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Estrogen regulation of Pak1 and FKHR pathways in breast cancer cells

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Abstract Stimulation of p21-activated kinase-1 (Pak1) and estradiol-estrogen receptor-α in mammary cancer cells promotes cell survival. We sought to determine whether estrogen stimulates the Pak1 pathway. We found that estrogen rapidly activated Pak1 kinase activity in a phosphatidylinositol 3-kinase-insensitive manner. Furthermore, estrogen induced phosphorylation and perinuclear localization of the cell survival forkhead transcription factor FKHR in the cytoplasm in a Pak1-dependent manner. In addition, Pak1 directly interacted with FKHR and phosphorylated it. The noticed phosphorylation-dependent exclusion of FKHR from the nucleus impaired the ability of FKHR to activate its target Fas ligand promoter containing the FKHR binding motif (FRE) in cells treated with estrogen or expressing catalytically active Pak1. In contrast, expression of the dominant-negative auto-inhibitory domain of Pak1 (Pak amino acids 83–149) promoted the ability of FKHR to activate transcription from FRE. Together, these results identify a novel signaling pathway linking estrogen action to Pak1 signaling, and Pak1 to FKHR, suggesting that Pak1 is an important mediator of estrogen's cell survival functions.

Key words: Breast cancer cell; Signaling; Estrogen; Cell survival

1. Introduction

P21-activated kinase (Pak1), a well characterized serine/threonine kinase [1,2] and effector protein of phosphatidylinositol 3-kinase (PI-3K) [1], mediates the cellular effect of polypeptide growth factor on cell motility [3], invasiveness [4], anchorage-independent growth [5,6], and cell survival of human breast cancer cells. Pak1 is an effector of small GTPase cdc42 and Rac1 [7] and binding of activated GTPase to Pak1 regulates the phosphorylation and kinase activity of Pak1. Activation of Pak1 by diverse signals leads to autophosphorylation at several sites, including threonine 423 (T423) within the activation loop of kinase domain [2,5]. Pak1 also modulates the activation status of signaling pathways such as mitogen-activated protein kinase (MAPK) and p38MAPK [8]. The development of human breast cancer is promoted by estrogen (E2) stimulation of mammary epithelial cell growth [9]. The biological effects of E2 are mediated by its binding to the structurally and functionally distinct estrogen receptors (ER) α and β. ERα is the major ER in the mammary epithelium [10]. Upon the binding of E2 to ERα, ligand-activated ERα translocates to the nucleus, and stimulates gene transcription, thus promoting the growth of breast cancer cells [11]. In addition to the transcription effect of E2, it is increasingly accepted that ligand-activated ERα regulates a series of non-genomic events in the cytoplasm [12] including the activation of PI-3K [13]. Because both Pak1 and ERα have been implicated in the progression of breast cancer and because Pak1 is downstream of PI-3K, we sought to determine whether Pak1 has a function in ER signaling and whether E2 utilizes Pak1 to facilitate cell survival.

The consistent involvement of members of the cell survival forkhead transcription factor (FKHR) in chromosomal translocations found in cancer suggests that FKHR plays an important role in regulation of cellular proliferation and differentiation [14]. Several recent studies have shown that FKHR promotes the expression of pro-apoptotic genes such as Fas ligand (FasL) and FKHR phosphorylation triggers its nuclear exclusion and inhibition of pro-apoptotic function [14–16]. The cell survival signaling kinase AKT has been shown to phosphorylate FKHR and support cell survival in mammalian cells [17–19]. Since Pak1 is downstream of AKT and since both Pak1 and FKHR are components of the cell survival pathway, here we investigated whether Pak1 could modulate the functions of FKHR in relation to E2 signaling in breast cancer cells.

2. Materials and methods

2.1. Cell cultures, transfection, cell extracts and reagents

Human breast cancer cells MCF-7 were cultured in phenol red-free medium supplemented with 5% charcoal stripped serum (DCC serum). For E2 treatment cells were E2-deprived for 48 h before treatment. For preparation of cell extracts, cells were washed three times with phosphate-buffered saline and lysed in buffer (50 mM Tris–HCl, pH 7.5; 120 mM NaCl; 0.5% NP-40; 100 mM NaF; 2 mM $NaVO_5$; 1 mM phenylmethylsulfonyl fluoride; 10 μg/ml leupeptin; 10 μg/ml aprotinin) for 30 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 10% SDS–polyacrylamide gel, transferred to nitrocellulose, and probed with appropriate antibodies. Antibodies against phospho-AKT-473, total AKT, phospho-FKHR-Ser-256 and total FKHR were purchased from Cell signaling Technology, USA. Anti-Pak1 antibody was from Neomarker, USA. Transfection was performed using the Fugene-6 kit (Roche Biochemical) as per the manufacturer's instruction.

2.2. Promoter-reporter assay

FKHR-Luc (FRE-Luc) plasmid and wild-type (WT) FKHR were obtained from Michael Greenberg (Harvard Medical School, Boston, MA, USA) [20]. For promoter assay cells were transiently cotransfected with a reporter construct and β-galactosidase. Cells were harvested with passive lysis buffer (Promega) and measured for luciferase activity.

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2.3. In vitro transcription and translation

In vitro transcription and translation of a desired protein were performed using the Transcription-Translation system (Promega) as described [21]. One μg of test plasmid was in vitro translated in the presence of ^{35}S in a reaction volume of 50 μl. The reaction was diluted to 1 ml with NP-40 lysis buffer and an aliquot (250 μl) was used for each glutathione *S*-transferase (GST) pull-down assay. Translation was verified by running 20 μl of reaction on SDS–PAGE and autoradiography.

2.4. GST pull-down and in vitro kinase assays

GST pull-down assays were performed by incubating equal amounts of GST, GST-AF2, GST-WT Pak1, GST-WT FKHR, proteins immobilized to glutathione beads Sepharose (Amersham) with in vitro translated ^{35}S-labeled protein [21]. The mixture was incubated for 2 h at 4°C, washed with NP-40 lysis buffer, bound proteins were eluted with 2×SDS buffer, separated on SDS–PAGE and developed by fluorography. In vitro kinase assay was performed using immunoprecipitation with anti-Pak1 antibody for 4 h at 4°C followed by kinase reaction in kinase buffer (20 mM HEPES pH 7.2; 10 mM $MgCl_2$; 10 mM $MnCl_2$; 1 mM dithiothreitol) containing 10 μCi of [γ-^{32}P]ATP and 25 μM of cold ATP. Reaction was performed in a volume of 40 μl for 30 min at 30°C

2.5. Immunofluorescence and confocal imaging

MCF-7 cells were plated on glass coverslips in six-well culture plates. When the cells were approximately 50% confluent, they were serum-starved for 36 h. Alternatively, 30% confluent MCF-7 cells were maintained in 5% DCC serum for 48 h, then were treated with β-estradiol (10^{-9} M) for 30 min with or without pretreatment with the anti-estrogen ICI 182780 (10^{-8} M) for 1 h. Cells were rinsed in phosphate-buffered saline, fixed in cold 100% methanol for 6 min, then blocked and incubated with primary antibodies against FKHR and myc-tagged followed by fluorochrome-tagged secondary antibodies. Confocal analysis were performed using an LSM510 Zeiss microscope [3–5].

3. Results and discussion

Since E2 has been shown to activate PI-3K and Akt as well as promote cell survival, we wondered whether E2 could also activate Pak1, a downstream target of PI-3K. To explore this possibility, ER-positive MCF-7 cells were treated with E2 in the presence of Ly 294002 (a specific inhibitor of PI-3K) or ICI 182780 (a pure anti-estrogen). E2 stimulation of cells was accompanied by a rapid stimulation of Pak1 activity that was blocked by ICI 182780 but not by Ly 294002 (Fig. 1A). However, ICI 182780 blocked E2-mediated Akt stimulation. E2 also activated Pak1 activity in murine mammary glands cultured in vitro (Fig. 1B). These observations suggested that E2 activates Pak1 activity in a PI-3K-independent manner.

To understand the significance of E2-induced activation of Pak1, a cytoplasmic kinase, we hypothesized that E2 utilizes Pak1 signaling to promote cell survival by phosphorylating FKHR, which was previously shown to be phosphorylated via the PI-3K/Akt pathway [17–19]. Stimulation by E2 in MCF-7 cells was accompanied by increased phosphorylation of FKHR on Ser-256, and this effect was inhibited by ICI 182780 (Fig. 2A). Using confocal microscopy, we further demonstrated that E2 induces perinuclear cytoplasmic localization (green) of the phosphorylated FKHR as compared to non-stimulated or ICI 182780-pretreated cells in which phospho-FKHR is within the nucleus (Fig. 2B). To demonstrate the impact of Pak1 signaling on FKHR subcellular distribution, we showed that transient expression of Myc-tagged (red) WT Pak1 but not dominant-negative K299R Pak1 [4] enhances the level of phosphorylated FKHR (green) in the cytoplasm of MCF-7 cells (Fig. 2C). Interestingly, phosphorylated

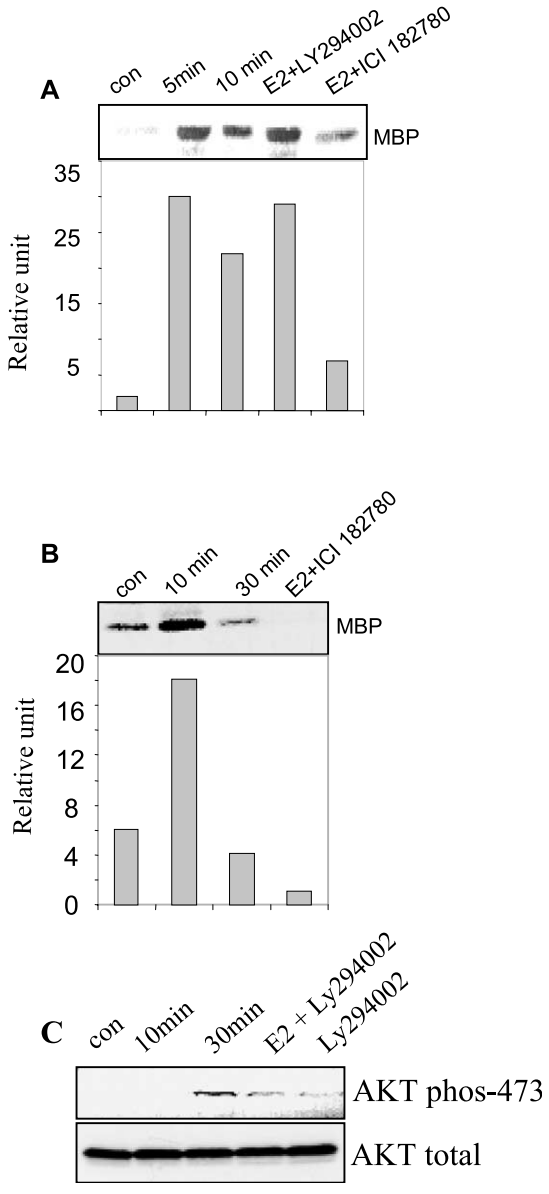


Fig. 1. E2 stimulation of the Pak1 activity. A: MCF-7 breast cancer cells were grown in phenol red-free Dulbecco's modified Eagle's medium–F12 (1:1) supplemented with 5% charcoal-stripped fetal calf serum for 48 h. MCF-7 cells were treated with 10^{-9} M E2 in the presence or absence of ICI 182780 (10^{-8} M) or LY294002 (20 μM) for 10 min, and Pak1 kinase activity was assayed using myelin basic protein (MBP) as a substrate. B: E2 regulation of Pak1 kinase activity in explanted mouse mammary gland tissues of mice using MBP as a substrate ($n=3$). C: MCF-7 cells treated with or without Ly 294002 (20 μM, lanes 4 and 5) were stimulated with E2 (10^{-9} M for 30 min (lanes 3 and 4) or 10 min (lane 2)). Cell lysates were immunoblotted with the indicated antibodies.

FKHR was also co-localized with WT Pak1 as shown by the development of yellow pixels due to overlapping of red and green pixels (Fig. 2C). Transfection of dominant-negative Pak1 (K299RLL, red) also inhibited the ability of E2 to redistribute phosphorylated FKHR (green) from the nucleus to the cytoplasm (Fig. 2C).

To determine whether the observed association between Myc-Pak1 and FKHR was direct or mediated via other proteins, we examined the ability of in vitro translated FKHR protein to bind with GST WT Pak1. As shown in Fig. 3A,

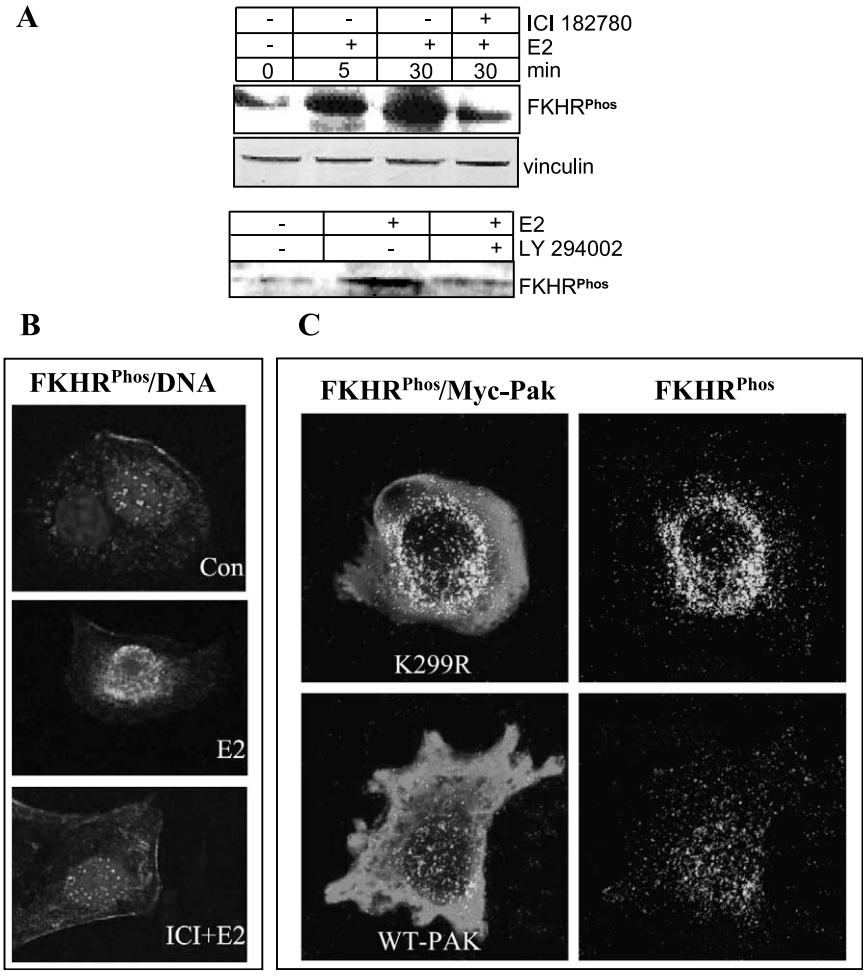
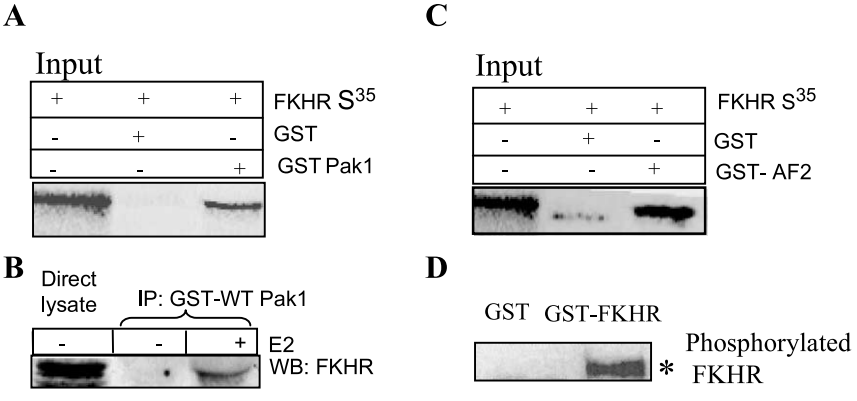


Fig. 2. E2 regulation of the FKHR pathway. A: E2 induced FKHR phosphorylation. MCF-7 cells were treated of 10^{-9} M E2 with or without ICI 182780 (10^{-8} M) or LY294002 (20 μM) for the indicated times. Cell lysates were prepared and were resolved on a 10% SDS–polyacrylamide gel, transferred to nitrocellulose, and probed with an antibody against FKHR Ser-256. The blot was reprobed with a vinculin antibody. B: E2 regulation of FKHR subcellular distribution. MCF-7 cells were grown in phenol red-free medium with 5% charcoal-stripped fetal calf serum for 48 h. Cells were treated with 10^{-9} M E2 in the presence or absence of ICI 182780 (10^{-8} M) for 10 min. Cells grown on glass coverslips were fixed (without permeabilization) at −20°C for 6 min. Confocal analysis was used to determine the FKHR localization using anti-phosphorylated Ser-256 FKHR antibody. Each image represents Z sections at the same cellular level and magnification. Confocal analysis was performed using a Zeiss laser scanning confocal microscope and the established methods [22], involving processing of the same section for each detector (the two excitations corresponding to 546 and 488 nm) and comparing pixel by pixel. Co-localization of two proteins is demonstrated by the development of yellow color due to red and green overlapped pixels. C: MCF-7 cells were transfected with Myc-tagged wild-type (WT) or dominant-negative K299R (DN) Pak1, and cells were treated with or without 10^{-9} M E2 (30 min), and analyzed by indirect immunofluorescence and confocal microscopy using antibodies directed against Myc (for Pak1, red) and phosphorylated Ser-256 FKHR (green). For controls, cells were treated only with the secondary antibodies ($n = 3$).



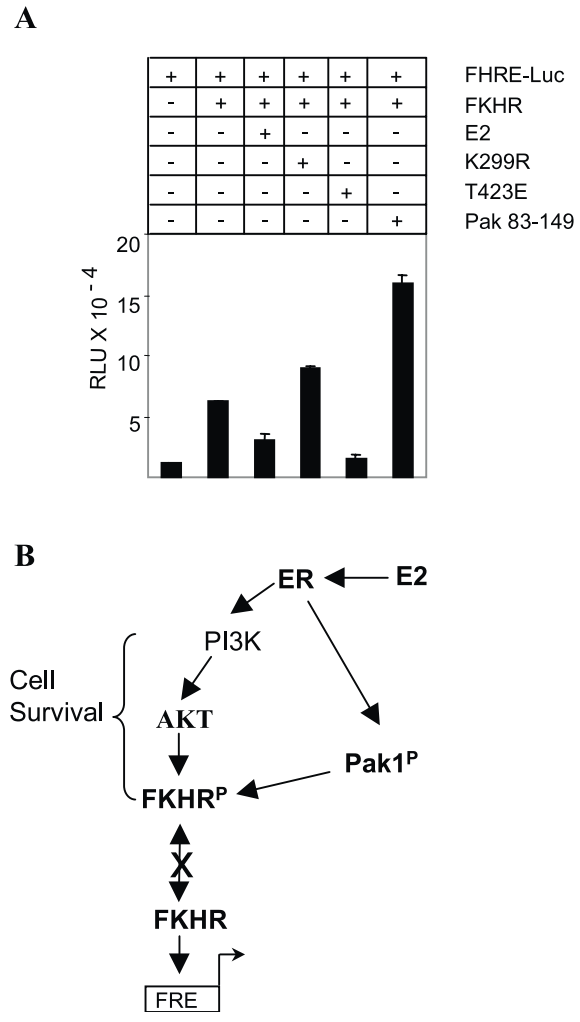


Fig. 4. Estradiol and Pak1 signaling modulation of FKHR target reporter system. A: Effect of E2 and Pak1 expression on FKHR-induced transactivation of FasL-Luc activity. MCF-7 cells were co-transfected with 50 ng each of FasL-Luc reporter with or without 0.5 μg of catalytically active T423E Pak1 or dominant-negative Pak1 inhibitory fragment 89–143. Some cells were treated with 10^{-9} M E2 for 16 h. After 36 h, cells were lysed to measure the Luc activity ($n = 4$). B: Schematic representation of our results presented here.

FKHR interacted with GST-Pak1, but not with GST alone in GST pull-down assays. However, GST pull-down assays using cell extracts from untreated or E2 (10^{-9} M for 30 min)treated MCF-7 cells demonstrated that cellular FKHR interacted with GST-WT Pak1 only in an E2-dependent manner (Fig. 3B), suggesting that E2-mediated modification of FKHR may be important for its binding to Pak1. To understand ER regulation of FKHR, GST pull-down assays were performed. FKHR interacted strongly with GST-AF2 but not with GST alone (Fig. 3C). Next we determined whether FKHR is a substrate of Pak1 by performing in vitro kinase assays with purified Pak1 enzyme and GST-FKHR as a substrate. Pak1 efficiently phosphorylated GST-FKHR (Fig. 3D).

Phosphorylation of FKHR is known to promote both its exclusion from the nucleus and its cell survival function, thus impairing the ability of FKHR to transactivate its target genes [18–20]. Next, we examined the effects of E2 and Pak1 expression on the ability of FKHR to activate a FasL promoter containing the FKHR binding motif (FRE) [20]. As shown in Fig. 4A, pretreatment with E2 as well as expression of catalytically active Pak1 (T423E) prevented the stimulation of FasL-Luc activity by FKHR. In contrast, expression of the dominant-negative auto-inhibitory domain of Pak1 (Pak amino acids 89–143) [22] promoted the ability of FKHR to activate transcription from the FRE-containing reporter system (Fig. 4A), suggesting that Pak1 signaling might impair pro-apoptotic functions of FKHR.

Our findings suggest that E2 activates Pak1 signaling in an ER-dependent manner without involving PI-3K. Findings that FKHR can interacts directly with Pak1 and can be phosphorylated by both E2 and Pak1 have a significant impact on FKHR translocation and thereby on cell survival. These results suggest a model (Fig. 4B) wherein Pak1 regulation of FKHR phosphorylation may support cell survival by impairing its pro-apoptotic nuclear function due to exclusion of phosphorylated FKHR from the nucleus and/or by preventing the translocation of FKHR from the cytoplasm to the nucleus. In summary, we provide new evidence that Pak1 is a signaling component of E2 action, Pak1 influences the cell survival FKHR, and Pak1 acts as an important mediator of the cell survival function of E2.

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Fig. 3. Pak1 interaction and phosphorylation of FKHR. A: Direct interaction of FKHR with Pak1. GST pull-down assays were performed by incubating equal amounts of GST, Pak1 proteins immobilized to GST-Sepharose beads (Amersham) with in vitro translated ^{35}S-labeled FKHR. The mixture was incubated for 2 h at 4°C, washed with NP-40 lysis buffer, bound proteins were eluted with 2×SDS buffer, separated on SDS–PAGE and developed by fluorography. B: Interaction of GST-Pak1 with FKHR from MCF-7 cells treated with or without 10^{-9} M E2 (30 min before cell lysis). C: Direct interaction of FKHR with the ligand binding domain of ER (AF2 domain). AF2 domain immobilized with GST- Sepharose beads with in vitro translated ^{35}S-labeled FKHR. D: Pak1 induced FKHR phosphorylation. Purified Pak1 and GST-FKHR were used for in vitro phosphorylation of GST-FKHR (right panel) ($n = 3$).

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