P21-activated kinase-1 phosphorylates and transactivates estrogen receptor- α and promotes hyperplasia in mammary epithelium

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Stimulation of p21-activated kinase-1 (Pak1) induces cytoskeleton reorganization and signaling pathways in mammary cancer cells. Here, we show that inhibition of Pak1 kinase activity by a dominant-negative fragment or by short interference RNA markedly reduced the estrogen receptor- α (ER) transactivation functions. To understand the role of Pak1 in mammary glands, we developed a murine model expressing constitutively active Thr423 glutamic acid Pak1 driven by the β -lactoglobulin promoter. We show that mammary glands from these mice developed widespread hyperplasia associated with apocrine metaplasia and lobuloalveolar hyperdevelopment during lactation. Mammary tissues with active Pak1 also exhibited an increased activation of mitogen-activated protein kinase and stimulated transactivation functions of the ER and expression of endogenous ER target genes. Furthermore, Pak1 directly phosphorylated the activation function-2 domain of the ER at the N-terminal residue Ser305, and its mutation to Ala (S305A) abolished the Pak1-mediated phosphorylation and transactivation functions of the ER, while its mutation to glutamic acid (S305E) promoted transactivation activity of ER. These findings reveal a novel role for the Pak1-ER pathway in promoting hyperplasia in mammary epithelium.

Keywords: estrogen receptor/hyperplasia/mammary gland/Pak1/transactivation

Introduction

The molecular mechanisms underlying normal mammary gland development and the early stages of mammary tumor development are not well understood but are believed to involve a growth factor-triggered signaling cascade that leads to the activation of protein kinases. One such protein kinase is p21-activated kinase (Pak1), a Ser/Thr kinase (Sells *et al.*, 1997; Tappon and Hall, 1997; Bagrodia and Cerione, 1999; Kumar and Vadlamudi, 2002). Pak1 is an effector of the small GTPases Cdc42 and Rac1 (Manser *et al.*, 1994). Pak1 has been shown to mediate the cellular effects of polypeptide growth factors on the motility and invasiveness of human breast cancer cells (Adam *et al.*, 1998, 2000; Vadlamudi *et al.*, 2000).

Activation of Pak1 by diverse signals led to autophosphorylation at several sites, including Thr423 (T423) within the activation loop of the kinase. 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 (Vadlamudi *et al.*, 2000). Hyperactivation of the Pak1 pathway has also been shown to promote anchorage-independent growth of human breast cancer cells (Vadlamudi *et al.*, 2000). In spite of Pak1 regulation of phenotypic changes in breast cancer cells, its involvement in the estrogen receptor- α (ER) pathway and in mammary gland development remains unknown.

Mammary gland growth and maturation consist of a series of highly ordered events involving interactions among several distinct cell types, which are regulated by the complex interactions among many steroid hormones and growth factors (Hennighausen and Robinson, 1998). In 4-week-old mice, mammary glands become increasingly sensitized to increases in ovarian hormones, which signal the terminal end-buds (club-shaped epithelial structures) to grow away from the nipple region to fill the fat pad. During this rapid but tightly regulated growth phase, an extensive network of epithelial and ductal tree-like branching develops. When the expanding mammary ductal mass reaches the limits of its fat pad, quiescent terminal end ducts and alveolar buds permanently replace the terminal end-bud structures. At the onset of pregnancy, rapid epithelial cell proliferation begins again, resulting in additional ductal branching and lobuloalveolar growth from the ductal skeleton. These alveoli are the functional units of milk production at lactation, the time at which the gland is fully differentiated. After lactation ceases, the gland undergoes massive apoptosis and restructuring, leading to involution and return of the primary ductal structures (Daniels and Siberstein, 1987).

A balance between cell proliferation, cell differentiation and cell death in the stem cell population and throughout the mammary gland is critical for normal development. In contrast, perturbations in this balance can contribute to carcinogenesis. Conditions that up-regulate cell proliferation or down-regulate apoptosis can lead to the accumulation of mutations, which contribute to the subsequent development of breast cancer. Atypical ductal hyperplasia is an abnormal ductal epithelial proliferative stage that can become more aggressive and eventually fill the lumen of the duct; thus, it is considered a precursor of the development of ductal carcinoma *in situ* (Harvell *et al.*, 2000; Mehta *et al.*, 2001). The susceptibility of normal mammary glands to tumorigenesis is influenced by the gland's development, particularly during puberty and pregnancy, stages that are characterized by marked alterations in cell proliferation and differentiation (Russo and Russo, 1982). Protein kinases are the largest class of

Fig. 1. Pak1 is required for optimum stimulation of the ER pathway. (A) Schematic representation of Pak1. Crib motif, amino acids 70–87 and autoinhibitory domain (AID), amino acids 83–149. (B) Upper three panels show RT-PCR analysis. MCF-7 cells (clones 10 and 17) expressing Pak1 inhibitor, amino acids 83–149, inhibitory fragment. Lower panel shows western blotting and the ER level in MCF-7 cells expressing Pak1 inhibitor. (C) Pak1 inhibitor blocks stimulation of ERE-driven transcription. Cells from clones 10 and 17 were transfected with 0.25 μ g of the ERE-luc reporter and treated with or without E2 (1 nM) ($n = 4$). (D) Dominant-negative Pak1 blocks stimulation of endogenous ER target gene product PR in MCF-7 cells. Stable MCF-7 clones expressing pcDNA or Pak1 inhibitor (clones 10 and 17) were treated with or without E2 for 24 h, and cell lysates were immunoblotted with anti-PR antibodies ($n = 3$). (E) Growth rates of MCF-7/vector cells and MCF-7 clones 10 and 17 in response to E2 ($n = 2$).

genes known to regulate growth, differentiation and development in eukaryotic organisms. Perturbations in the regulated expression or function of protein kinases or their associated signaling pathways can lead to malignant transformation of the breast. For example, overexpression of several tyrosine kinases has been shown to contribute to the development of tumorigenic potential in both humans and animal model systems (Alimandi *et al.*, 1995; Bagheri-Yarmand *et al.*, 2001). To determine the role of Pak1 in the pathophysiology of mammary gland development, we explored Pak1 regulation of the ER pathway using tissue culture models and transgenic (TG) mice expressing activated Pak1 in mammary glands.

Results

Pak1 signaling in endogenous ER target gene expression

To explore the role of Pak1 signaling in the regulation of the ER pathway, we sought to determine the role of Pak1 in the induction of ER responsive element (ERE)-driven transcription and expression of the endogenous ER target gene, such as the progesterone receptor (PR). We used well characterized MCF-7 clones 10 and 17, which

ectopically express an auto-inhibitory domain of Pak1 amino acids 83–149 that does not interact with GTPases (Zhao *et al.*, 1998; Bagheri-Yarmand *et al.*, 2001) (Figure 1A and B). It was interesting that inhibition of the Pak1 pathway in MCF-7 cells substantially suppressed the ability of 17- β -estradiol (E2) to stimulate ERE-driven transcription (Figure 1C). Consistent with this finding, E2 did not effectively induce the expression of PR isoforms in MCF-7 clones 10 and 17 (Figure 1D). There was also a marked reduction in the baseline level of PR expression in clones 10 and 17 (Figure 1D). The inhibitory effects of the Pak1 inhibitor were not due to a reduction in ER content (Figure 1B). Furthermore, we found that Pak1 signaling was also required for the optimal proliferation of MCF-7 cells, as expression of the Pak1 auto-inhibitory, amino acids 83–149, fragment suppressed the ability of estrogen to stimulate the growth of MCF-7 cells (Figure 1E). Together, these findings suggested that the Pak1 pathway might have an important role in the biology of ER in breast cancer cells.

To further validate the significance of Pak1 signaling in optimal ER functioning, we next knocked down endogenous Pak1 expression by using the recently emerged short interference RNA (siRNA) methodology (Elbashir *et al.*, 2001; Yu *et al.*, 2002). The efficacy of siRNA methodology was established by showing the down-regulation of both GFP-tagged Pak1 and endogenous Pak1 by Pak1-specific siRNA using western blotting and confocal microscopy (Figure 2A–C). The Pak1-specific siRNA had no effect on the levels of Pak2 and Pak3 as determined by specific antibodies. Interestingly, the noticed knock down of Pak1 expression by Pak1-siRNA but not by control siRNA was accompanied by a significant reduced stimulation of the ER transactivation activity and ER target gene pS2 expression by estrogen (Figure 2D and E). Together, results from Figures 1 and 2 suggest that the Pak1 pathway might be required for optimal ER action.

Generation of TG mice expressing T423E Pak1 in mammary gland

To elucidate the functional significance of the observed Pak1 regulation of ER signaling, we first determined the pattern of Pak1 expression in murine mammary glands. Immunohistochemical staining of mammary glands with an anti-Pak1 antibody showed the expression of Pak1 during all the stages of mammary gland development, with particularly strong expression during pregnancy and lactation. Pak1 was found predominantly in the ductal and alveolar epithelium, with low levels in myoepithelial cells. Pak1 expression was mostly confined to the cytoplasm of the epithelial cells of the ducts and alveoli (Figure 3A). These observations suggest that Pak1 might have an important role in mammary gland development during pregnancy and lactation.

To determine whether deregulation of Pak1 signaling in mammary glands produces a pathologic state and contributes to abnormalities during the process of mammary gland development, we expressed the constitutively active T423E Pak1 mutant in murine mammary glands during pregnancy and lactation, which was driven by using the β -lactoglobulin (BLG) promoter (Lundgren *et al.*, 1997; Figure 3B). In the targeted Pak1 construct, Thr423 was replaced by glutamic acid (E), rendering it constitutively

negatively charged and thus, mimic activated Pak1 (Sells *et al.*, 1997; Vadlamudi *et al.*, 2000). A myc-tagged T423E Pak1 cDNA was cloned into the vector pBJ41 under the control of the ovine BLG promoter. Of 21 mice

produced from injected fertilized oocytes, we identified five positive founder mice by Southern blotting (Figure 3C). These five mice were bred with B6DF1/J and recorded as different lines. Expression of the T423E Pak1 transgene was determined by amplifying the region spanning the myc tag and the N-terminus of Pak1 with RT-PCR. As expected, a 345 bp transgene band was detected in the TG mice but not in the wild-type mice, as verified by subsequent Southern blotting (Figure 3D). Of the five TG lines we established, lines 13 and 14 had the highest levels of transgene expression and were used in subsequent analyses. Immunohistochemical staining of c-myc tag revealed a strong signal from lactating TG mammary glands. In contrast, stage-matched wild-type mammary glands showed only a background level of staining (Figure 3E). Next we analyzed the signaling status of various major pathways. As expected, mammary gland lysates of TG mice had a higher level of Pak1 kinase activity than did age-matched and developing stage-matched wild-type mice (Figure 3F). To examine the functionality of the catalytically active Pak1 transgene *in vivo*, we examined the activation status of p42/44MAPK and p38MAPK, two downstream targets of Pak1 (Vadlamudi *et al.*, 2000). Interestingly, the phosphorylation levels of p42/44MAPK and p38MAPK in Pak1-TG mice were 3- to 5-fold higher than the levels present in the age- and stage-matched wild-type mice (Figure 3G). Together, these results establish the functionality of the expressed Pak1 transgene in mammary glands during pregnancy and lactation.

Pak1 stimulation of hyperplasia

To determine the effect of Pak1 hyperactivation on the morphogenesis of mammary glands, we performed hematoxylin and eosin (H&E) staining (Figure 4A–I). Normally, during pregnancy, the mammary glands grow quickly, and the ducts branch out and form lobules and alveoli. There was no obvious difference in the mammary glands between pregnant TG and wild-type mice (data not shown). However, in the lactating TG mice, we observed drastic morphologic changes in the mammary glands. Normally, a lactating mammary gland is composed of branching ductal networks and alveoli that fill up an entire whole fat pad. Accordingly, both the ductal and lobuloalveolar epithelia of day 12 lactating mammary glands of wild-type mice were smooth and single layered (Figure 4A, D and G). However, lactating age-matched mammary glands from Pak1-TG mice exhibited hyperplasia characterized by ductal epithelium up to three to five layers thick, either in a diffused pattern or papillary form (Figure 4B and C). The ductal epithelial cells also

Fig. 2. Knock down of Pak1 by siRNA reduces ER-transactivation functions. (A–D) MCF-7 cells were cotransfected with 10 nM Pak1 siRNA or control Pak1, or GAPDH siRNAs and GFP-Pak1. After 48 h, the expression of exogenous GFP-Pak1 and the endogenous Pak1 was determined by western blotting (A and B) and fluorescence microscopy (C). Upper panel, GFP-Pak1-transfected cells are shown in green ($n = 4$). (D and E) Reduction in ERE-driven luciferase activity and pS2 gene expression by Pak1-siRNA. MCF-7 cells were cotransfected with 10 nM Pak1 or control siRNA and ERE-luc for 48 h, and treated with or without 1 nM E2 for 16 h before analyzing the status of ERE-luc activity or pS2 mRNA expression ($n = 2$). Bar = 10 μ m.

Fig. 3. Generation of active Pak1-TG mice. **(A)** Expression of Pak1 during various stages of mammary gland development as revealed by immunohistochemistry. The lower panels show myoepithelial cells identified by the mouse keratin-5 marker (MK-5). **(B)** Structure of the T423E Pak1 transgene. c-myc-tagged T423E-mutated Pak1 was fused to the ovine BLG promoter. PBD, p21-binding domain. **(C)** Identification of the TG founder mice by Southern blotting. Tail DNA (5 μ g) was digested with *Bam*HI, resolved on 0.7% agarose gel and probed with a Pak1 cDNA probe. The transgene band is 2.2 kb. **(D)** Expression of transgene as determined by RT-PCR followed by Southern blotting. Three Pak1-TG mice on day 12 of lactation and two wild-type mice were analyzed on the same day of lactation. **(E)** Expression of transgene on day 12 of lactating mammary glands as revealed by immunohistochemistry using anti-c-myc-tag antibody. An age-matched wild-type mammary gland was used as a control. **(F)** Pak1 kinase activity analysis. Protein lysates from the indicated days of pregnancy or lactation were immunoprecipitated with Pak1 antibody and then *in vitro* kinase assay was performed using myelin basic protein (MBP) as a substrate. **(G)** Western blotting of mammary lysates showed an increased level of phosphorylated p38MAPK and MAPK in the Pak1-TG mice, with an unchanged level of total p38 and MAPK. Bar = 40 μ m.

possessed vacuoles, which indicated an active milk production (Figure 4C), a phenomenon generally not seen in the ductal epithelium. In addition to having clear evidence of ductal hyperplasia, the alveolar epithelium frequently protruded into the lumen and was often multilayered, a characteristic feature of hyperplasia (Figure 4E, F, H and I). Another prominent feature of the TG mammary glands was the presence of red-tipped alveolar epithelial protrusions (Figure 4E, F, H and I), a characteristic feature of apocrine metaplasia, which has been noticed in fibrotic cystosis of mammary glands (Schnitt and Connolly, 2000). The ductal and lobuloalveolar hyperplasia were confirmed by immuno-

staining of epithelial boundaries for β -catenin, a tight-junction protein (Figure 4J–P). Furthermore, both the ductal and lobuloalveolar hyperplasia in Pak1-TG mammary glands contained hyperplastic cells with two or three nuclei (Figure 4K and O), compared with mammary glands from the age- and stage-matched wild-type mice (Figure 4J and M).

Abnormal proliferation during lactation

To reveal the cytologic basis for the observed hyperplasia and hyperploidy in the mammary glands of the Pak1-TG mice, we analyzed the status of cell proliferation by BrdU labeling during pregnancy and

Fig. 4. Active Pak1-TG mice developed mammary gland hyperplasia. H&E staining showing ductal and lobuloalveolar hyperplasia in mammary glands from day 12 lactating Pak1-TG mice. (A, D and G) Smooth, single-layer ductal and alveolar epithelium from wild-type mice. (B and C) Ductal epithelium of Pak1-TG mice showed multi-layered hyperplasia in either a diffuse pattern (B) or a papillar shape (C). Many vacuoles are seen in the ductal epithelial cells. (E, F, H and I) Alveolar epithelium from Pak1-TG mice showing long, often multi-layered protrusions into the lumen. The apical portion of the protrusions often stained bright red. (H and I) Amplifications of the squared portions of (D–F), respectively. (J and M) β-catenin staining of ductal and alveolar epithelium from wild-type mice. (K, N and O) β-catenin staining showing multi-layered hyperplasia in the ductal and alveolar epithelium from Pak1-TG mice. (L and P) are the quantitation of the ductal and alveolar hyperplasia, respectively ($n = 6$, $**P < 0.01$). Note the presence of cells with more than one nucleus in Pak1-TG mice. WT, wild type mice; TG, active Pak1-TG mice. Bar = 20 μ m.

lactation. There was no significant difference in the BrdU-labeling rate in the mammary glands of the pregnant Pak1-TG and wild-type mice (data not shown). After the second day of lactation, the mammary fat pad is normally filled with glandular tissues, epithelial cells enter into the G_0 quiescent state and proliferation is rarely detected. Interestingly, lactating mammary glands from the Pak1-TG mice had significantly more proliferating cells than did age-matched wild-type mammary glands (Figure 5A). These findings suggest that hyperplasia in mammary glands of Pak1-TG mice may be linked with enhanced cell division during lactation.

Pak1 regulation of ER target genes

Because ERs have been linked with hyperplastic ductal breast disease and breast cancer (Harvell *et al.*, 2000; Mehta *et al.*, 2001), we hypothesized that catalytically active Pak1 transgene-induced hyperplasia may involve the ER pathway. Subsequently, we examined the expression of ER target genes, such as PR, cathepsin D and the anti-apoptosis gene *Bcl-2* in mammary glands. Normally, PR is expressed in the nuclei of most ductal epithelial cells in virgin mice, and its expression level progressively decreases after pregnancy and is barely detectable during lactation (Shyamala *et al.*, 1990; Seagroves *et al.*, 2000). The mammary glands of Pak1-TG mice during pregnancy

Fig. 5. Abnormal proliferation and expression of ER target genes in transgenic mammary glands. (A) Active Pak1-TG mice show increased BrdU labeling during lactation. Animals on day 12 of lactation were injected with 50 mg/kg BrdU 2 h before being sacrificed, and positive-labeled cells were revealed by immunohistochemistry. Right panel shows the quantitation of the positive cells found per 1000 cells ($n = 6$). Bar = 10 μ m. (B) PR immunostaining of mammary glands obtained from wild-type mice and Pak1-TG mice at days 15 and 18 of pregnancy. Quantitation of the PR-positive cells is shown in the right panels ($n = 6$). (C) Cathepsin D expression as detected by using *in situ* hybridization. Pak1-TG mammary glands and wild-type mammary glands at day 15 of pregnancy were probed with antisense or sense control probes. (D) Immunoblot analysis of lysates from day 15 of pregnancy and day 12 of lactation revealed an increase in the Bcl-2 level in transgenic mammary glands compared with the wild-type controls. Bar = 20 μ m.

expressed significantly more PR-positive cells than did mammary glands from age- and stage-matched wild-type mice (Figure 5B). However, we did not find a significant difference in the percentage of PR-positive cells during lactation between the wild-type and TG mammary glands (data not shown). This was probably due to the overwhelmingly tight control of PR transcription during lactation, which makes PR difficult to detect. Nonetheless, we could readily detect more mRNA expression of cathepsin D, an ER-regulated gene, during pregnancy in Pak1-TG mice than in wild-type mice (Figure 5C). In addition, the level of Bcl-2 was also substantially higher during pregnancy and lactation in Pak1-TG mice than in wild-type control mice (Figure 5D).

These findings provide evidence that Pak1 upregulated ER target genes and that ER may have been a downstream target of Pak1, leading to the observed ductal and lobuloalveolar hyperplasia in mammary glands.

Pak1 regulation of ER-driven transcription

To explain the observed upregulation of ER target genes in Pak1-TG mice, we hypothesized that Pak1 signaling regulates ER-driven transcription in mammary epithelium. To determine the effect of Pak1 on the ER pathway, we used an ERE reporter system to examine the ability of Pak1 to influence the transcriptional activity of the ER. Cotransfection of MCF-7 human mammary epithelial cancer cells with ERE-luciferase (ERE-luc) and a

Fig. 6. Pak1 stimulated ER signaling by regulation of the AF2 domain function in MCF-7 human breast cancer cells. (A) Pak1 stimulated ERE activity in the absence of ligand. MCF-7 cells were transfected with 0.25 μ g of ERE-luc reporter with or without 0.5 μ g of Pak1-T423E or pCMV, treated with or without 1.0 nM E2 for 16 h ($n = 4$). (B) Pak1 activates AF2 in the absence or presence of E2. MCF-7 cells were cotransfected with 0.25 μ g each of gal4-AF2 and gal4-Luc (containing five gal4 binding domains), with or without 0.5 μ g of T423E Pak1, wild-type Pak1, or pCMV. As a positive control, cells were treated with 1×10^{-9} M E2 for 16 h ($n = 4$). (C) Pak1 stimulates the ER target gene promoter activity. MCF-7 cells were transfected with 0.1 μ g of pS2-CAT or myc-luc reporter with or without 0.5 μ g of wild-type Pak1 or pCMV. Chloramphenicol acetyltransferase and ERE-luc activities were measured. (D) Pak1 stimulates ER target gene expression. MCF-7 clone expressing HA-tagged T423E Pak1 were treated with 1.0 μ g/ml doxycycline to induce the expression of Pak1. Expression of pS2 was assessed by northern blotting ($n = 4$).

catalytically active Pak1 gene (T423E), but not with a control vector (pCMV), stimulated ERE-driven transcription of the reporter gene with or without stimulation by estrogen (Figure 6A). To assess the ER dependency of the noticed ER transactivation function, these studies were extended to ER-negative HeLa cells. As expected, there was no effect of Pak1 on ERE-driven transactivation in the absence of ER co-expression (data not shown). To determine whether Pak1 signaling can directly stimulate the AF2 domain of ER (independent of changes in the DNA-binding activity of the ER), we used the galactosidase 4-luciferase (gal4-luc) assay system (Mazumdar *et al.*, 2001). This system involves the transient transfection of two plasmids. We used gal4-AF2, which contains the ligand-binding domain of ER, and a gal4-luc reporter; activation of the luc reporter requires stimulation of the AF2 domain by E2. The AF2 domain-dependent luc activity was significantly higher in cells cotransfected with dominant-active T423E or wild-type Pak1 than in cells cotransfected with the control vector pCMV, either with or without estrogen stimulation (Figure 6B). Next we sought to determine whether Pak1 signaling influenced ERE-dependent transcription from natural promoters such as the pS2 and c-Myc (Dubik and Shiu, 1988). Our results indicated that Pak1, but not control vector pCMV, stimulated transcription from both promoters (Figure 6C).

Accordingly, inducible expression of HA-tagged T423E Pak1 in the well characterized MCF-7 clone (Vadlamudi *et al.*, 2000) also upregulated the expression of pS2 in the absence of E2 treatment (Figure 6D).

Pak1 phosphorylation of ER

To evaluate the biochemical basis of Pak1 regulation of ER, we sought to determine whether Pak1 phosphorylates ER. To that end, we assessed the ability of T7-tagged Pak1 immunoprecipitated from MCF-7 cells to phosphorylate ER *in vitro*. MCF-7 cells were transfected with T7-pcDNA or T7-T423E-Pak1, and cell lysates were immunoprecipitated with an anti-T7 antibody. Half of the material was examined for expression of T7-tagged Pak1 using western blotting with an anti-T7 monoclonal antibody (Figure 7A, upper panel). The other half of the T7-immunoprecipitated material was used to phosphorylate recombinant ER (Figure 7A, lower panel). We found 3.5 $\times$ more phosphorylation of recombinant ER in the T7-Pak1 immunoprecipitate than in the T7-pcDNA immunoprecipitate.

To determine whether Pak1 interacts with ER, we examined the ability of the *in vitro* translated ER protein to bind GST-Pak1. ER interacted with GST-Pak1, but not with GST alone, in GST pull-down assays (Figure 7B). To confirm the interaction of Pak1 with ER, we determined the effectiveness of GST-Pak1 to precipitate T7-tagged ER by using lysates prepared from HeLa cells transfected with T7-tagged ER (Figure 7C). Because several Pak isoforms exist, we examined whether ER interacts with *in vitro* translated Pak1, Pak2 and Pak3. It was interesting that Pak1, but not Pak2 or Pak3, interacted with both the AF1 and AF2 domains of ER (Figure 7D), suggesting that only Pak1 interacts with ER. To demonstrate the association between the endogenous Pak1 and ER, we co-immunoprecipitated Pak1 with an anti-ER antibody using the mouse uterus lysate, as uterus has high levels of both ER (Bergman *et al.*, 1987) and Pak1 (Moore *et al.*, 2000). Results show that Pak1 interacts with ER *in vivo* (Figure 7E).

We next identified the ER binding domain of Pak1. We created a series of GST fusion proteins of various Pak1 domains and performed a GST pull-down assay (Figure 7F). There was no specific binding of ER with the kinase domain, the Cdc42/Rac1 interactive binding (CRIB) domain, and the auto-inhibitory domain of Pak1. However, N-terminal fusion proteins containing amino acids 1–132 and 1–75 showed significant binding to the ER (Figure 7F), indicating that an ER binding site is located in the N-terminal 75 amino acids, a region shown to be involved in Nck binding to Pak1 (Lu *et al.*, 1997).

Identification of the Pak1 phosphorylation site in ER

To identify the functional domain of ER that may be phosphorylated by Pak1, we examined the ability of purified Pak1 enzyme to phosphorylate GST fusion proteins with different domains of ER (Krust *et al.*, 1986). Pak1 specifically phosphorylated the AF2 domain (amino acids 301–552) of ER but failed to phosphorylate other domains (Figure 8A). This is an unexpected finding, as the AF2 domain has been previously shown to be phosphorylated in a ligand-dependent manner (Le Goff

Fig. 7. Pak1 interaction with and phosphorylation of ER. (A) Pak1 phosphorylation of ER. MCF-7 cells were transfected with T7-pcDNA or T7-Pak1, and cell lysates were immunoprecipitated with anti-T7 antibody. Half of the material was used to confirm the expression of Pak1 by western blotting with an anti-T7 mAb (upper panel). The other half of the material was subjected to an *in vitro* kinase assay using 1 μ g of recombinant ER (Affinity Bioreagents Inc.) in the presence of [γ - 32 P]ATP (lower panel). (B) Interaction of ER with Pak1. GST pull-down assay showing the association of Pak1 with the *in vitro*-translated ER. (C) GST-Pak1 interacts with T7-ER precipitated from HeLa cells transfected with T7-tagged ER (right lane). Purified GST-Pak1 and TNT-T7-ER were used as positive controls for Pak1 and ER, respectively. Residual cleavage of GST-Pak1 resulted in a modest detection of the cleaved Pak1 in lane 4. NS, non-specific. (D) Interaction of ER domains with Pak isoforms. Pak1, Pak2 and Pak3 cDNAs were transcribed and translated *in vitro*, and then incubated with GST-AF1, GST-AF2, or GST and analyzed by the GST pull-down assay. (E) Interaction between endogenous Pak1 and ER. Lysates from virgin mouse uterus (500 μ g) were IP-down with an anti-ER antibody or normal mouse IgG, and sequentially immunoblotted with Pak1 and ER antibodies. (F) Upper panel, Pak1 fusion proteins were incubated with *in vitro* translated ER, and binding was analyzed by the GST pull-down assay. The ER binding is within the Nck binding domain amino acids 1–75 (lane 5). Middle panel, the loading control of various ER constructs shown with Ponceau staining; lower panel, a schematic illustration of the various regions of Pak1–GST construct. 1, GST vector alone; 2, full-length Pak1; 3, C-terminal fragment (kinase domain); 4, N-terminal fragment; 5, Nck-binding domain; 6, CRIB domain; and 7, inhibitory domain.

et al., 1994). A careful analysis of the AF2 domain indicated that there are two consensus Pak1 Ser phosphorylation sites, one at KKXS305 and another at RXXS518. Although there is an additional consensus Pak1 phosphorylation site (RXXS167) in the AF1 domain, which has been shown previously to be phosphorylated by pp⁹⁰rsk1 and Akt (Joel *et al.*, 1998; Campbell *et al.*, 2001), it was obviously not phosphorylated by Pak1 (Figure 8A).

To map the Pak1 phosphorylation sites in the AF2 domain of ER, we next mutated two potential Pak1 phosphorylation sites in ER. Mutation of ER Ser305 to Ala (designated as S305A) completely abolished the ability of Pak1 to phosphorylate ER, whereas mutation of ER Ser518 to Ala (designated as S518A) had no effect (Figure 8B). To examine the effect of Pak1 phosphorylation of ER *in vivo*, ER-negative HeLa cells were cotransfected with Pak1 and T7-ER wild-type or T7-ER S305A, and cells were metabolically labeled with [γ - 32 P]orthophosphoric acid. Cell lysates were immunoprecipitated with an anti-ER mAb and analyzed by autoradiography. Results indicated that Pak1 only phosphorylated the wild-type ER but not mutant ER (Figure 8C). These experiments confirmed that Ser305 of ER is the Pak1 phosphorylation site. To verify that this phosphorylation site also exists in mouse ER, we com-

pared the amino acid sequence of this region between human and mouse ER. As expected, both the phosphorylation site (Ser309 in mouse) and motif are conserved in human and mouse ER (Figure 8D). To determine the significance of Ser305 phosphorylation in the mechanism of ER action, we examined the transcriptional activity of S305A ER. S305A mutation totally blocked both the Pak1-stimulated ligand-independent activation and sensitization of ER (Figure 9A). To evaluate whether the constitutive negative charge at the Ser305 (due to its phosphorylation) is responsible for the noticed enhancement of transactivation activity of ER, we next mutated the Ser305 to glutamic acid (E). As expected, the S305E ER mutant was a potent activator of ERE-driven transcription as compared with that of wild-type ER (Figure 9B). Together, these findings suggest that Pak1 phosphorylation of the ER at Ser305 is important for transactivation functions of ER by the Pak1 pathway.

Because Pak1 activation in mammary epithelium was accompanied by upregulation of MAPK activation and because the AF1 domain of ER was shown to be phosphorylated on S118 by MAPK (Kato *et al.*, 1995), we examined the status of ER S118 phosphorylation by using a well characterized antibody that specifically recognizes ER phosphorylated on S118 (Ali *et al.*, 1993; Chen *et al.*, 2000). Interestingly, mammary epithelium

Fig. 8. Pak1 regulates ER transactivation functions via Ser305 phosphorylation. (A) Upper panel, GST construct of five various functional domains of ER were incubated with purified Pak1 enzyme in the presence of [32 P]ATP; the AF2 domain, which has two consensus Pak1 phosphorylation sites, was strongly phosphorylated. Lower panel, loading control stained with Ponceau, right panel shows a schematic diagram of the various GST-ER domain constructs (Krust *et al.*, 1986). A/B, AF1 domain; C, DNA-binding domain; D, hinge; E, AF2/ligand-binding domain; F, region for modulation of function. (B) S305A mutation (right lane), but not S518A (middle lane), blocked phosphorylation of AF2 by Pak1. Upper panel shows the Pak1 kinase control and the lower panel shows the GST protein loading control. (C) *In vivo* phosphorylation of ER at S305 by Pak1. HeLa cells were cotransfected with T423E-Pak1 and T7-wild-type or T7-S305A ER for 36 h and incubated with [γ - 32 P]ATP overnight. Cell lysates were immunoprecipitated with ER antibody, resolved by SDS-PAGE and transferred to membranes. After autoradiography, the membranes were blotted with ER antibody. (D) Comparison of the partial amino acid sequence of mouse and human ER shows that the Pak1 phosphorylation site S305 (Mouse S309), as well as the consensus motif KKXS (boxed), is conserved in human and mouse ER.

from Pak1-TG mice (pregnancy day 15) did not exhibit detectable phosphorylation of ER on S118 (data not shown). Together, these findings suggest that the Pak1 pathway might have a role in the biology of ER in breast cancer cells.

Discussion

We undertook this study to elucidate the role of Pak1 in the pathophysiology of mammary epithelial cells by using breast cancer cells and TG mice expressing catalytically active Pak1 in mammary glands. Our results showed that the Pak1 pathway is required for the optimal transactivation functions of ER, and that Pak1 kinase activity in mammary glands is developmentally regulated. It was interesting that Pak1-TG mice developed severe ductal and lobuloalveolar hyperplasia associated with abnormal proliferation during lactation. Often, breast diseases are linked with deregulation of estrogen or its receptors (e.g. human cystic and fibrotic mammary gland hyperplasia). Administration of estrogen and TG expression of aroma-

Fig. 9. Transactivation activity of ER S305 mutants. HeLa cells were cotransfected with 0.25 μ g of ERE-luc reporter, 0.5 μ g of wild-type Pak1 and 0.5 μ g of wild-type ER, S305A ER or S305E ER, with or without 1 nM E2 stimulation ($n = 3$). (A) S305A mutation of ER blocked activation by Pak1. (B) S305E mutation of ER resulted in stimulation of transactivation activity in the absence of estrogen treatment and was further increased upon 1 nM E2 treatment ($n = 3$).

tase to locally promote estrogen synthesis in mammary glands have been shown to induce hyperplastic changes (Tekmal *et al.*, 1996; Kirma *et al.*, 2001). However, the role of the ER in mammary hyperplasia and tumorigenesis, independent of its natural ligand, is poorly understood. Increased cell proliferation accompanied the observed stimulation of mammary hyperplasia in Pak1-TG mice. Furthermore, mammary glands from the Pak1-TG mice exhibited a significant elevation in the levels of expression of ER target gene products. This is important because it raises the possibility that Pak1 could activate the ER in the absence of estrogen and in principle could contribute to the development of hormone independence.

Besides the natural ligand estrogen, several signaling molecules including Akt, protein kinase A and pp 90 rsk1 have been shown to phosphorylate the AF1 domain of ER, which contributes the transactivation function of ER (Bunone *et al.*, 1996; Joel *et al.*, 1998; Chen *et al.*, 1999; Campbell *et al.*, 2001). However, both phosphorylation and transactivation functions of the AF2 domain of ER have been shown to be regulated in a ligand-dependent manner (Le Goff *et al.*, 1994). In this context, we have shown that Pak1 phosphorylates the AF2 but not the AF1 domain in the absence of estrogen. The small region of ER, amino acids 302–339, that contains Pak1 phosphorylation site S305 has been shown to be an extra activation domain in the N-terminal portion of AF2 (Pierrat *et al.*, 1994). More recently, it was shown that Lys residues at 302 and 303, just one amino acid away from S305, could be acetylated by the co-activator protein CBP/p300 complex, thus transactivating ER in the absence of ligand (Wang *et al.*, 2001). Taken together, these findings imply that bringing a negative charge to this small region by either phosphorylation or acetylation is important in the ligand-independent activation and sensitization of ER.

Our observations that Pak1 activation in mammary epithelium was accompanied by upregulation of MAPK activation and that Pak1 failed to phosphorylate the AF1 domain of the ER that contained a MAPK consensus site (S118) are important, suggesting that Pak1-triggered MAPK may not be involved in the phosphorylation of

ER. This is also supported by the finding that showed a lack of ER phosphorylation on S118 in mammary glands from Pak1-TG mice. In this context, it is possible that MAPK activation in Pak1-TG mice could be involved in conferring anchorage-independent phenotype to the mammary epithelial cells, as has been shown in MCF-7 breast cancer cells (Vadlamudi *et al.*, 2000). It is also possible that the MAPK pathway may cooperate with the Pak1-ER pathway in the development of the observed hyperplasia in mammary glands.

In summary, ectopic expression of catalytically active Pak1 in murine mammary glands led to the development of hyperplasia. We also found evidence that closely links Pak1-induced hyperplasia with the ability of Pak1 to directly phosphorylate and regulate the AF2 domain of ER at residue Ser305. In addition, a functional Pak1 pathway was required for optimal expression of endogenous ER target genes and ER-mediated cell proliferation. Therefore, our findings suggest a model in which Pak1 may play an important mechanistic role in the regulation of expression of ER target genes and hyperplasia in mammary epithelial cells. Future long-term studies are planned to evaluate the potential development of mammary gland tumors in Pak1-TG mice.

Materials and methods

Generation of TG mice and genotyping

A myc-tagged constitutively kinase active T423E Pak1 mutant cDNA containing T423E was cloned into the pBJ41 vector under the control of the ovine BLG promoter (Lundgren *et al.*, 1997). Linearized transgene fragments were injected into the fertilized oocytes of B6DF1/J mice. The founder mice were genotyped by Southern blotting with 5 µg of tail DNA digested with *Bam*HI. Positive mice have a 2.2 kb fragment insertion. Mice having exogenous genes were bred with the same strain of wild-type mice but were recorded as different lines. The F₂- and F₃-generation mice were used for phenotype analysis. Genotyping was performed using PCR to amplify the region spanning the myc tag and Pak1 transgene. The sequences of primers were: forward, 5'-CATCTCTGAAGAGGATCTGTCTAGCG-3' and backward, 5'-GGGTTTTTCTCTGCTCCGACTTA-3'.

Histology and immunohistochemistry

The number 4 right inguinal mammary glands were removed and fixed with Bouin's fixatives and subsequently processed to paraffin sections, routinely stained with H&E. Immunohistochemistry was performed using indirect-enzyme labeling method as described previously (Wang *et al.*, 1998). The first antibodies and dilutions used were as follows: rabbit anti-Pak1 (1:50), rabbit anti-β-catenin (1:100), mouse anti-c-myc tag (1:50; NeoMarkers), and rabbit anti-PR (1:50; Dako). Controls included antigen pre-absorption of the primary antibodies or using the same dilution of pre-immune serum from same species.

Histomorphometry and statistical analysis

Quantitation of the hyperplasia in the mammary ductal and alveolar epithelium was done by stereological analysis. For each case, five fields of 20× photographs were quantitated by overlapping on transparency net. For quantitation of hyperplasia, the area of hyperplasia was divided by the total area of epithelium of the field, and presented as percentages. The differences between wild-type and TG mice were analyzed by *t*-test.

Plasmid construction

Wild-type myc-tagged Pak1, GST-Pak1 and catalytically-active Pak1 T423E constructs have been described previously (Vadlamudi *et al.*, 2000). Pak2 construct in pcDNA3.1 vector was purchased from Invitrogen Inc. GST-Pak3 construct was a gift from Dr Hisataka Sabe, Japan. GST fusion proteins containing various Pak1 domains were constructed by PCR amplification and subcloning into *Eco*RI and *Xho*I sites of pGEX5 vector and have been described previously (Li *et al.*, 2002). GST fusion proteins containing various ER domains have been

described previously (Kumar *et al.*, 2002). Site-directed point mutations were performed by using Quickchange kit (Stratagene) according to the manufacturer's instructions. Primer sequences are: ERS305A, CATGATCAAACGCTCTAAGAAGAAGCGCCTGGCCTTGTCCTGAC and its complementary; and ERS305E, CAAACGCTCTAAGAAGAAGCAACTGGCCTTGTCCTGACG and its complementary. GST proteins were purified using GST beads (Pharmacia, Inc.) according to the manufacturer's instructions.

GST pull-down assay, immunoprecipitation and in vitro kinase assays

In vitro transcription and translation of the Pak1, Pak2 and Pak3 proteins were performed using the TNT transcription-translation system (Promega, Inc.) according to the manufacturer's instructions. Immunoprecipitation studies were performed as previously described (Mazumdar *et al.*, 2001) and resolved by 12% SDS-PAGE. *In vitro* kinase assays using purified ER protein were performed in HEPES buffer (50 mM HEPES, 10 mM MgCl₂, 2 mM MnCl₂, 1 mM DTT) containing immunoprecipitated T7-tagged T423E Pak1 enzyme, 10 µCi [γ -³²P]ATP and 25 µM cold ATP. The reaction was performed in a volume of 40 µl and was incubated for 30 min at 30°C.

In situ hybridization

For *in situ* hybridization, mouse mammary gland tissues were excised out and fixed with 4% paraformaldehyde and processed routinely to paraffin sections as described previously (Mazumdar *et al.*, 2001). Sense-probe hybridization was used for background control.

RT-PCR, northern and Southern hybridization

RT-PCR was performed using the Access RT-PCR system (Promega) according to the manufacturer's instructions. Northern and Southern blotting was performed as previously described (Kumar *et al.*, 2002).

Cell culture, reporter assay, cell growth assay and RNA interference

MCF-7 cells were maintained in DMEM/F12 (1:1) supplemented with 10% FCS. For reporter gene transient transfections, cells were cultured in minimal essential medium, without phenol red and containing 3% DCC serum for 24 h and then transfected with plasmids using Eugene-6 reagents. The activity of the β-galactosidase reporter was used to correct the transfection efficiencies (Mazumdar *et al.*, 2001). For cell growth assay, cells were grown in phenol red-free medium supplemented with 5% charcoal-stripped serum in 6-well plates and treated with 0.1 nM estrogen for the indicated times.

For Pak1 RNAi, the siRNA was synthesized using the Silencer siRNA construction kit (Ambion, Texas) according to the manufacturer's instructions. The targeted sequence is between 988 and 1007 of DDBJ/EMBL/GenBank accession No. U24152. The Pak1 siRNA sequences are: sense, UCUGUAUACACACGGUCUGTT; and antisense, CAGACC-GUGUGUAUACAGATT. Control siRNA sequences are sense, CCC-UAAACCAUGGUUCUAATT; and antisense, UUAGAACCAUGGU-UUAGGGTT. GAPDH siRNA primers were obtained from the Ambion kit.

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