Estrogen receptor activation at serine 305 is sufficient to upregulate cyclin D1 in breast cancer cells

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Abstract **Recent studies have shown that p21-activated kinase 1 (Pak1) phosphorylates estrogen receptor- (ER) at Ser 305 and also promotes its transactivation function. Here, we sought to investigate whether substitution of serine 305 in ER with glutamic acid (ER-S305E), which mimics the phosphorylation state, would influence the status of ER-target genes. To explore this possibility, we generated clones overexpressing ER-S305E in ER-negative MDA-MB-231 cells and analyzed the status of ER-regulated genes using a gene array. Results indicated that the expression of ER-S305E is sufficient to upregulate the expression of a few but not all ER-regulated genes, i.e., cyclin D1 and zinc finger protein 147 (estrogen-responsive finger protein), while there was no significant change in the expression of remaining genes on the array. In addition, we found an increased expression as well as nuclear accumulation of cyclin D1 protein in MDA-MB-231 cells expressing ER-S305E as compared to the level of cyclin D1 in MDA-MB-231 cells expressing WT-ER or pcDNA. Furthermore, ER-S305E, but not mutation of ER-S305 to alanine, enhanced the cyclin D1 promoter activity. These findings suggest that ER activation at S305 is sufficient to upregulate the expression of cyclin D1, an ER-regulated gene that is implicated in the progression of breast cancer. Phosphorylation of ER by Pak1 or its upstream regulators could upregulate the expression of a subset of ER-target genes in a ligand-independent manner and hence, might contribute toward the development of hormone independence in breast cancer cells.**

Keywords: Breast cancer; Estrogen receptor; Estrogen; p21-activated kinase 1; Cyclin D1

1. Introduction

The small GTPases, including Cdc42 and Rac1, have been implicated in the regulation of mammalian cell morphology and motility [1]. More specifically, Rac1 induces cortical actin polymerization seen as membrane ruffling and lamellipodia, and Cdc42 induces the formation of peripheral actin microspikes and filopodia [2–4]. The small GTPases regulate the formation of cytoskeletal structures via a family of serine/threonine kinases known as p21-activated kinases (Paks). Activation of Pak1 is accompanied by the disassembly of stress fibers and focal adhesion complexes as well as maintenance of the integrity of the motile leading edge [5,6]. Pak1 is activated by a number of extracellular signals, including heregulin (HRG) and epidermal growth factor (EGF), which are potent inducers of Pak1 activity and cell motility of breast cancer cells [7,8]. Activation of Pak1 involves autophosphorylation of several sites, including threonine 423 (T423) within the auto-inhibitory loop of the kinase [9]. Accordingly, Pak1 phosphorylation at T423 has been linked with its activation, as substitution of the acidic residue glutamic acid (E) at this site yields a constitutively active T423E Pak1 enzyme [9,10]. In addition to its cytoskeleton effects, Pak1 also activates JNK and ERK kinases, and thus influences nuclear signaling [11,12].

Recent studies suggest that in addition to cell motility, Pak1 is also involved in breast cancer progression. Adam et al. [13] have shown a mechanistic role for Pak1 activation in the increased cell invasion of breast cancer cells by HRG. Furthermore, expression of a kinase-dead Pak1 mutant in the highly invasive breast cancer cell lines MDA-MB-435 and MDA-MB-231 led to stabilization of stress fibers, enhanced cell spreading and reduction in invasiveness [14]. Conversely, hyperactivation of the Pak1 pathway by conditional expression of catalytically active T423E Pak1 in the non-invasive breast cancer cell line MCF-7 promotes cell migration and anchorage-independent growth [15]. Furthermore, increased Pak1 activity correlates well with the invasiveness of human breast cancer cells and tumors [15]. Emerging data suggest that Pak1 may be overexpressed in human cancers. For example, Pak1 gene amplification has been also reported in ovarian [16] and breast [17] cancers. Furthermore, Pak1 protein has been shown to be upregulated in ovarian tumors [16] and breast cancer [15,18,19].

Overexpression of cyclin D1 has been noted in over 50% of human breast tumors of all histological types [20–22]. Cyclin D1 overexpression is found at the earliest stages of breast cancer progression such as ductal carcinoma in situ and maintained in all stages of metastasis [23]. The expression of cyclin D1 is regulated by diverse signaling cascades [20–23]. In addition, upregulation of cyclin D1 by estrogen receptor-α (ERα) signaling is accompanied by an increased proliferative response in breast cancer cells [24,25], as ERα-stimulated proliferation could be effectively blocked by antisense cyclin D1 [25]. Upregulation of cyclin D1 expression has also been found in hyperplastic mammary glands and proliferative human breast disease [26,27]. Together, these observations suggest that cyclin D1 may constitute an important downstream

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target of diverse upstream signals with a role in mammary gland development and tumorigenesis.

More recently, Pak1 has been shown to directly phosphorylate ERα at Ser305 and to promote its transactivation functions [28]. Additionally, expression of kinase-active T423E Pak1 transgene in mammary glands induces hyperplasia in the mammary epithelium [28], a phenotype manifested by several other oncogenes including cyclin D1 [20]. To directly implicate the ERα-S305 in the biology of breast cancer cells, here we investigated whether activation of ERα by replacing serine with glutamic acid at residue 305 can upregulate estrogen signaling pathway genes using an estrogen signaling pathway gene array.

2. Materials and methods

2.1. Cell cultures and reagents

MDA-MB-231 human breast cancer cells were maintained in Dulbecco's modified Eagle's medium F12 (1:1) supplemented with 10% fetal calf serum. Antibodies were purchased from the following companies: anti-cyclin D1 from the Santa Cruz Biotechnology, Santa Cruz, CA, and anti-Vinculin from Sigma. The anti-T7 epitope antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).

2.2. Generation of stable cell lines

MDA-MB-231 cells stably expressing pcDNA, T7-ERα-S305E and T7-WT-ERα were generated by transfecting pcDNA, pcDNA-T7-ERα-S305E and pcDNA-T7-WT-ERα by using the calcium phosphate method. Forty eight hours after transfection, cells were selected by G418 (1 mg/ml) selection. Approximately 50 clones were isolated. Twenty clones were screened for the selection of individual clones.

2.3. Cell extracts and immunoblotting assays

For preparation of cell extracts, cells were washed 3× with phosphate-buffered saline and lysed in buffer (50 mM Tris–HCl, pH 7.5; 120 mM NaCl; 1% Triton X-100; 100 mM NaF; 200 mM NaVO5; 1 mM PMSF and protease inhibitor cocktail (Gibco)) for 15 min on ice. The lysates were centrifuged in an Eppendorf centrifuge at 4 °C for 15 min. Cell lysates containing equal amounts of protein were resolved on a 10% SDS–PAGE, transferred to nitrocellulose and probed with the appropriate antibodies, using an ECL method. Vinculin expression was used as protein loading control.

2.4. RNA isolation and cDNA array analysis

Total RNA was isolated using TRIzol reagent (Invitrogen) following the manufacturer's protocol. Aliquots of RNA (5–10 μg) were used to analyze gene expression of estrogen signaling pathway (Cat # hGEA9914010) by GE array technology as per the manufacturer's instructions (SuperArray, Inc., Bethesda, MD). Relative amounts of mRNA transcripts were quantified using Molecular Imager System and GE Array Analyzer version 1.3 software. The relative amount of a given gene transcript was estimated by background substraction and normalizing the signal with the signal derived from β-actin.

2.5. Immunofluorescence and confocal imaging

Cells were plated on glass coverslips in six-well culture plates. After 24 h, the cells were rinsed in PBS, fixed in 4% paraformaldehyde and then processed for routine immunofluorescent localization of cyclin D1, T7-tag and F-actin (using Alexa-568-labeled Phalloidin, Molecular Probes, Inc., Eugene, OR). The DNA was visualized by counterstaining with ToPro3 (Molecular Probes). Fluorescent labeling was visualized using a Zeiss LSM 510 microscope and a 63× objective.

2.6. Cyclin D1 promoter–reporter assays

Subconfluent MDA-MB-231 cells cultured in six-well plates were transiently cotransfected with 0.5 μg of −1745 bp cyclin D1 promoter–luciferase reporter construct [19], 1 μg of WT-ER, ERα-S305E and ERα-S305A [28], and 10 ng of β-galactosidase using the FuGENE-6 reagent according to manufacturer's protocol (Roche Molecular Biochemicals, Indianapolis, IN). Twenty four hours after transfection, cells were lysed with passive-lysis buffer and luciferase assay was performed using Luciferase Reporter Assay Kit (Promega, Madison, WI). β-Galactosidase activity was used to normalize the transfection. Each transfection was performed in triplicate wells [19].

2.7. Statistical analysis

Statistical analysis was done using Student's *t*-test and values with $p < 0.05$ were considered statistically significant.

3. Results and discussion

Earlier work from this laboratory has shown that Pak1 phosphorylates ERα at Ser305 and promotes transactivation functions of ER [28] and that Pak1 regulates cyclin D1 expression in mammary epithelial and breast cancer cell lines [19]. Since ER signaling has been shown to upregulate cyclin D1 expression [24,25] and because an ER serine 305 to glutamic acid mutant (ER-S305E) mimics Pak1 phosphorylation, we tested the possibility whether Pak1 activation of ERα-S305E is sufficient to influence the status of estrogen-inducible genes. To address this hypothesis, we first generated stable clones from ER-negative MDA-MB-231 cells expressing T7-tagged ERα-S305E or T7-WT-ERα or pcDNA. We characterized four individual pcDNA-T7-ERα-S305E clones, three individual pcDNA control vectors and T7-WT-ERα expressing clones. Immunoblot analysis of the cell lysates from exponentially growing cells revealed the expression of T7-ERα in ERα-S305E and WT-ERα stable clones (Fig. 1).

The effect of ERα-S305E substitution on ER-responsive genes was studied using a human estrogen signaling pathway gene array. Interestingly, of the 26 genes analyzed (Table 1), expression of ERα-S305E significantly enhanced only the expression of cyclin D1 and estrogen-responsive finger protein [29], compared to the pcDNA clones, and there was no significant change on other genes spotted on the array (Fig. 2). These results suggested that ERα-S305E expression is sufficient to upregulate the expression of selected ER-regulated gene such as cyclin D1 in a ligand-independent manner.

We next examined the expression of cyclin D1 protein in ERα-S305E clones. Western blot analysis showed that overexpression of ERα-S305E was accompanied by a 7–8-fold upregulation of cyclin D1 protein expression compared to the levels of cyclin D1 in the control clones (Fig. 3). To gain an insight about the functional consequence of ER activation at residue 305 and cyclin D1 expression, we determined the subcellular distribution of cyclin D1 in ERα-S305E and WT-ERα clones by quantitative confocal scanning microscopy. Significant induction of cyclin D1 expression was observed in ERα-S305E clones compared to the WT-ERα and pcDNA control clones, and induced cyclin D1 predominantly accumulates

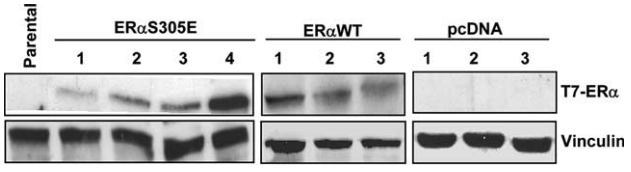

Fig. 1. Characterization of stable clones expressing ERα-S305E. Total cell lysates from ERα-S305E, WT-ERα and pcDNA expressing clones were analyzed by Western blotting with an anti-T7 monoclonal antibody. Vinculin was used as a loading control.

Table 1
Estrogen-regulated genes in the SuperArray

Position	UniGene	GeneBank	Symbol	Description	Gene name
1	Hs.79241	M14745	BCL2	B-cell CLL/lymphoma 2	bcl-2
2	Hs.194143	U68041	BRCA1	Breast cancer 1, early onset	BRCA1
3	Hs.79572	M11233	CTSD	Cathepsin D (lysosomal aspartyl protease)	Cathepsin D
4	N/A	L08752	PUC18	PUC18 bacterial plasmid DNA	PUC18
5	Hs.25647	V01512	FOS	Human cellular oncogene c-fos	c-fos
6	Hs.78465	J04111	JUN	v-jun avian sarcoma virus 17 oncogene homolog	c-jun
7	Hs.79070	X00364	MYC	v-myc avian myelocytomatosis viral oncogene homolog	c-myc
8	Hs.30888	AB007618	COX7A2L	Cytochrome *c* oxidase subunit VIIa polypeptide 2 like	COX7RP
9	Hs.82932	M64349	CCND1	Cyclin D1 (PRAD1: parathyroid adenomatosis 1)	Cyclin D1
10	Hs.9222	AB007619	EBAG9	Estrogen receptor binding site associated, antigen, 9	EBAG9
11	Hs.288061	X00351	ACTB	β-Actin	β-Actin
12	Hs.1579	D21205	ZNF147	Zinc finger protein 147 (estrogen-responsive finger protein)	EFP
13	Hs.2230	X04571	EGF	Epidermal growth factor (β-urogastrone)	EGF
14	Hs.77432	X00588	EGFR	Epidermal growth factor receptor	EGFR
15	Hs.1657	NM_000125	ESR1	Estrogen receptor α	ER α
16	Hs.103504	AB006590	ESR2	Estrogen receptor β	ER β
17	Hs.144321	NM_002128	HMG1	High mobility group protein 1	HMG1
18	Hs.169476	M33197	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
19	Hs.37003	NM_005343	HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog	H-ras
20	Hs.182265	NM_002276	KRT19	Keratin 19	Keratin 19
21	Hs.2905	M15716	PGR	Progesterone receptor	PR
22	Hs.1905	M29386	PRL	Prolactin	PRL
23	Hs.1406	NM_003225	TFF1	Trefoil factor 1 (breast cancer, estrogen-inducible sequence expressed in)	pS2
24	Hs.115256	AF015950	TERT	Telomerase reverse transcriptase	Telomerase
25	Hs.170009	NM_003236	TGFA	Transforming growth factor, α	TGF-α
26	Hs.194679	NM_003881	WISP2	WNT1 inducible signaling pathway protein 2	WISP2

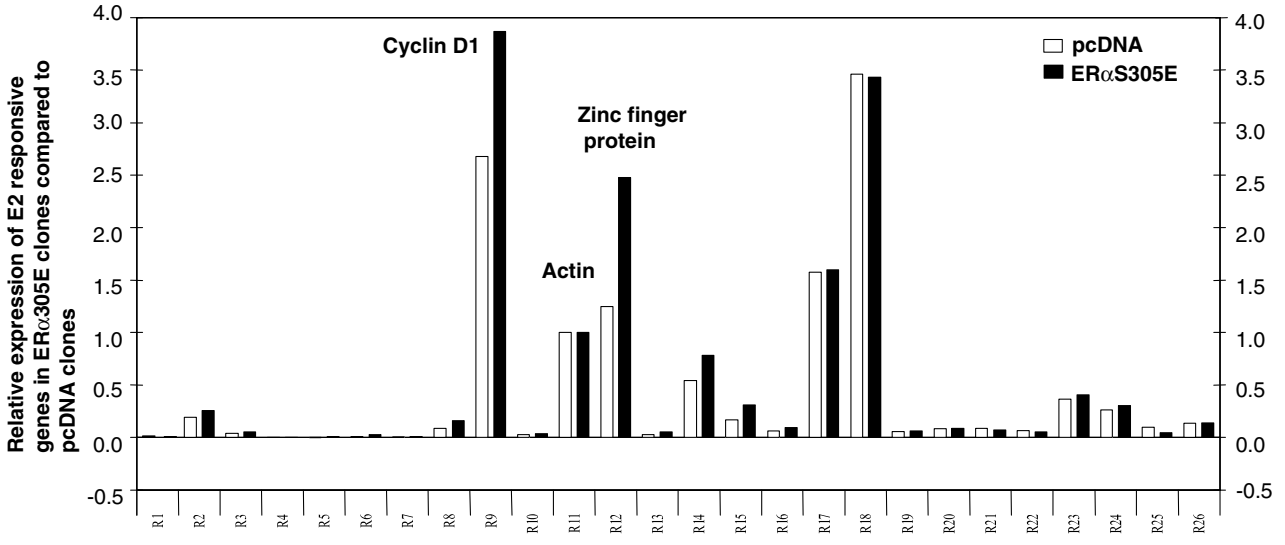

Fig. 2. Effect of ERα-S305E on estrogen-responsive genes. Total RNA was isolated from pcDNA and ERα-S305E expressing clones. Aliquots of 5 μg RNA were taken and hybridized to the cDNA array of estrogen-responsive signaling pathway as per the manufacturer's instructions ([hGEA9912080], SuperArray, Bethesda, MD). Quantitation of the cDNA array analysis in pcDNA and ERα-S305E clones using a Molecular Imager System and GE Array Analyzer version 1.3 software.

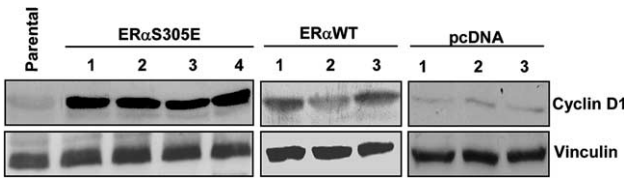

Fig. 3. ERα-S305E upregulates cyclin D1 expression. Total cell lysates from ERα-S305E, WT-ERα and pcDNA expressing clones were analyzed for cyclin D1 by Western blotting with an anti-cyclin D1 monoclonal antibody. Vinculin was used as a loading control.

in the nucleus (Fig. 4, upper panel). Transgene expression in ERα-S305E and WT-ERα clones was validated using confocal microscopy (Fig. 4, bottom panel).

Because ERα-S305E stimulated the expression of cyclin D1 (this study), we further studied the effect of ERα-S305E on cyclin D1-luciferase reporter driven promoter activity. As expected, the ERα-S305E mutant, but not ERα-S305 to alanine mutant (S305A) [28], resulted in a significant induction of cyclin D1 promoter activity as compared to the WT-ERα (Fig. 5A). Together, these findings suggest that ERα phosphorylation

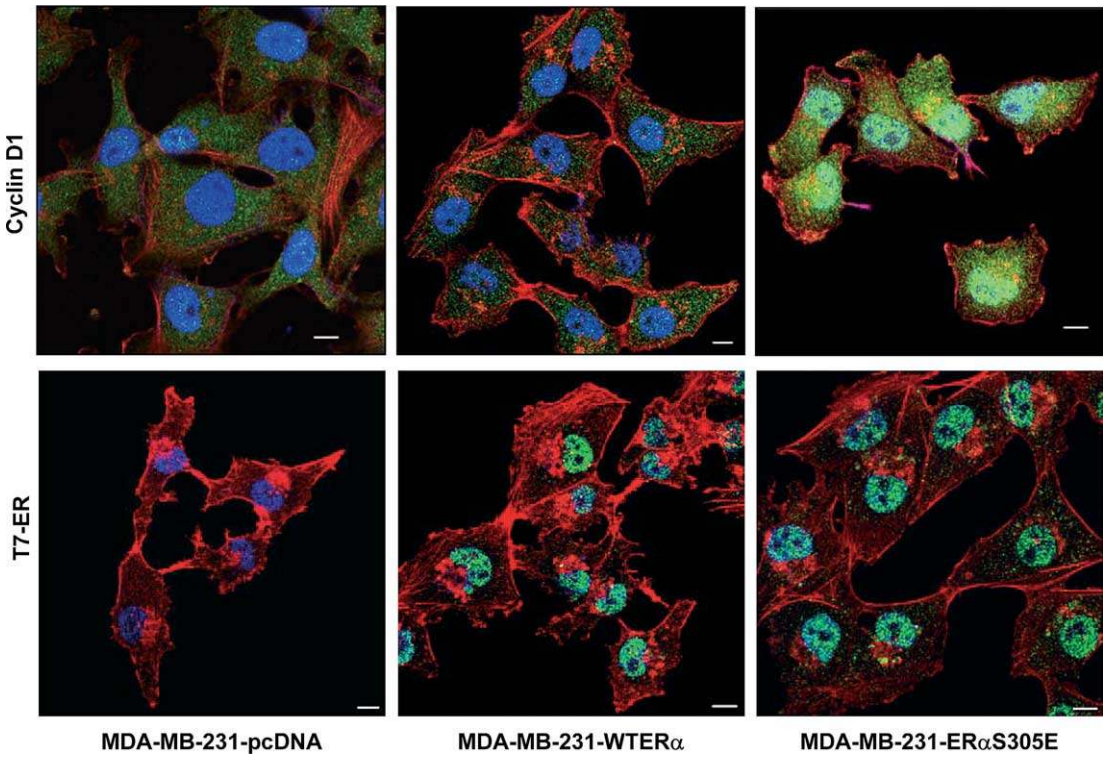

Fig. 4. Effect of ERα-S305E on cyclin D1 expression and localization. MDA-MB-231 cells stably expressing pcDNA, WT-ERα and ERα-S305E were fixed in 4% paraformaldehyde and immunofluorescently stained for endogenous cyclin D1 (green), T7-tag (green), actin (red) and counterstained for nuclear DNA (blue) (63× magnification).

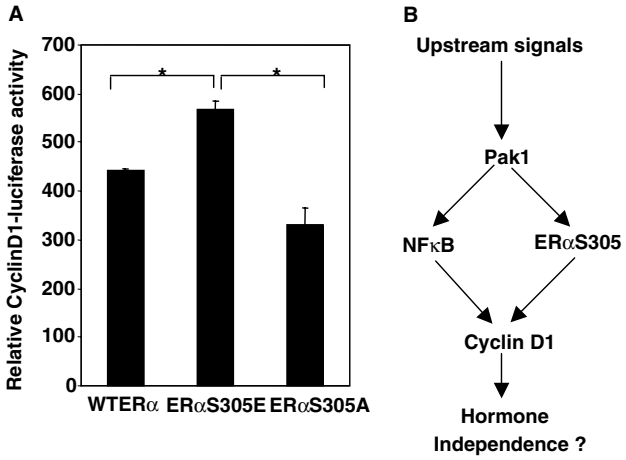

Fig. 5. Effect of ERα-S305E and ERα-S305A on cyclin D1 promoter activity. (A) MDA-MB-231 cells were cotransfected with 0.5 μg of cyclin D1-luciferase reporter, 1.0 μg of WT-ERα, ERα-S305E or ERα-S305A and 10 ng of β-galactosidase. After 24 h, the cells were lysed and luciferase activity was measured. The activity was normalized with β-galactosidase activity. $^{*}P < 0.05$. (B) Schematic representation of the proposed working model.

at Ser305 by Pak1 is important for transactivation functions of the ER pathway via Pak1.

In addition to its natural ligand estrogen, ER has also been shown to be activated by a variety of growth factors [30,31] and because growth factors have been implicated in the development of hormone independence [30,31], our findings presented here suggest a model wherein upstream signals such as growth factors leading to Pak1 activation may potentially activate ER at Ser305 and thus regulate the expression and nuclear functions of cyclin D1. We have recently shown that these functions also can be regulated via NF-κB by Pak1 [19] (Fig. 5B). Since Pak1 regulation of ER at S305 is independent of estrogen [28], it is very likely that this noted ligand-independent increase in cyclin D1 expression might contribute towards the development of hormone-independent phenotypes.

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