PHOSPHORYLATION-DEPENDENT REGULATION OF STABILITY AND TRANSFORMING POTENTIAL OF ETS TRANSCRIPTIONAL FACTOR ESE-1 by p21-ACTIVATED KINASE 1

Bramanandam Manavathi¹, Suresh K. Rayala¹, and Rakesh Kumar¹,²*

¹Molecular & Cellular Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas, 77030, USA; ²Molecular & Cellular Biology, Baylor College of Medicine, Houston, Texas 77030, USA.

Running title: Signaling-mediated breast epithelial cell transformation

*To whom correspondence should be addressed: E-mail: rkumar@mdanderson.org

Differential phosphorylation of transcription factors by signal transduction pathways play an important role in regulation of gene expression and functions. ESE-1 is an epithelium-specific ETS transcription factor that transforms human breast epithelial cells through a serine- and aspartic acid-rich domain (SAR) by an unknown cytoplasmic mechanism. Here we found that a signaling kinase, p21-activated kinase-1 (Pak1), interacts with and phosphorylates ESE-1. Interestingly, Pak1 selectively phosphorylates ESE-1 at Ser207, which is located within the SAR domain. A Ser207Ala substitution in ESE-1 reduced its ability to transform breast cancer cells. We also found that ESE-1 is a labile protein and by interacting with F-box binding protein β-TrCP, undergoes ubiquitin-dependent proteolysis. Intriguingly, Pak1 phosphorylation inactive mutant ESE-1S207A is more unstable than either wild type ESE-1 or its Pak1 phosphorylation mimetic mutant i.e., ESE-1S207E. These findings provide novel insights into the mechanism of transformation potential of ESE-1 and discovered that ESE-1 functions are coordinately regulated by Pak1 phosphorylation and β-TrCP–dependent ubiquitin-proteosome pathways.

The ETS family of transcription factors plays critical roles in the normal and pathologic processes important for growth and development (1). In addition to regulating cell proliferation, differentiation, apoptosis, migration, and epithelial mesenchymal transitions (EMT) during normal development, ETS proteins, when their levels and activity are deregulated, also contribute to the initiation and progression of human cancers (2-4). In general, ETS family transcription factors are defined by a highly conserved 85-amino-acid motif called the ETS domain that binds to a core recognition sequence (GGAA/T) located on promoters of their target genes (5). The epithelium-specific ETS (ESE) family, which is part of the ETS family and includes ESE-1, ESE-2, ESE-3, and PDEF, has been implicated in epithelial cell differentiation (6).

ESE-1 (also known as ERT, Jen, ESX, or Elf3) was initially identified as a regulator of epithelial cell differentiation owing to its ability to regulate expression of epithelial cell markers in keratinocytes (7;8), bronchial (9), and retina (10). As a transcription factor, ESE-1 regulates a variety of genes, such as Her-2 in breast epithelial cells (11), MIP3α in colon (12), and transforming growth factor-beta type II receptor (13). ESE-1 also regulates genes involved in inflammation, such as angiopoietin-1 and cyclooxygenase-2 (14;15). Interestingly, Her-2–mediated pathway regulates ESE-1 expression, and ESE-1 regulates Her-2 expression by binding...
at the Her-2 promoter (11;16). Accumulating evidence suggests that overexpression of several ETS genes, including PEA3, ESE-1, ETS-1, and PDGF, is associated with breast cancer (17). Recent reports suggest that ESE-1 transforms human breast epithelial MCF12A cells through the serine– and aspartic acid–rich (SAR) domain by an unknown cytoplasmic mechanism (18). Furthermore, overexpression of ESE-1 in MCF12A cells confers a motile phenotype to these cells similar to Rho, Rac, Cdc42 GTPases’s displayed motile phenotype (19).

In general, unstable eukaryotic proteins are encoded by short destruction sequences called “degrons,” including the PEST domain, DSGXXG motif which can be recognized and targeted for ubiquitin proteolysis (20;21). The Skp1/Cul1/F-box protein (SCF) complex E3-ubiquitin ligase targets many proteins for proteolysis in diverse cellular contexts (22). β-TrCP, a WD40 repeat-containing F-box protein of SCFβ-TrCP, recognizes the doubly phosphorylated DSG motif (DpSGφXpS, where φ represents a hydrophobic amino acid and X represents any amino acid) in various SCFβ-TrCP target proteins (23). The phosphoserine residues within the DSG motif are essential for target proteins to interact with β-TrCP (22). Although ETS transcription factors are unstable proteins with short half-lives, the molecular mechanisms underlying the degradation of ETS proteins and the influence of phosphorylation on this process remain elusive (24).

In recent years, it has become increasingly evident that the ETS transcription factors are targets of signaling pathways (25;26). In particular, the MAPK pathway has been intimately linked with diverse regulatory events involving ETS-domain proteins (25). Phosphorylation of ETS proteins at particular serine or threonine residues has been shown to affect their transactivation potential, DNA-binding activity, interaction with coregulatory partners, or subcellular localization (2). In brief, signaling pathways may regulate the activity and functions of ETS proteins in an important manner.

P21-activated kinase 1 (Pak1), an evolutionary conserved family of serine/threonine kinases, was initially identified as effector of Rac1 and Cdc42, and as being involved in formation of lamellipodia and membrane ruffles and required for cell motility (27-30). It has been shown that expression of a catalytically active Pak1 mutant stimulates anchorage-independent growth of breast cancer cells (31). Recent findings suggest that Pak1 promotes the process of tumorigenesis by phosphorylating DLC1 (30;32) and epithelial to mesenchymal transition by phosphorylating Snail (33). Further, Pak1 hyperactivation is sufficient for tumor formation in the murine mammary gland (34). Since ESE-1 and Pak1 exhibit overlapping functions such as cell motility, EMT, and tumorigenesis, and because the mechanism of ESE-1-mediated transformation remains unknown, here we investigated the possibilities that Pak1 is an upstream kinase of ESE-1 and that Pak1 phosphorylation of ESE-1 regulates the stability and transforming ability of ESE-1.

**EXPERIMENTAL PROCEDURES**

**Plasmid constructions** - The following plasmids were constructed for expressing glutathione S-transferase (GST) fusion proteins in *Escherichia coli* DE3. pGST-ESE-1 (encoding GST-ESE-1; ESE-1 amino acids 1-371), pED1 [encoding GST-ESE-1(1-159); ESE-1 amino acids 1-159], pED2 [encoding GST-ESE-1(128-159); ESE-1 amino acids 128-159], pED3 (encoding GST-ESE-1(274-371); ESE-1 amino acids 274-371), pED4 (encoding GST-ESE-1(128-159); ESE-1 amino acids 128-159), pED5 (encoding GST-ESE-1(159-371); ESE-1 amino acids 159-371), and pED5 (encoding GST-ESE-1(1-259); ESE-1 amino acids 1-259) were constructed by inserting an EcoRI-NotI–cleaved polymerase chain reaction (PCR)–derived fragment, using pCGN2-ESX...
as a template, to the same sites of pGEX5X1 (Amersham Biotech, Sweden). pGST-ESE-1-S207A (encoding the serine 207 to alanine mutant of GST-ESE-1) and pGST-ESE-1-S254A (encoding the serine 254 to alanine mutant of GST-ESE-1) with site-directed mutations at indicated amino acids were mutated by site-directed mutagenesis using a quick-change mutagenesis kit (Stratagene, Cedar Creek, TX).

The following plasmids were constructed for use in mammalian cell transfections. T7-tagged ESE-1 (encoding T7-ESE-1; ESE-1 amino acids 1-371) was constructed by inserting an EcoRI-NotI-cleaved PCR-derived fragment into the same sites of pcDNA3.1C (Invitrogen, Carlbad, CA). pcDNA-ESE-1-S207A (encoding the serine 207 to alanine mutant of T7-ESE-1) and pcDNA-ESE-1-S207E (encoding the serine 207 to aspartic acid mutant of T7-ESE-1) were derived from pcDNA-ESE-1, with site-directed mutations at indicated amino acids by site-directed mutagenesis using a quick-change mutagenesis kit (Stratagene, Cedar Creek, TX).

**Cell culture and reagents** - Human breast cancer cell lines MCF-7 and ZR75 were obtained from the American Type Culture Collection (Manassas, VA, USA) and were maintained in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and F12 medium supplemented with 10% fetal calf serum. MCF12A human breast epithelial cells (American Type Culture Collection) were maintained in Ham’s F12/Dulbecco’s modified Eagle’s medium containing 100 ng/ml of cholera toxin (Gibco/BRL, USA), 0.5 µg/ml hydrocortisone (Sigma Chemical Co., St. Louis, MO), 10 µg/ml bovine insulin (Sigma), 20 ng/ml epidermal growth factor (EGF; Sigma), and 5% horse serum (Gibco/BRL). Polyclonal ESE-1 antibody was obtained from Abcam, MA, USA. Mouse antibody against T7 was purchased from Novagen (Madison, WI), and rabbit antibody against T7 was purchased from Bethyl Lab (Montgomery, TX). Vinculin antibody was purchased from Sigma (Saint Louis, MO). Antibody against Pak1 was purchased from Cell Signaling Technology (Beverly, MA).

**In vitro Pak1 kinase assay** - Using GST-ESE-1 fusion protein as a substrate and bacterially purified GST-Pak1 as an enzyme, we performed in vitro kinase assays in HEPES buffer (50 mM HEPES, 10 mM MgCl₂, 2 mM MnCl₂, 1 mM dithiothreitol) containing 100 ng of purified GST-Pak1 enzyme, 10 µCi of [γ-³²P]ATP, and 25 µM cold ATP. Dynein light chain 1 (DLC1) was used as positive control and GST as negative control. GST proteins were purified by using glutathione Sepharose (Amersham Biosciences, Sweden) according to the manufacturer’s instructions. The reaction was carried out in a volume of 30 µl for 30 min at 30°C and then stopped by the addition of 10 µl of 4× sodium dodecyl sulfate (SDS) buffer. We resolved the reaction products by SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed the results by autoradiography.

**Stable cell lines** - Nontransformed but immortalized MCF12A human mammary epithelial cells were transfected by using the FuGene reagent (Roche Molecular Biochemicals, Indianapolis, IN) with 5 µg of pcDNA3.1C, pcDNA-ESE-1, pcDNA-ESE-1-S207A, or pcDNA-ESE-1-S207E in 60-mm dishes. Twenty-four hours after transfection, the medium was replaced with fresh Ham’s medium as well as 500 µg/ml of G418 to select for geneticin-resistant cells. After 12 days of selection, pools of G418-resistant cells, denoted as pcDNA3.1C, WT-ESE-1 (wild type), ESE-1-S207A, and ESE-1-S207E, were replated, expanded, and maintained in complete medium containing 200 µg/ml G418. For experiments, log phase cells between passages 4 and 10 were utilized.

**Cell extracts, immunoblotting, and immunoprecipitation** - Cells were grown in
Ham’s F12/Dulbecco’s modified Eagle’s medium containing all components already described. To prepare cell extracts, cells were washed three times with phosphate-buffered saline (PBS) and then subjected to lysis in NP-40 lysis buffer (50 mM Tris-HCl, pH 8.0; 137 mM NaCl; 10% glycerol; 1% Nonidet P-40; 50 mM NaF; 1× protease inhibitor mixture (Roche Biochemical); 1 mM sodium vanadate) for 15 min on ice. The lysates were subjected to centrifugation in an Eppendorf centrifuge at 4°C for 15 min. Cell lysates containing equal amounts of protein (~100 µg) were resolved by SDS-PAGE (10%-12% acrylamide), transferred to nitrocellulose membranes, probed with the appropriate antibodies, and developed by using the enhanced chemiluminescence method. Immunoprecipitation was performed for 4 h at 4°C using 1 µg of antibody/mg of protein as previously reported (35).

**Glutathione S-transferase pull-down assay -** *In vitro* transcription and translation of Pak1 was done by using a T7-TNT kit (Promega Biosciences, San Luis Obispo, CA), where 1 µg cDNA in pcDNA3.1 vector was translated in the presence of [35S]methionine in a reaction volume of 50 µl. The reaction mixture was diluted to 1 ml with GST binding buffer (25 mM Tris-HCl, pH 8.0; 50 mM NaCl; 10% glycerol; 0.1% NP-40). An equal aliquot was used for each GST pull-down assay. Translation and product size were verified by subjecting 2 µl of the reaction mixture to SDS-PAGE and autoradiography. The GST pull-down assays were done by incubating equal amounts of GST, GST-tagged full-length proteins, and GST-tagged deletion constructs immobilized on glutathione Sepharose beads with *in vitro* translated [35S]-labeled protein to which the binding was being tested. Bound proteins were isolated by incubating the mixture for 2 h at 4°C in GST binding buffer, washing five times with GST binding buffer, eluting the proteins with 2X SDS buffer, and separating them by SDS-PAGE. The bound proteins were then visualized by autoradiography.

**siRNA Transfection and RT-PCR** - Pak1-specific small interfering RNA (siRNA; Cell Signaling Technology), ESE-1 siRNA, and control nonspecific siRNA (Dharmacon, Lafayette, CO) were purchased. siRNA transfections were performed by using 100 nM of a pool of four individual RNAi against Pak1, ESE-1, or control nonspecific siRNA and 4 µl of oligofectamine (Invitrogen, Carlbald, CA) according to the manufacturer’s protocol in six-well plates. Forty-eight hours were allowed to elapse after transfection to allow efficient silencing of the gene. Reverse transcription-PCR (RT-PCR) for ESE-1 was done using Access RT-PCR kit (Promega) using specific primers shown below: FP: 5’AAAAGAAACACGACCATGACCTAGCA’3 RP: 5’ GTTCCAAACCTCAGTTCCGAC 3’ T7-FP: 5’CAGCAAATGGGTCGGGATC 3’

**Soft agar assays -** Soft-agar colony-growth assays were done as previously described (36). Briefly, 1 mL of 0.6% Difco agar in DMEM medium was layered onto tissue culture plates. Test cells (1x10^4) mixed with 1 mL of 0.36% bactoagar solution in DMEM were layered on top of the 0.6% bactoagar layer and plates were incubated at 37°C in 5% CO₂ for 21 days and colonies were counted.

**Immunofluorescence and confocal microscopy studies** - We determined the cellular localization of proteins by indirect immunofluorescence using Olympus FV300 laser scanning confocal microscope as described previously (36). Briefly, cells grown on glass coverslips were fixed in 4% paraformaldehyde at 30°C for 4 min. The cells were incubated with primary antibodies for 1 h, washed three times in PBS, and then incubated with secondary antibodies conjugated with Alexa 546 (red), Alexa 633 (blue), or Alexa 488 (green) dye from...
Molecular Probes (Eugene, OR). The DNA dye Topro-3 (Molecular Probes, Invitrogen, Carlbad CA) was used to co-stain the DNA (blue). Cells treated only with the secondary antibody served as controls.

RESULTS

Pak1 phosphorylates ESE-1 both in vitro and in vivo - To explore the possibility that Pak1 phosphorylates ESE-1, we first performed an in vitro Pak1 kinase assay by incubating recombinant GST-ESE-1 and purified Pak1 enzyme. The DLC1 protein, an established Pak1 substrate (32) and GST were used as positive and negative controls, respectively. The results showed that the Pak1 enzyme could phosphorylate GST-ESE-1 but not GST alone (Fig. 1A). Further, we also observed a strong dose-dependent enhancement of ESE-1 phosphorylation by Pak1 (Fig. 1B), suggesting that Pak1 effectively phosphorylates ESE-1 in vitro. Next we examined the status of ESE-1 phosphorylation in vivo. To that end, MCF7 cells were metabolically labeled with [32P]orthophosphoric acid, and the status of ESE-1 phosphorylation was determined after treatment with physiological signal that activate Pak1 (29) such as serum. Signal (serum) that activates Pak1 increased the phosphorylation of transfected epitope-tagged ESE-1 (Fig. 1C). To further confirm the direct involvement of Pak1 in ESE-1 phosphorylation, we cotransfected either control siRNA or Pak1 siRNA, along with T7-ESE-1, into MCF7 cells, and then cells were labeled metabolically with [32P]orthophosphoric acid. Under modest knock down conditions of Pak1 (Fig 1D, lower panel), phosphorylation of ESE-1 was reduced significantly (Fig. 1D). Together, these results suggest that Pak1 efficiently phosphorylates ESE-1 both in vitro and in vivo.

Pak1 phosphorylates ESE-1 at serine 207 in the SAR domain - After confirming that Pak1 phosphorylates ESE-1 both in vitro and in vivo, we next mapped the potential Pak1 phosphorylation sites in ESE-1. ESE-1 protein contains several functional domains, including the pointed, transactivation (TAD), SAR, AT hook, and ETS domains (Fig. 2A). It is known that Pak1 is a serine/threonine kinase which phosphorylates a number of substrates on serine/threonine residues, preferring preceding basic residues such as (K/R)(R/X)(S/T) (28;29). Analysis of the ESE-1 amino acid sequence identified several potential putative Pak1 phosphorylation sites (Fig. 2A). To map the functional Pak1 phosphorylation site in ESE-1, we next generated a series of GST-fused ESE-1 deletions and assayed each one for its phosphorylation by the Pak1 enzyme. Results indicated that the Pak1 phosphorylation site on ESE-1 is located in the region between amino acids 159 and 259 (Fig. 2A). Examination of this region of ESE-1 revealed the presence of two potential Pak1 phosphorylation sites, i.e., serine 207 and serine 254. Next, single point mutation of serine 254 to alanine did not affect the phosphorylation of ESE-1 by Pak1, whereas point mutation of ESE-1 at serine 207 to alanine completely abolished Pak1 phosphorylation of ESE-1 as demonstrated by in vitro Pak1 kinase assay, isolating serine 207 as the only Pak1 phosphorylation site in ESE-1 (Fig. 2B). To further confirm the in vitro kinase assay results in vivo, we subcloned Pak1 phosphorylation mutant of ESE-1, such as GST-ESE-1S207A, into mammalian expression vector pcDNA3.1C, resulting in pcDNA-ESE-1S207A, which expresses T7-tagged ESE-1S207A in mammalian cells. MCF7 cells were transfected with either wild-type T7-ESE-1 or T7-ESE-1S207A, or control pcDNA vector and then were metabolically labeled with [32P]orthophosphoric acid. Immunoprecipitation of the cell lysates with a T7 monoclonal antibody indicated a substantial reduction of the phosphorylation of ESE-1S207A as compared to intense...
phosphorylation of the wild-type T7-ESE-1, and thus strengthening the serine 207 as the Pak1 phosphorylation site in ESE-1 (Fig. 2C). Further to confirm that phosphorylation at ser 207 on ESE-1 is Pak1 dependent or not, we have silenced the Pak1 using Pak1 siRNA in either wild type ESE-1 or ESE-1S207A expressing MCF7 cells. Under effective knock down of Pak1, substantial reduction in the $[^{32}\text{P}]$ signal of wild type ESE-1 but not ESE-1S207A indicating that phosphorylation at serine 207 is indeed Pak1 dependent (Fig. 2D). Interestingly, we found that ESE-1 serine 207 is well conserved in human, mouse, and rat (Fig. 2E). All together these results suggest that Pak1 phosphorylate ESE-1 at serine 207 both in vivo and in vitro.

**Pak1 interacts with ESE-1 in breast cancer cells** - Although ESE-1 is a transcriptional factor, it localizes predominantly in the cytoplasm in breast cancer cells (18) and since Pak1 is also localized predominantly in the cytoplasm (28), we next explored the possibility of interaction between ESE-1 and Pak1 in breast cancer cells. Results from the GST pull-down assay showed that in vitro translated ESE-1 efficiently interacted with GST-Pak1 (Fig. 3A). Conversely, in vitro-translated Pak1 protein also interacted with GST-ESE-1 (Fig. 3B). To further confirm this interaction in a physiologic setting, lysates of ZR75 cells grown in 10% serum were subjected to immunoprecipitation using a Pak1 antibody. Results showed that ESE-1 is coprecipitated with Pak1 but not with control IgG (Fig. 3C). Since the available ESE-1 antibodies and the one used here are not good for immunoprecipitation, we were unable to conduct a reverse immunoprecipitation using an ESE-1 antibody. To further strengthen this observation, we carried out confocal microscopy studies using ZR75 breast cancer cells by double staining for transiently transfected myc-Pak1 and endogenous ESE-1. As shown in Figure 3D, transfected myc-Pak1 colocalises with endogenous ESE-1 which is predominantly localized in the cytoplasm.

Next, we defined the minimal region of Pak1 required for its interaction with ESE-1. Pak1 has several important domains which include N-terminal domain (AA 1-132), Cdc42/Rac1 interactive binding (CRIB) domain (AA 52-132), PIX binding domain (AA 132-270) and C-terminal kinase domain (AA 270-545) (28). These C- and N-terminal Pak1 deletion constructs were used and expressed as GST-fusion proteins; they were then subjected to GST pull down assays with the $^{35}$S-labeled ESE-1. The results suggested that ESE-1 binds to region comprising amino acids 1 to 270, which include N-terminal domain and PIX binding domain, but did not binds to the C-terminal kinase domain (AA 270-545) of Pak1 (Fig. 3E). Conversely, to define the binding region(s) of ESE-1 that are important for Pak1 interaction, we used a series of GST-fused ESE-1 deletion constructs. Results of the GST pull down assays indicated that ESE-1 uses amino acids 159-371, which contain the SAR, AT hook and ETS domains, to interact with Pak1 (Fig.3F). Taken together, these results suggest that ESE-1 interacts with Pak1 both in vitro and in vivo.

**Pak1 phosphorylation promotes ESE-1 transforming ability** - Since overexpression of either ESE-1 or SAR domain of ESE-1 alone in MCF12A mammary epithelial cells has been shown to be transforming in nature (18) and the fact that Pak1 phosphorylates ESE-1 at serine 207 which is located in the SAR domain, raised the possibility that phosphorylation of ESE-1 by Pak1 may be required for its transformation ability. To address this question, we generated stable MCF12A cells expressing either wild-type T7-ESE-1 or its Pak1 phosphorylation mutants. Then the expression of T7-tagged wild type ESE-1 and its phosphorylation mutants such as T7-ESE-1S207A and T7-ESE-1S207E mutants was determined by western analysis (Fig. 4A) and also by RT-PCR analysis (Fig. 4B). Next we have assayed the transformation ability of each of
these stable clones by an anchorage-independent-based colony formation assay under control siRNA or Pak1 siRNA knock down conditions. Typical colonies were photographed after 21 days and the numbers of colonies counted (Fig. 4C). In agreement with the previous reports (18), the cells expressing vector alone generated small clusters, whereas T7-ESE-1 stable transfectants formed larger colonies, indicating the transforming ability of ESE-1 in MCF12A cells (Fig. 4C). Interestingly, we also found that the number of transformed colonies was markedly reduced (~50%) in the serine 207 to alanine mutants (i.e., T7-ESE-1S207A), while the serine to glutamic acid mutants (i.e., T7-ESE-1S207E) formed more colonies than the wild-type T7-ESE-1 stable transfectants (Fig. 4D) under control siRNA transfected clones. Whereas silencing of Pak1 markedly reduced the number of transformed colonies by wild type ESE-1 but not either of Pak1 phosphorylation mutants indicating the dependence of Pak1 on ESE-1 transforming ability (Fig. 4D, 2nd row). All together these results indicate that Pak1 phosphorylation of ESE-1 affects its transformation ability at least in a significant manner.

Since, Pak1 phosphorylation mutant such as ESE-1S207A exhibits inefficient transforming ability in MCF12A cells (Fig. 4D) than wild type ESE-1, we reasoned that Pak1 phosphorylation might protect ESE-1. Therefore we evaluated the half-life of wild type and its Pak1 phosphorylation mutants, such as ESE-1S207A and ESE-1S207E in presence of protein synthesis inhibitor, cycloheximide. Interestingly, the half-life of wild type ESE-1 appears to be ~5 hr whereas Pak1 mutant, ESE-1S207A has lesser half-life which is about 3 hr (Fig. 5C). However, ESE-1S207E showed comparatively more stable (half life about 8-10 h) than either wild type or ESE-1-S207A. To confirm these findings, we carried out pulse-chase experiments using MCF7 cells by transfecting ESE-1 or its mutants in presence of [35S]methionine. In consistent with previous results (Fig. 5C), the results showed that indeed Pak1 phosphorylation mutant such as ESE-1 S207A is more short lived than either wild type or Pak1 phosphorylation mimetic mutant, such as ESE-1S207E (Fig. 5D). These results indicated that Pak1 phosphorylation may inhibit ESE-1 degradation.

Next we examined the status of endogenous ESE-1 in ZR75 cells under Pak1 silencing conditions, using Pak1 siRNA, in presence of protein synthesis inhibitor, cycloheximide. Cells were transfected with either control siRNA or Pak1 siRNA, grown for 48 h and then followed cycloheximide treatment for different time points as indicated in Fig. 5E. Results indicated that...
ESE-1 half life appears to be about 6h in control siRNA treated cells however silencing of Pak1 reduced the half life of ESE-1 from 6 h to ~3 h (Fig. 5E). Taken together, these results concluded that Pak1 phosphorylation inhibits the degradation of ESE-1, implying a role for Pak1 in promoting the tumorigenic functions of ESE-1 due to an overall increased ESE-1 level.

**ESE-1 interacts with β-TrCP and undergoes β-TrCP-dependent ubiquitination** - To further understand the mechanism of ESE-1 degradation, we searched for the presence of critical destruction elements in ESE-1 and reasoned that the potential regulation of such motifs by ESE-1-Ser207 phosphorylation by Pak1. A careful analysis of the ESE-1 amino acid sequence using the EMBOSS GUIv1.14 motif program revealed the presence of a potential PEST sequence (PEST score >5 is considered as a potential PEST sequence; PEST score for ESE-1 is 12.32) with 16 amino acid residues between amino acids 208 and 225 in the SAR domain (Fig. 6A). Proteins containing PEST sequences are known to undergo proteolytic degradation (21). Interestingly, we also found two putative β-TrCP interacting motifs or F-box (DSGXXX) or DDGXXD-like sequences in ESE-1, one such potential motif, DSGGSD, spanning between amino acids 211 and 216, located in close proximity to the Pak1 phosphorylation site, i.e., serine 207 (Fig. 6A), whereas another one, such as DSTLAS spanning between amino acids 24 and 26 is located in the pointed domain of ESE-1 (Fig. 6A). Ikk-β, β-catenin and Snail have been shown to contain serine/threonine sites in their F-box (37;38) and to interact with β-TrCP, and the stability of these proteins was influenced by the phosphorylation status of their serine residues. To explore the possibility of β-TrCP interaction with ESE-1, we cotransfected T7-ESE-1 and myc-β-TrCP into MCF-7 cells and carried out a coimmunoprecipitation assay using the T7 antibody. We found that β-TrCP is coimmunoprecipitated with T7-tagged ESE-1 (Fig. 6B). Further we also observed barely detectable levels of ESE-1 protein in MCF7 cells when we exogenously expressed myc-tagged β-TrCP but not in control myc-tagged (CMV) vector (Fig. 6C), indicating the role of β-TrCP in ESE-1 degradation. To further confirm this observation, we have silenced the endogenous β-TrCP in MCF7 cells using β-TrCP siRNA in MCF7 cells. Results showed that silencing of β-TrCP enhanced the ESE-1 protein level compared to control siRNA transfected cells (Fig. 6D). Since Pak1 phosphorylation inhibits ESE-1 degradation and Pak1 phosphorylation mutant such as ESE-1S207A has less half-life, it’s possible that ESE-1-Ser207Ala may be more prone to undergo ubiquitination. To test this notion, we cotransfected β-TrCP along with either wild type ESE-1 or ESE-1S207A or ESE-1S207E into MCF7 cells and examined the ubiquitination of each protein. We observed that both wild type ESE-1 and ESE-1S207A are readily conjugated to polyubiquitin chains in MCF7 cells in presence of β-TrCP expression (Fig. 6E, lane 3 and 5), however the ubiquitination of ESE-1S207E is significantly reduced (Fig. 6E, lane 6 and 7). All together these results suggest that ESE-1 undergoes β-TrCP mediated proteosomal degradation and Pak1 phosphorylation inhibits ESE-1 degradation.

**Pak1-ESE-1 activates MAPK pathway** - Since expression of a catalytically active Pak1 mutant stimulates anchorage-independent growth of breast cancer cells in soft agar in a preferential MAPK-sensitive manner (31) and overexpression of ESE-1 activates MAPK (19), we reasoned that Pak1 stabilizes ESE-1 which may in turn leads to the activation of MAPK. Indeed, expression of T7-ESE-1 in MCF7 cells activated MAPK by 3 fold; while the levels of the MAPK activation were reduced upon ESE-1-Ser207Ala expression (Fig. 7A). Furthermore, knockdown of either
ESE-1 or Pak1 also resulted significant reduction in the level of MAPK activation as compared to cells transfected with the control siRNA (Fig. 7B). Taken together these results suggested that a role of Pak1 is an upstream regulator of ESE-1 signaling in MAPK activation.

**Pak1 phosphorylation of ESE-1 modulates its transcriptional potential**- Since ESE-1 is a transcriptional factor, we next examined the effect of Pak1 phosphorylation on its transactivation potential. To address this issue, we used a previously described and widely used transient cotransfection assay utilizing a Cox-2 luciferase reporter, which contains ESE-1 binding elements (15). Transfection of T7-ESE-1 into MCF12A cells activated the Cox-2 reporter gene by several folds and cotransfection of Pak1 further increased it in a dose dependent manner (Fig. 8A). We next examined the ESE-1 transactivation potential by cotransfecting either wild-type ESE-1 or its Pak1 phosphorylation mutants along with Cox-2 luciferase reporter plasmid into MCF12A cells. The luciferase assay results suggested that Ser207 to Glu207 substitution (T7-ESE-1 S207E) enhanced the transcriptional potential of ESE-1 more efficiently than wild-type ESE-1, whereas the Ser207 to alanine mutant (i.e., T7-ESE-1-S207A) displayed markedly less reporter activity than wild-type ESE-1 (Fig. 8B). To validate the noted activation of ESE-1 by Pak1 in vivo, we next depleted the endogenous Pak1 using Pak1-specific siRNA in MCF7 cells and examined the status of endogenous Cox-2 by reverse transcriptase PCR analysis. We found that depletion of either Pak1 or ESE-1 significantly reduced the expression of Cox-2 compared to the cells transfected with control siRNA (Fig. 8C). Together, these findings suggest that Pak1 phosphorylation of ESE-1 affects its transcriptional activity.

**DISCUSSION**

Transcription factor ESE-1 has been shown to transform human mammary epithelial cells by an unknown cytoplasmic mechanism. The primary goal of this study was to define the upstream regulators that control ESE-1–transforming functions. Since Pak1 and ESE-1 exhibit overlapping functions, we hypothesized that Pak1 could be an upstream regulator of ESE-1. As reported here, we found that Pak1 directly interacts with and phosphorylates ESE-1, and this phosphorylation critically modulates the transformation functions of ESE-1. We also discovered that ESE-1 interacts with β-TrCP and is targeted to ubiquitin-proteosomal degradation, and that this process is prevented by Pak1 phosphorylation.

Several lines of evidence suggest that Pak1 phosphorylates ESE-1 at Ser207, which is located in the SAR domain of ESE-1. This domain consists of 50 amino acids, rich in serine and aspartic acid residues, and is unique, as it found only in ESE-1 but not in its homologues such as ESE-2 and ESE-3; thus, it provides specificity to the noted Pak1-ESE-1 connection. Despite the fact that many ETS transcription factors are targeted by signaling pathways such as MAP, JNK, and PI3 kinases and are controlled primarily by phosphorylation (25;26), this is the first evidence of Pak1 phosphorylation of an ETS family member. Pak1 regulates the functions of divergent cellular key proteins through phosphorylation, including histone H3, a component of nucleosome (39), estrogen receptor (40), and SHARP, repressor of Notch signaling (41), and also promotes mammary tumorigenesis (32;34). Since overexpression of ESE-1 transforms breast epithelial cells (18), it is possible that the earlier reported Pak1 hyperactivation–driven tumorigenesis may be influenced, at least in part, by ESE-1. We found that Pak1 and ESE-1 colocalize in the cytoplasmic compartment of breast cancer cells, and that substitution of Ser207 with
alanine significantly reduces the transformation ability of ESE-1, whereas gain-of-function mutant Ser207Glu (ESE-1-S207E) enhances such transformation in MCF12A cells. Oncogenic cell transformation is correlated with growth factor independence, often due to establishment of endogenous loops feeding into MAPK activation. We found that Pak1 and ESE-1 pathway is required for optimal MAPK kinase activities in breast cancer cells. Since catalytically active Pak1 mutant stimulates anchorage-independent growth of breast cancer cells in soft agar in a preferential MAPK-sensitive manner (31), it’s possible that these events may be influenced by Pak1 phosphorylation of ESE-1.

Another notable finding here is the effect of Pak1 phosphorylation in preventing degradation and conferring stability to ESE-1. Here we provide the evidence that phosphorylation of ESE-1 by Pak1 stabilizes ESE-1, as demonstrated in breast cancer cell lines on transfected ESE-1 as well as on endogenous ESE-1 levels (Fig. 5). In this context, many unstable eukaryotic transcription factors are characterized by an overlapping degradation region between the transactivation domain and the “degron” (degradation signal) sequence (20). ESE-1 contains two potential F-box–like motifs (DSGXXXS), one at the pointed domain (24 DSTLAS 26) and another at the SAR domain (212 DSGGSD 216). Notably, the second DSG motif was found as part of the PEST domain (amino acids 208 to 225), which is located in the SAR domain. Compelling evidence suggests that phosphorylated serine or threonine residues located in the F-box are associated with β-TrCP– or F-box–interacting proteins and are targeted to a ubiquitin-proteosomal pathway for degradation (37). Since Kanemori et al. provided the evidence that degradation motifs need not be serine but instead negatively charged amino acids such as aspartic acid, which give a net negative charge to the protein to enable association with β-TrCP and progression to the proteosomal degradation pathway (42), our results suggest that β-TrCP may binds to ESE-1 through these motifs and undergoes ubiquitination. It is possible that the Pak1 phosphorylation site exists in close proximity to the DSG motif in the SAR domain, and thus that phosphorylation prevents the association of β-TrCP with ESE-1 at this site. This may be the reason why Pak1 phosphorylation mutant of ESE-1 such as ESE-1S207A has less half-life than either wild type or its Pak1 phosphorylation mimetic mutant i.e., ESE-1207E.

Since ESE-1 initially defined as a transcriptional factor for several critical genes (11, 12, 13, 14, 15), we have verified its transcriptional activity using Cox-2 as a model. In agreement with previous reports, we also demonstrated that ESE-1 transactivate Cox-2 luc activity however in breast cancer cell lines (Fig. 8). Because Pak1 phosphorylation affects ESE-1’s stability, ESE-1S207A exhibited less transcriptional activity than either wild type or its phosphor mimetic mutant suggesting the Pak1 phosphorylation of ESE-1 impacts on its over all functions in breast cancer cells.

Our finding that Pak1 phosphorylation of ESE-1 enhances its transformation functions is interesting because it now links with cytoplasmic Pak1 signaling. Early reports also suggested a either Pak1 or ESE-1 expression leads to breast tumorigenesis however the linkage between these two proteins is not studied (18;31;34). Consistent with these reports our findings collectively support the notion that Pak1 is a critical upstream regulator of ESE-1/MAPK pathway. In conclusion, findings presented here reveal a novel regulatory mechanism wherein Pak1 phosphorylation and ubiquitin-proteosomal degradation coordinate control the level of ESE-1, and consequently its role in transformation by enhancing the steady state levels of a transforming gene product (Fig. 7D). Furthermore, these findings provide an
example of signaling convergence on the ESE-1 ubiquitination system.

Reference List

1. Oikawa, T. and Yamada, T. (2003) *Gene* **303**, 11-34
2. Sharrocks, A. D. (2001) *Nat.Rev.Mol.Cell Biol.* **2**, 827-837
3. Hsu, T., Trojanowska, M., and Watson, D. K. (2004) *J.Cell Biochem.* **91**, 896-903
4. Dittmer, J. and Nordheim, A. (1998) *Biochim.Biophys.Acta* **1377**, F1-11
5. Graves, B. J. and Petersen, J. M. (1998) *Adv.Cancer Res.* **75**, 1-55
6. Feldman, R. J., Sementchenko, V. I., and Watson, D. K. (2003) *Anticancer Res.* **23**, 2125-2131
7. Cabral, A., Fischer, D. F., Vermeij, W. P., and Backendorf, C. (2003) *J.Biol.Chem.* **278**, 17792-17799
8. Oettgen, P., Alani, R. M., Barcinski, M. A., Brown, L., Akbarali, Y., Boltax, J., Kunsch, C., Munger, K., and Libermann, T. A. (1997) *Mol.Cell Biol.* **17**, 4419-4433
9. Reddy, S. P., Vuong, H., and Adisesiah, P. (2003) *J.Biol.Chem.* **278**, 21378-21387
10. Jobling, A. I., Fang, Z., Koleski, D., and Tymms, M. J. (2002) *Invest Ophthalmol.Vis.Sci.* **43**, 3530-3537
11. Scott, G. K., Chang, C. H., Erny, K. M., Xu, F., Fredericks, W. J., Rauscher, F. J., III, Thor, A. D., and Benz, C. C. (2000) *Oncogene* **19**, 6490-6502
12. Kwon, J. H., Keates, S., Simeonidis, S., Grall, F., Libermann, T. A., and Keates, A. C. (2003) *J.Biol.Chem.* **278**, 875-884
13. Choi, S. G., Yi, Y., Kim, Y. S., Kato, M., Chang, J., Chung, H. W., Hahn, K. B., Yang, H. K., Rhee, H. H., Bang, Y. J., and Kim, S. J. (1998) *J.Biol.Chem.* **273**, 110-117
14. Brown, C., Gaspar, J., Pettit, A., Lee, R., Gu, X., Wang, H., Manning, C., Voland, C., Goldring, S. R., Goldring, M. B., Libermann, T. A., Gravallese, E. M., and Oettgen, P. (2004) *J.Biol.Chem.* **279**, 12794-12803
15. Grall, F. T., Prall, W. C., Wei, W., Gu, X., Cho, J. Y., Choy, B. K., Zerbini, L. F., Inan, M. S., Goldring, S. R., Gravallese, E. M., Goldring, M. B., Oettgen, P., and Libermann, T. A. (2005) *FEBS J.* **272**, 1676-1687
16. Neve, R. M., Ylstra, B., Chang, C. H., Albertson, D. G., and Benz, C. C. (2002) *Oncogene* **21**, 3934-3938
17. Kurrpios, N. A., Sabolic, N. A., Shepherd, T. G., Fidalgo, G. M., and Hassell, J. A. (2003) *J.Mammary.Gland.Biol.Neoplasia.* **8**, 177-190
18. Prescott, J. D., Koto, K. S., Singh, M., and Gutierrez-Hartmann, A. (2004) *Mol.Cell Biol.* **24**, 5548-5564
19. Schedin, P. J., Eckel-Mahan, K. L., McDaniel, S. M., Prescott, J. D., Brodsky, K. S., Tentler, J. J., and Gutierrez-Hartmann, A. (2004) *Oncogene* **23**, 1766-1779
20. Muratani, M. and Tansey, W. P. (2003) *Nat.Rev.Mol.Cell Biol.* **4**, 192-201
21. Rechsteiner, M. and Rogers, S. W. (1996) *Trends Biochem.Sci.* **21**, 267-271
22. Cardozo, T. and Pagano, M. (2004) *Nat.Rev.Mol.Cell Biol.* **5**, 739-751
23. Fuchs, S. Y., Spiegelman, V. S., and Kumar, K. G. (2004) *Oncogene* **23**, 2028-2036
24. Li, R., Pei, H., and Watson, D. K. (2000) *Oncogene* **19**, 6514-6523
25. Wasylyk, B., Hagman, J., and Gutierrez-Hartmann, A. (1998) *Trends Biochem.Sci.* **23**, 213-216
26. Yordy, J. S. and Muise-Helmericks, R. C. (2000) *Oncogene* **19**, 6503-6513
27. Bagrodia, S. and Cerione, R. A. (1999) *Trends Cell Biol.* **9**, 350-355
28. Kumar, R. and Vadlamudi, R. K. (2002) *J. Cell Physiol* **193**, 133-144
29. Bokoch, G. M. (2003) *Annu. Rev. Biochem.* **72**, 743-781
30. Kumar, R., Gururaj, A. E., and Barnes, C. J. (2006) *Nat. Rev. Cancer* **6**, 459-471
31. Vadlamudi, R. K., Adam, L., Wang, R. A., Mandal, M., Nguyen, D., Sahin, A., Chernoff, J., Hung, M. C., and Kumar, R. (2000) *J. Biol. Chem.* **275**, 36238-36244
32. Vadlamudi, R. K., Bagheri-Yarmand, R., Yang, Z., Balasenthil, S., Nguyen, D., Sahin, A. A., den Hollander, P., and Kumar, R. (2004) *Cancer Cell* **5**, 575-585
33. Yang, Z. B., Rayala, S., Nguyen, D., Vadlamudi, R. K., Chen, S., and Kumar, R. (2005) *Cancer Research* **65**, 3179-3184
34. Wang, R. A., Zhang, H., Balasenthil, S., Medina, D., and Kumar, R. (2005) *Oncogene*
35. Manavathi, B., Nair, S. S., Wang, R. A., Kumar, R., and Vadlamudi, R. K. (2005) *Cancer Research* **65**, 5571-5577
36. Vadlamudi, R. K., Manavathi, B., Balasenthil, S., Nair, S. S., Yang, Z. B., Sahin, A. A., and Kumar, R. (2005) *Cancer Research* **65**, 7724-7732
37. Karin, M., Cao, Y., Greten, F. R., and Li, Z. W. (2002) *Nat. Rev. Cancer* **2**, 301-310
38. Zhou, B. P., Deng, J., Xia, W., Xu, J., Li, Y. M., Gunduz, M., and Hung, M. C. (2004) *Nat. Cell Biol.* **6**, 931-940
39. Li, F., Adam, L., Vadlamudi, R. K., Zhou, H., Sen, S., Chernoff, J., Mandal, M., and Kumar, R. (2002) *EMBO Rep.* **3**, 767-773
40. Wang, R. A., Mazumdar, A., Vadlamudi, R. K., and Kumar, R. (2002) *EMBO J.* **21**, 5437-5447
41. Vadlamudi, R. K., Manavathi, B., Singh, R. R., Nguyen, D., Li, F., and Kumar, R. (2005) *Oncogene* **24**, 4591-4596
42. Kanemori, Y., Uto, K., and Sagata, N. (2005) *Proc. Natl. Acad. Sci. U.S.A* **102**, 6279-6284
43. Wiese, C. and Galande, S. (2001) *Biotechniques* **30**, 960-963

**Acknowledgments**

This study was supported by the NIH grants CA90970, and CA65746 (to RK). We thank Dr. Gutierrez-Hartmann, University of North Colorado Health Sciences Center, Denver, Colorado, for providing pCGN2-ESX plasmid, and Dr. M. C. Hung, The University of Texas M. D. Anderson Cancer Center, Houston, TX, for providing pCMV-wt β-TrCP plasmid.

**Footnotes**

SiRNA- small interfering RNA, SDS-PAGE, sodium dedocylsulphate polyacrylamide gel electrophoresis, Co-IP- coimmunoprecipitation.
Figure 1. Pak1 phosphorylates ESE-1 both in vitro and in vivo. A, In vitro phosphorylation of ESE-1. An in vitro kinase assay shows phosphorylation of purified GST-ESE-1 by purified Pak1 enzyme in presence of [γ-32P] ATP. GST-DLC1 and GST served as positive and negative controls, respectively. The lower panels are Ponceau S–stained blots showing GST-ESE-1, GST-DLC1, and GST. Asterisk illustrates the [32P] signal corresponds to protein bands on Ponceau S–stained blots. B, Increasing amounts of GST-ESE-1 were incubated with Pak1 enzyme, and in vitro kinase assay was repeated as above. C, In vivo phosphorylation of ESE-1. Following 24 h transfection of T7 tagged ESE-1, MCF7 cells were serum starved for 24 h and labeled with [32P] orthophosphoric acid overnight. Cells were then treated with serum (10%) for 30 min. Cell lysates were immunoprecipitated with T7 antibody, resolved by SDS-PAGE, and transferred to nitrocelullose membranes. After autoradiography, the same membranes were probed with a T7 antibody (lower panel). D, Following 48 h post transfection of either control siRNA or Pak1 siRNA, MCF7 cells were transfected with T7-ESE-1 and cells were grown for 24 h and then, cells were labeled with [32P]orthophosphoric acid as above. Phosphorylation status of T7-ESE-1 was determined by immunoprecipitation followed by autoradiography. An aliquot of total lysate was analyzed by western blot to check the knockdown of Pak1. Vinculin served as protein loading control.

Figure 2. Identification of the Pak-1 phosphorylation site in ESE-1. A, Schematic representation of functional domains of ESE-1 and putative Pak1 phosphorylation sites in ESE-1 (upper panel). AA, amino acids. In vitro kinase assays were performed using GST-fused ESE-1 fragments as substrates and Pak1 as the enzyme (middle panel). The lower panels are Ponceau S–stained blots showing GST-tagged ESE-1 deletion fragments, GST-DLC1, and GST. Asterisk illustrates the [32P] signal corresponds to protein bands on Ponceau S–stained blots. B, In vitro Pak1 kinase assay shows that Pak1 phosphorylates either WT-GST-ESE-1 or GST-ESE-1S254A but not GST-ESE-1S207A (Upper panel). The lower panels are Ponceau S–stained blots showing GST-tagged ESE-1 mutants, GST-DLC1, and GST. C, In vivo phosphorylation of wild-type ESE-1 and its Pak1 phosphorylation mutant, T7-ESE-1-S207A in MCF7 cells in presence of [32P] orthophosphoric acid (lower panel). Protein band intensities were quantified by Image quant 5.1 software. D, Effect of Pak1 silencing by Pak1 siRNA on in vivo phosphorylation of ESE-1. Following 48 h post transfection of either control siRNA or Pak1 siRNA, MCF7 cells were transfected with T7-ESE-1 or T7-ESE-1S207A. Eighteen hours post transfection of either WT-ESE-1 or ESE-1S207A, cells were labeled with [32P] orthophosphoric acid as above, and the phosphorylation status of ESE-1 was determined by immunoprecipitation followed by autoradiography. An aliquot of total lysate was analyzed by western blot to check the knockdown of Pak1. E, Conservation of serine 207 in ESE-1 among human, mouse and rat is shown in red.

Figure 3. Pak1 interacts with ESE-1 both in vitro and in vivo. A-B, Reciprocal interaction between Pak1 and ESE-1 demonstrated by in vitro translation combined GST pull-down assays. The lower panels are Ponceau S–stained blots showing either GST-ESE-1 or GST-Pak1 and GST. C, Interaction of ESE-1 with Pak1 in ZR75 cells. Co-immunoprecipitation (Co-IP) was performed with either control immunoglobulin IgG or Pak1 antibody using lysates of ZR75 cells. Cell lysates were mixed with 1XSDS dye which lacks β-mercaptoethanol to keep the IgG chains (heavy and light chains) intact before loading onto 8% PAGE gel as described (43). Then, immunoblots were analyzed by either Pak1 or ESE-1 antibody. D, Co-localization of endogenous ESE-1 with transfected myc-tagged Pak1 in ZR75 cells. Colocalization between ESE-1 and Pak1 appears in
yellow. Bar 10µm. Indicates with a white box area is blow ups and shown in the right side of the panel. E-F, Mapping the minimal interacting regions between Pak1 and ESE-1 by GST pulldown assay described in methods section.

Figure 4. Phosphorylation of ESE-1 by Pak1 promotes its transformation potential. A, Western blot analysis shows the overexpression of ESE-1 and its Pak1 phosphorylation mutants in MCF12A cells. B, Total RNA was extracted from either pcDNA or wild type or mutant ESE-1 stably expressing in MCF12A cells and synthesis of ESE-1 mRNA was analyzed by RT-PCR (right panel). Lanes 2, 3 and 4 show the ~400bp PCR product which spans between T7 tag and N-terminal region of ESE-1. C, Anchorage-independent growth potentials of wild-type ESE-1 and its mutants under control siRNA or Pak1 siRNA treated conditions. Number of colonies formed on soft agar after 21 days. Representative phase images obtained at 21 days in soft agar using a 10x (top row) objective are shown. Western blot presented underneath soft agar pictures is silencing of Pak1 in either pcDNA or WT ESE-1 or ESE-1S207A or ESE-1S207E stably expressing MCF12A cells. D, Graph represents the number of colonies formed on soft agar by either pcDNA or wild type or mutant ESE-1 MCF12A transformants in either control siRNA or Pak1 siRNA treated conditions. Significant levels were determined by the two-sided t-test. Wild type T7-ESE-1 vs T7-ESE-1S207A ($P = 0.03$) and wild type T7-ESE-1 vs T7-ESE-1S207E ($P = 0.01$).

Figure 5. ESE-1 undergoes proteosomal degradation. A, Effect of proteosomal inhibitor MG132 on the stability of ESE-1. Following 24 h transfection of ESE-1 into MCF7 cells, cells were treated with MG132 (10 µM) for the indicated times and then cell lysates were analyzed by western blotting using T7 antibody. Vinculin served as loading control. B, MCF7 cells transfected with T7-ESE-1 were left untreated (second lane) or treated with protein synthesis inhibitor, cycloheximide (CHX) (25 µg/ml) for 8 h (third lane) or with MG132 (10 µM) for 30 min followed by CHX for 8 h (fifth lane) or with MG132 alone for 8 h (fourth lane), as indicated. Cell extracts were subjected to anti-T7 (upper panel) or anti-vinculin western blot. C, Following 24 h transfection of either wild type T7-ESE-1 or its mutants, MCF7 cells treated with CHX (25 µg/ml) for indicated times and then cells lysates were analyzed by western blotting using T7 antibody or vinculin (left panel). The amount of ESE-1 protein at each time point was quantified on a PhosphorImager and normalized relative to the amount of ESE-1 present in cells at 0 h; results are plotted (right panel). D, Twenty hours after transfection, cells were pulse-labeled with [$^{35}$S] methionine for 2 h and then chased for the indicated length of time in presence of CHX. Cell lysates were immunoprecipitated with an anti-T7 antibody. The resulting T7 immunoprecipitates were separated by SDS-PAGE and visualized by autoradiography (left panel). The amount of labeled ESE-1 protein at each time point was quantified on a PhosphorImager and normalized relative to the amount of radiolabeled ESE-1 present in cells following the 0 h chase; results are plotted (right panel). E, ZR75 cells were transfected with either control siRNA or ESE-1 siRNA. Forty-eight hours following transfection, cells were treated with cycloheximide (25 µg/ml) for indicated times and then cell lysates were analyzed by western blotting using ESE-1 and Pak1 antibodies, respectively; results are plotted (right panel). Vinculin served as loading control.

Figure 6. ESE-1 interacts with β-TrCP and follows β-TrCP/ubiquitin-mediated proteosome degradation. A, Schematic representation of ESE-1 functional domains. Identification of PEST sequence between amino acids 208 and 225 in the SAR domain of ESE-1 (PEST score is 12.32). PEST score >5 is a potential PEST sequence. Alignment of DSG motifs found in ESE-1 with known DSG and DDG motifs. B, Co-IP and western blot shows the interaction of transfected T7-
ESE-1 with myc-tagged β-TrCP in MCF7 cells. C, Western blot analysis shows the ESE-1 protein levels after expression of myc-tagged β-TrCP in MCF7 cells. Following 24 h transfection cells were lysed and subjected to Co-IP with T7 and probed with indicated antibodies. D, Western blot analysis shows the increased levels of ESE-1 protein after silencing β-TRCP by β-TRCP siRNA in MCF7 cells. Following 48 h transfection with either control siRNA or β-TRCP siRNA, cells were lysed and subjected to SDS-PAGE, probed with indicated antibodies. E, Co-IP and western blot analysis shows the polyubiquitination of ESE-1 and its mutants in MCF7 cells. MCF7 cells were transfected with either wild type T7-ESE-1 or its mutants alone or along with myc-β-TrCP. Following 24 h transfection cells were lysed and subjected to Co-IP with T7 and probed with indicated antibodies.

Figure 7. Pak1-ESE-1 activates MAPK. A, Western blot analysis shows activation of MAPK by expression of either wild type ESE-1 or mutant ESE-1 in MCF7 cells. B, Western blot analysis shows the reduced activation of MAPK by knock down of either Pak1 or ESE-1 by Pak1 siRNA or ESE-1 siRNA, respectively in MCF7 cells.

Figure 8. Phosphorylation of ESE-1 by Pak1 modulates its transcriptional activity. (A) MCF12A cells were cotransfected with Cox-2 reporter plasmid, T7-ESE-1, and increasing amounts of wild-type Pak1. Following 18 h of transfection, cells were subjected to lysis and luciferase activity was measured. (B) MCF12A cells were cotransfected with Cox-2 reporter plasmid and either pcDNA alone or wild-type T7-ESE-1 or T7-ESE-1-S207A or T7-ESE-1-S207E. Eighteen hours following transfection, cells were subjected to lysis and luciferase activity was measured. (C) MCF7 cells were transfected with control siRNA, Pak1 siRNA, or ESE-1 siRNA. Forty-eight hours after transfection, total RNA was extracted and analyzed by reverse-transcriptase PCR using Cox-2 or ESE-1-specific primers (left panel).
Fig. 1

A

| 1 | 2 | 3 |
|---|---|---|
| + | + | + Pak1 |
| + | - | - GST-ESE-1 |
| - | + | - GST-DLC1 |
| - | - | + GST |

[B] Ponceau S stain

[GST-ESE-1] [32P] signal

C

| 1 | 2 | 3 |
|---|---|---|
| + | + | + T7 ESE-1 |
| + | - | - serum |

[32P] signal

WB:T7 (ESE-1)

B

| 1 | 2 | 3 |
|---|---|---|
| + | + | + Pak1 |

[32P] signal

Ponceau S stain

GST-ESE-1

D

| 1 | 2 | 3 |
|---|---|---|
| + | + | + T7 ESE-1 |
| + | - | - Con siRNA |
| - | + | + Pak1 siRNA |

[32P] signal

T7 (ESE-1)

WB

Pak1

Vinculin
Fig. 2

A

| GST ESE-1(1-159) | Pak1 Pho. |
|------------------|-----------|
| + + + + + + + | - - - - - - |
| GST ESE-1(128-159) | - - - - - - |
| GST ESE-1(274-371) | - - - - - - |
| GST ESE-1(1-259) | + - - - - - |

B

| GSTWT ESE1 | Pak1 Pho. |
|-----------|-----------|
| + + + + + + | - - - - - - |
| GST ESE1 S207A | - - - - - - |
| GST ESE1 S254A | - - - - - - |

C

T7 ESE-1 S207A
T7 ESE-1

D

| WT T7 ESE-1 | Pak1 siRNA | Con siRNA |
|-------------|------------|-----------|
| + + + + + + | - - - - + + |

E

Human ESE-1 199 -AGT GASRS SHSSDSG- 213
Mouse ESE-1 ARTATPQ SHASDSG
Rat ESE-1 SGDTPQ SHSSDSG

Serine 207
Fig. 4

A

pcDNA pool
WT ESE-1 pool 1
ESE-1S207A pool 2
ESE-1S207E pool 2

WB

1 2 3 4 lane

vinculin

T7

B

MW pcDNA WT ESE-1 ESE-1S207A ESE-1S207E

400bp

400bp

100bp

1 2 3 4 5 lane

FP
RP
(~400bp)

C

pcDNA WT ESE-1 ESE-1S207A ESE-1S207E

Con. siRNA Pak1 siRNA

Pak1 Vinculin

Con. siRNA Pak1 siRNA

Con. siRNA Pak1 siRNA

Con. siRNA Pak1 siRNA

Con. siRNA Pak1 siRNA

D

Number of colonies on soft agar

Control siRNA Pak1 siRNA

P=0.02

P=0.03

P=0.01

pCDNA WT ESE-1 ESE-1S207A ESE-1S207E pCDNA WT ESE-1 ESE-1S207A ESE-1S207E
Fig. 6

A

| 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|
| N | ESE-1 | ESE-1 | ESE-1 | ESE-1 | ESE-1 | ESE-1 |
| 1 | Pointed | TAD | SAR | AT | ETS | C |
| N | PEST | SAR (AA 190–239) | PEST (AA 208–225) |

ESE-1 209 SSDGGSGDVD 218
ESE-1 21 SSDSTLASVP 30

DSG motif consensus: DSGXXS

DDG motif consensus: DDGXXD

B

| IP: T7 | + | + | myc-β-TrCP |
|---|---|---|---|
| + | + | T7 ESE-1 |
| - | - | pcDNA |

\[ \text{Myoglobin} \]

\[ \text{Myc} \]

\[ \text{IgG} \]

\[ \text{T7} \]

1 2 3 lane

C

| - | + | - | CMV-vector |
|---|---|---|---|
| + | - | - | myc-β-TrCP |
| + | + | + | T7-ESE-1 |

\[ \text{T7-ESE-1} \]

\[ \text{Non-specific} \]

\[ \text{Myc- β-TRCP} \]

vinculin

1 2 3 lane

D

| - | + | Con.siRNA |
|---|---|---|
| + | - | β-TrCP siRNA |

\[ \text{ESE-1} \]

\[ \text{β-TrCP} \]

\[ \text{Vinculin} \]

1 2 lane

E

| IP-T7 | + | - | + | - | + | - | myc β-TrCP |
|---|---|---|---|---|---|---|---|
| + | + | - | + | - | - | - | T7-ESE-1S207E |
| + | - | - | - | - | - | - | T7-ESE-1S207A |
| - | - | + | + | - | - | - | WT T7-ESE-1 |

\[ \text{Ub} \]

\[ \text{T7} \]

\[ \text{myc β-TrCP} \]

1 2 3 4 5 6 7 lane
Fig. 8

A

Cox-2 Luc

ESE-1

WT-Pak1

B

Cox-2 Luc

pcDNA

WT ESE-1

ESE-1 S207A

ESE-1 S207E

C

Con siRNA

Pak1 siRNA

ESE-1 siRNA

RT-PCR

ESE-1 (~170bp)

Actin (~400bp)

Cox-2 (~350bp)

Fold change

4.6 3.2 1

by guest on March 24, 2020http://www.jbc.org/Downloaded from
Phosphorylation-dependent regulation of stability and transforming potential of ETS transcriptional factor ESE-1 by p21-activated kinase 1
Bramanandam Manavathi, Suresh K. Rayala and Rakesh Kumar

J. Biol. Chem. published online May 9, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M702309200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts