42°C. To detect TIF2 or AIB1 transcript, the membranes were re-hybridized with ^{32}P-labeled TIF2 or AIB1 probe after dehybridization. Quantitative analysis was performed using a BAS-2000 analyzer (Fuji film, Japan) followed by exposure on X-ray film (Kodak XAR-5).

RT-PCR. The first strand of cDNA was synthesized from 1 μg total RNA using Avian myeloblastosis virus (AMV) reverse transcriptase (TaKaRa biomedicals, Japan) in a final volume of 20 μl. Subsequent PCR was performed as follows: 96°C for 5 min (initial denaturation step), 35-cycle amplification at 96°C for 30 s (denaturing), 55°C for 45 s (annealing), and 68°C for 1 min (extension), and additive incubation at 72°C for 5 min (final elongation step), with 1 μl of RT reaction product. To estimate the contamination of genomic DNA, RT-PCR was carried out in the same way as in the S7 clone except no transcriptase. The primers used were 5′-ACGACTCACTATAGGGAGACC-3′ (N-terminal 5′-primer), 5′-CTCCCTTCACAGTTCACAAC-3′ (N-terminal 3′-primer), 5′-GCCCAGATGCAGATGAGCTC-3′ (C-terminal 5′-primer), and 5′-GCGAGCTCTAGCATTTAGGTGACAC-3′ (C-terminal 3′-primer). PCR products were electrophoresed on 1.5% agarose gel with estimation of product length from a 1-kb DNA ladder marker (Life Technologies).

Transfection assay. Cells (2–3 $\times$ 10^5) were seeded onto 60-mm culture dishes and cultured for 2 days in DMEM containing 10% FBS. After washing twice with phenol red-free DMEM, cells were transfected with a CAT reporter vector containing a consensus estrogen-responsive element (ERE) fused with the sequence from rabbit β-globin promoter ("ERE-G-CAT"; 27), with LipofectAMINE (Life Technologies). After transfection (15–24 h), cells were washed with phenol red-free DMEM and given medium A containing E_2 at the indicated concentrations. Culture was maintained for 36–48 h, then cells were scraped, collected and lysed by the freeze–thaw cycle technique (27). The concentration of synthesized CAT protein in cell lysates was measured by a commercial ELISA kit (Boehringer-Mannheim).

RESULTS

Establishment of the stable transformant cell line overexpressing human SRC-1. To establish the stable transformant cells overexpressing SRC-1, we selected the human breast adenocarcinoma MCF-7 cell, since it is well known that 17β-estradiol (E_2) potentiates the growth of MCF-7 (28–30). MCF-7 cells were transfected with human SRC-1a expression vectors and cultured in the presence of G418 for the selection of stable transformants. One of the clones, designated S7 hereafter, expressed a 4.5-kb transcript, which is the putative length of the transfected human SRC-1 cDNA plus poly (A)+ sequence (lane 2 in Fig. 1A). In this S7 clone, endogenous SRC-1 transcripts were detected as doublets of 7-9 kb in length, as observed in control C1 clone transfected with the parent control vector pcDNA3 (lane 1 in Fig. 1A). RT-PCR with a set of primers corresponding the 5′- and 3′-untranslated regions, or SRC-1 expression vector, further confirmed the expression of exogenous human SRC-1 transcripts (Fig. 1B, lane 1 and lane 2), indicating integration of the exogenous SRC-1 into the chromatin. Thus, taken together, these results show that the exogenous SRC-1 gene was

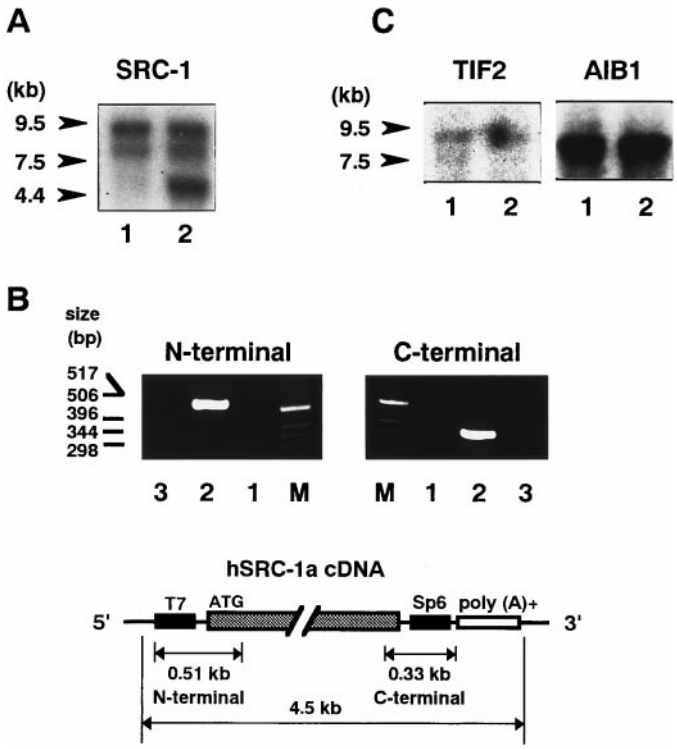

FIG. 1. Establishment of a stable transformant overexpressing human SRC-1. (A) A putative transcript of 4.5 kb derived from exogenous SRC-1 cDNA was detected in an SRC-1 transformant (S7 clone; lane 2), but not in a control cell (C1 clone; lane 1). (B) RT-PCR was carried out using the primers designed to synthesize 0.51-kb fragments containing a start codon ("N-terminal"), or 0.33-kb fragments containing a stop codon ("C-terminal"; see schematic figure) from exogenous SRC-1 cDNA. The PCR products in the S7 and C1 cells were shown in lane 2 and 1, respectively. M, DNA marker. Result of the reaction without reverse transcriptase is shown in lane 3. (C) Membranes used in A were further hybridized with the probe of either TIF2 cDNA or AIB1 cDNA and finally with GAPDH probe for normalization (data not shown).

integrated and expressed at significant levels in the S7 clone.

Expression of two other p160 nuclear receptor coactivators was not affected by SRC-1 overexpression. Recently Xu *et al.* reported that, in SRC-1 knock-out mice, loss of SRC-1 caused an increase in the expression of TIF2, a p160 coactivator family protein (20). This upregulation is thought to occur as a compensation for the loss of SRC-1 function in naive animals. Therefore, it is possible that the expressions of the other p160 coactivator members, such as TIF2/GRIP1 or AIB1/RAC3/ACTR, are constitutively downregulated due to overexpression of SRC-1 in the S7 clone. To test this possibility, we performed Northern blot analysis to examine the expressions of endogenous TIF2 and AIB1 genes in the S7 clone. Consequently, we found that, unlike in the SRC-1 knock-out mice, the expressions of the TIF2 and AIB1 genes were not significantly affected by SRC-1 overexpression in this clone (Fig. 1C).

SRC-1 overexpression enhanced E_2-responsiveness in gene expression. To examine the effect of overexpressed SRC-1 in E_2-induced transactivation function of estrogen receptors (ER), the E_2-responsiveness in gene expression was studied by examination of the endogenous gene expression and assessment of endogenous ER function by a transient expression assay. pS2 was used as the endogenous E_2 target gene, since this gene expression is shown to be under transcriptional control by E_2-bound ER via its gene promoter in MCF-7 cells (31, 32). Stimulation of pS2 gene expression by E_2 treatment (12 h) was seen in the C1 cells. As expected, the E_2 responsiveness was clearly potentiated 2-3 fold in the S7 cells (upper panels in Fig. 2A). Such potenti-

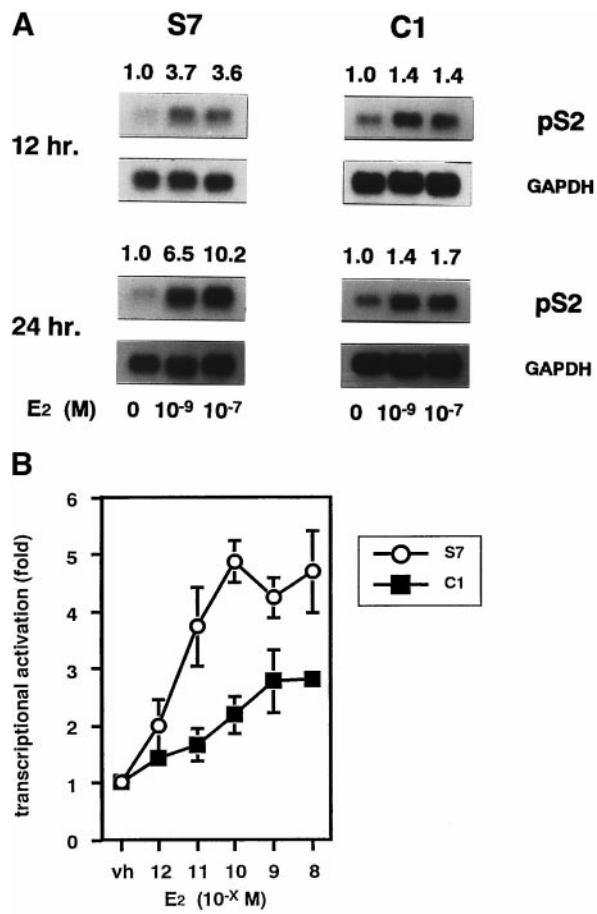

FIG. 2. Enhancement of E_2-induced gene expression by SRC-1 overexpression in MCF-7 cells. (A) S7 and C1 cells were treated with E_2 or ethanol (untreated control) for 12 or 24 h, then endogenous pS2 gene expression was assessed by Northern blot analysis. The E_2-induction ratio between E_2-treated and untreated cells was shown above the panel. (B) S7 (open circles) and C1 (solid squares) cells were transiently transfected with ERE-G-CAT and stimulated by E_2 at the indicated concentrations. The induced CAT protein was measured to assess the E_2-induced transactivation function of endogenous ER. The E_2-induction ratio of the CAT concentration between E_2-treated and untreated cells was calculated and taken as the result. Data are shown as the means ± SD of triplicate cultures.

ation by SRC-1 was more marked after 24 h of treatment (lower panels in Fig. 2A). To further assess the effects of SRC-1 overexpression in ER-mediated gene expression in the S7 clone, we examined the transactivation function of endogenous ER by a transient expression assay. An ERE-CAT reporter gene containing the rabbit β-globin promoter and a consensus estrogen response element (27) was transiently transfected into the S7 clone, and E_2-responsiveness was studied by monitoring CAT production with ELISA (Fig. 2B). In S7 clone cells, CAT protein production increased in an E_2-dose-dependent manner (EC_{50}; 3×10^{-12} M) and reached plateau at 10^{-10} M E_2 (approximately 5 fold over the vehicle control), while CAT production in the C1 cells had a lesser response to E_2 (EC50; 2×10^{-11} M), in agreement with the potentiated E_2-responsiveness in the endogenous pS2 gene expression. These findings suggested that SRC-1 overexpression augments E_2-responsiveness in gene expression, possibly through the enhanced transactivation function of ER by SRC-1 overexpression.

SRC-1 overexpression potentiated E_2-induced cell growth of MCF-7. We next examined the effect of the SRC-1 overexpression on E_2-induced growth of MCF-7 to clarify whether overexpressed SRC-1 augments E_2-induced cell growth, reflecting the potentiated transactivation function of endogenous ER by SRC-1. The S7 and C1 clones were cultured for 7 and 9 days in the absence or presence of E_2 at the indicated concentrations. Cell number was estimated by colorimetric assay (XTT assay) and the increased cell numbers are shown as ratios to the numbers of untreated cells. Stimulation of cell growth by E_2 resulted in a 30% increase in the control C1 cells (right panel in Fig. 3A). In S7 cells, this stimulatory effect of E_2 was more prominent, especially as a 100% increase when the cells were cultured for 7 days (left panel in Fig. 3A). Thus, these findings clearly indicated that SRC-1 plays a significant role in E_2-induced cell growth in MCF-7 cells.

Tamoxyfen (TAM), an estrogen-related compound, exhibits partial agonistic or antagonistic activity in a cell-type specific manner (33–35). Molecular dissection study of ERα shows that TAM blocks only the function of AF-2 (activation function-2) in the ligand-binding domain, but not AF-1 in the N-terminal A/B domain of ERα, so the agonistic action of TAM is supposed to be exerted via the AF-1 function of the TAM-bound ERα (33). Thus, we compared the action of TAM with E_2 in cell growth stimulation of the S7 clone cells, and found that an active form of TAM, 4-hydroxytamoxyfen (OHT), at 10^{-7} M had little effect on cell growth in either S7 or C1 cells (Fig. 3B). These findings indicated that TAM acts as an antagonist to cell growth, possibly inhibiting the SRC-1-mediated AF-2 function of ERs.

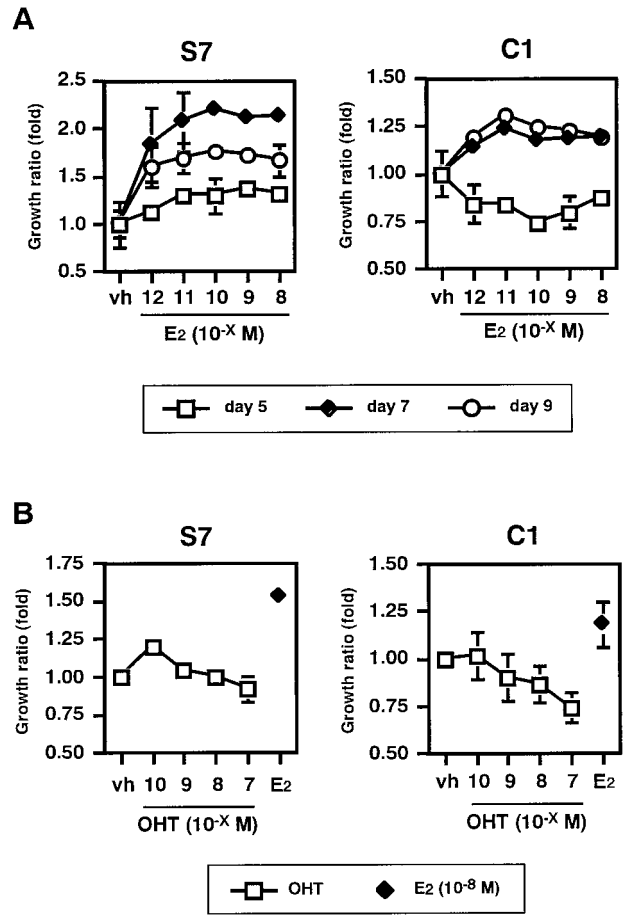

FIG. 3. SRC-1 overexpression potentiates E_2-stimulated cell growth of MCF-7 cells. The S7 and C1 cells were cultured on 24-well plates with ligands. After culturing for the indicated periods, cell growth was estimated by XTT assay. A shows the effect of E_2 on cell growth. vh, vehicle; solid diamonds, day 7; open circles, day 9. In B, open squares, OHT; solid diamond, E_2 (10^{-8} M). Effect of OHT on cell growth on day 9 is shown in B. Data are shown as the means ± SD of quadruplicate cultures.

DISCUSSION

E_2 is considered to control the development and function of female and male reproductive organs, and is also well known to stimulate development in some types of breast cancer. These E_2 actions are thought to be mediated by ERα (36, 37) and ERβ (38–40), which act as ligand-inducible transcriptional factors, by recruiting common coactivators such as CBP/p300 (41), SRC-1/TIF2 family proteins (6, 8, 35). Loss of the ERα gene in mice showed that it plays a crucial role in E_2 action on female reproductive organs (42). Therefore, it is of interest which ER coactivator is essential for ER function in E_2-induced development of normal tissues and E_2-dependent tumors. To address this issue, we examined SRC-1 function in E_2-induced cell growth in the breast cancer cell line MCF-7 by establishing a stable transformant overexpressing SRC-1. One trans-

formant (S7) expressed the putative exogenous SRC-1 transcript at significant levels, and E_2-induced transcriptional control of the endogenous gene and endogenous ERs were indeed potentiated (Fig. 2). These findings clearly support previous reports that ERα and ERβ require SRC-1 for ligand-induced transactivation *in vitro* (4, 40). Most notably, such potentiation by SRC-1 overexpression was also observed in the E_2-induced cell growth of MCF-7 cells. Thus, it is likely that SRC-1, which is a major ER coactivator in transcription control, plays a significant role in ER-mediated cell growth.

There is increasing evidence of nuclear receptor coactivators associating with AF-2 and AF-1 in ligand-dependent or -independent manners (40, 43, 44). Most of them, including SRC-1, act as common coactivators among nuclear receptors, and only a few that are specific for some nuclear receptors have been reported (19, 45). In this respect, it is remarkable that the loss of SRC-1 gene in mice causes a significant decrease in E_2-responsiveness of reproductive organs, presumably mediated by ERs (20). In agreement with these findings, we observed in this study that SRC-1 overexpression potentiates E_2-responsiveness in cell growth. However, since coactivators other than SRC-1/TIF2 family proteins can potentiate ER function (41, 46), it is not possible to exclude their significant functions in E_2-induced cell growth. Especially, we have recently shown that the cross-talk between estrogen and growth factor signalings is mediated through MAP kinase-mediated phosphorylation of human ERα AF-1 A/B domain (21), and furthermore the p68 RNA helicase protein was identified to act as a ERα AF-1-specific coactivator in a MAP kinase-mediated phosphorylation-dependent manner (22). Therefore, it is possible that the p68 coactivator plays a key role in the E_2-induced cell growth in response to stimulation by growth factors, which activate MAP kinase. Nevertheless, it is clear that SRC-1 is important for ER function to induce a particular set of E_2-target genes which control cell and tissue growth. Considering that the molecular mechanisms of E_2-induced development in breast cancer and reproductive organs are largely unknown, identification of the target genes controlled by the SRC-1-ER complex is of particular interest.

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