Pak1 (p21-activated kinase1), one of the evolutionarily conserved families of serine/threonine protein kinases, is a downstream effector of the activated Rho GTPases Rac1 and Cdc42. Over the years, Pak1 kinase activity has been implicated in a wide variety of cellular processes like cytoskeletal reorganization, cell growth, motility, morphogenesis and gene regulation by stimulation of signaling pathways (1). In addition to influencing various cytoplasmic functions, it has also been increasingly recognized that the Pak1 pathway also affects downstream nuclear events, presumably by the stimulation of mitogen-activated protein kinases (MAPKs) (2-4) and modulation of co-activator / co-repressor mediated gene regulation (5, 6).

Previous study from our laboratory showed that Pak1 binds and phosphorylates Histone 3.3 and that endogenous Pak1 repression of NFAT1 expression. These investigations provide proof-of-principle evidence that Pak1 could influence the expression of its putative chromatin targets in both positive and negative manner. Together, for the first time, these findings defined the NLSs of the Pak1, its association with chromatin and the resulting modulation of transcription, and thus opening new avenues to further the search for nuclear Pak1 functions and identify putative Pak1 interacting nuclear proteins.
localizes inside the nucleus of 18-24% of the interphase cells (7). In this context, it has been established that the nuclear localization of activated protein kinases such as MAP kinases (8-10), Brutons Tyrosine kinase (11) and Rsk-encoded Pnt kinase (12) is a common cellular event in growth factor-activated cells. Epidermal growth factor receptor (EGFR), a transmembrane glycoprotein with intrinsic tyrosine kinase activity, was found to localize in the nucleus and acts as a potential transcription co-activator in a kinase activity-independent manner (13). Pak6 has been shown to interact with the androgen receptor and translocates into the nucleus upon androgen stimulation (14). Another Pak family member Pak2 was also found to localize in the nucleus upon proteolysis with caspases, the resulting Pak2 fragment containing the nuclear localizing signal relocates in the nucleus and stimulates programmed cell death whereas the intact protein with nuclear export signal motif localizes in the cytoplasm (15).

In spite of emerging significance of Paks in the nuclear compartment, the role and nature of the potential Pak1 nuclear targets remains unknown. In the present study we investigated the nuclear localization of Pak1, identified its nuclear localizing signal sequences and also have obtained evidence of its association with chromatin. We have further investigated the association of Pak1 with the chromatin of two important genes, PFK M (muscle type isoform) and NFAT1 and the resulting modulation of their transcription in the cell. These investigations provide proof-of-principle evidence that Pak1 could influence the expression of its targets in both positive and negative manner.

Materials and Methods

Cell cultures and Reagents – MCF-7 and MDA-MB-435 human breast cancer cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM)-F12 (1:1) supplemented with 10% fetal calf serum. When cells were treated with EGF they were cultured in DMEM media without FBS for 24h prior to growth factor or serum treatment.

Sub-cellular protein extraction - The MCF-7 cellular components were sequentially extracted using a widely adopted biochemical fractionation and sequential extraction procedure (16-18) as “soluble” (with Nonidet P-40 buffer), “cytoskeletal / nucleoplasm-associated” (with Triton X100), “chromatin-associated” (with DNase treatment) and “nuclear matrix-associated” protein fractions. The purity of the isolated fractions was established by western blot analysis with antibodies against marker proteins like, poly-(ADP-ribose) polymerase (PARP) (BD Pharmingen, San Diego, CA) for chromatin, Lamin-B1 (Biotechnology Inc, Santa Cruz, CA) for nuclear matrix and Paxillin (BD Transduction labs, San Diego, CA) for cytoplasm. The presence of Pak1 in individual fractions was confirmed by western blot analysis with a Pak1 specific antibody (Cell Signaling, Beverly, MA).

Generation of Pak1 NLS mutants, Immunoflorescence and Confocal microscopy – Pak1 NLS mutants was generated using a Quick Change Kit (Stratagene, La Jolla, CA) and the lysine residues in the three NLSs of Pak1 were mutated to alanines using myc-tagged Pak1 as template. The primers used were

\[
\begin{align*}
\text{Pak1NLS1:} & \quad 5'-\text{CCAAACCCAGAGGAGGCAGCAGCAGCAGACCGATTTTACCGATCC}-3' \\
\text{Pak1NLS2:} & \quad 5'-\text{CGGAATACTGAGAAGCAGCAGCAGCAGCTAAAATGTCTGATGAGGAG}-3' \\
\text{Pak1NLS3:} & \quad 5'-\text{GTGAGTGTGGGCAGATCTGCTGCAGCAGCA}
\end{align*}
\]
TATACACGTTTGAG–3’ (Pak1 NLS3). The cellular localization of WT and NLS mutants of Pak1 on EGF treatment (100ng/mL) using indirect immunofluorescence by scanning confocal microscopy as described previously (19).

**Transient transfections and luciferase assay** – Transient transfections of plasmids into MCF-cells was done using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN). Luciferase assay was done using Promega luciferase assay system (Madison, WI).

**Chromatin Immunoprecipitation assay** - Serum-starved cells were stimulated with EGF (100 ng/mL for 45 min), cross-linked with formaldehyde (1% final concentration), and sonicated on ice to fragment the chromatin into an average length of 1-2 kilo bases. Supernatants in the sonicated lysates were diluted ten fold with chromatin-dilution buffer (0.01% sodium dodecyl sulfate (SDS), 1.1% Triton X100 and protease inhibitor cocktail). Chromatin solutions were immunoprecipitated with Pak1-specific antibody (Cell Signaling, Beverly, MA) at 4°C overnight. Protein A sepharose beads were added to the lysate to isolate the antibody-bound complexes. The beads were washed to remove non-specific binding and the antibody-bound chromatin was eluted. The eluate was “decrosslinked” by heating at 70°C for 6 h. RNase was added during this step to digest the RNA contamination. Samples were then treated with proteinase K for 1 h at 40°C to digest the proteins pulled down by immunoprecipitation, and finally, the DNA was extracted using the phenol chloroform method. Primers used to PCR amplify the PFK-M gene chromatin which was one of the Pak1 targets were 5’-TCTTCCAGGGAGAGCTGTGA-3’ and 5’-TGATCCTACAACACTGGGCGCATAGC-3’.

Primers to amplify NFAT1 gene chromatin were 5’-AACACAAAGTCCCCGTAAC-3’ and 5’-TTTGAATCCGAACGAGTGTC-3’.

**PFK-M expression by Western blotting, RT-PCR and Northern blot analysis** – Serum starved MCF-7 cells were treated with EGF (100ng/mL) and cell lysates were prepared using Triton X100 buffer (50mM Tris-HCl, pH 7.5, 100mM NaCl, 0.5% Triton X-100, 1X protease inhibitor mixture and 1mM sodium vanadate) for 15 min on ice. The lysates containing equal amount of proteins (100 µg) were resolved on SDS-polyacrylamide gel (8% acrylamide), transferred to nitrocellulose membrane, probed with PFK-M specific antibody and developed using enhanced chemiluminescence method (ECL). RNA from MCF-7 cells treated for different intervals of time was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), treated with DNAse for 15 min after which DNAse was inactivated by heating the samples at 65°C for 10 min. The PFK mRNA levels was analyzed by RT-PCR with PFK-M mRNA specific primer, 5’-GTTCCTGGGGATGCGTAAGAG-3’ and 5’-GCATGGTCTGAAGTGTCCAAG-3’.

For northern blot analysis 15 µg RNA of each sample were separated on 0.8% Agarose formaldehyde-formamide RNA gel and transferred to nitrocellulose membrane. The PCR product from the RTPCR analysis was isolated and labeled with [α-32P] dCTP and used as the probe in northern blot analysis. The RNA blot was also probed for GAPDH and used as control.

**Pak1 knockdown with RNAi and effect on PFK-M and NFAT1 expression** – MCF-7 cells were transfected with Pak1-specific RNAi (Cell signaling, Beverly, MA) or control RNAi using the transfection reagent Oligofectamine (Invitrogen, Carlsbad, CA) and after 36 h cells were transfection of Pak1 RNAi was repeated to ensure an effective knockdown of Pak1 expression. After 24h of transfection cells were cultured in serum free media for another 24 h and treated with EGF for 8h. RNA was extracted and the levels of PFK-M expression were checked by RT-PCR. The successful knockdown of Pak1 was
checked by western blot analysis with Pak1 antibody. The effect of pak1 knockdown on NFAT1 expression was checked both in MCF7 and MDA-MB-435 cells by RTPCR analysis and western blotting. 5’-GCCCCCTTGCTAGTCTCTCT-3’ and 5’-GGTGTAGGGGGAGAAGGTGT-3’ were used as primers for RTPCR and NFAT1 antibody (BD Biosciences, NJ) was used for western blot analysis.

RESULTS

Differential subcellular localization of Pak1 – Differential extraction of sub-cellular proteins and immunoblotting with Pak1-specific for Pak1 showed that Pak1 was present in almost all of the sub-cellular fractions with a major portion of total Pak1 being associated with the active chromatin (DNase-insensitive fraction) and also with the nuclear matrix. These results demonstrated that Pak1 could be localized in the nuclear localization, and thus, raising the possibility that it may be involved in transcription modulation. At least three Pak1 bands of different mobility could be detected in the western blot analysis (Fig. 1A), presumably due to different phosphorylated and active forms of Pak1. Of interest, Pak1 existed as a single band with a slightly higher electrophoretic mobility in the chromatin fraction (Fig. 1A, lane 4). These initial findings raised the possibility that active Pak1 may preferentially localize in the nucleus upon activating growth factor signaling.

Identification and functionality of the Pak1 nuclear localization signals - To identify possible nuclear localization signals (NLSs) in Pak1, which are responsible for its nuclear accumulation, the Pak1 amino acid sequence was screened with a NLS recognizing program, PSORTII (20, 21). This led to the identification of three potential signals in Pak1 and designated as NLS1, NLS2 and NLS3 (Fig. 1B). To investigate the functional significance of these NLSs, we selectively mutated each one by replacing the three basic lysine residues with alanines and used confocal scanning microscopy to evaluate the ability of the resulting myc-tagged Pak1 NLS mutants to localize in the nucleus upon stimulation by epidermal growth factor (EGF) (Fig. 1B). On average over 50 transiently transfected cells under each condition (wild type [WT] and the NLS mutants, with and without EGF treatment) were screened, and the numbers of cells with cytoplasmic and nuclear Pak1 were recorded. As illustrated in Fig. 1B, we found that Pak1 was predominantly localized in the cytoplasm when the cells were cultured in the absence of serum or external signals such as EGF. However, upon stimulation with EGF (100 ng/mL for 30 min), the levels of the nuclear Pak1 rose markedly. Mutation of any one of the putative NLSs was not sufficient to prevent cytoplasmic-to-nuclear translocation of Pak1, because the remaining two intact NLSs remained capable of causing nuclear import (data not shown). However, simultaneous mutation of two of NLS sites, NLS1+NLS2 or NLS2+NLS3, in Pak1 prevented the nuclear localization of Pak1 upon EGF stimulation, whereas mutation of NLS1+NLS3 had no inhibitory effect on the ability of Pak1 to translocate to the nucleus. Subsequent analysis of Pak1 double-NLS mutants suggested a superior role of NLS2 for the nuclear accumulation of Pak1. The fact that the mutation of NLS2 alone did not eliminate the nuclear localization of Pak1 indicated that NLS1 and NLS3 individually had weak nuclear localization effects. However, when both NLS1 and NLS3 were intact in Pak1, these sites could functionally compensate for the absence of NLS2 and may help in the noted nuclear translocation of Pak1. We also found that the presence of intact NLS1 or NLS3 alone with other two NLS sites mutated was not sufficient Pak1 was unable to the nucleus. The percentage of
cells with nuclear Pak1 (WT, double and triple NLS mutants) was quantitated and representative photomicrographs from confocal microscopy analysis are shown in Fig. 1C.

**Pak1 modulation of transcription** - To investigate the effect of Pak1 on gene transcription, we next cloned the full-length wild type (WT Pak1) and a catalytically active Pak1 (T423E) into the pSG424 vector. These constructs expressed WT and T423E mutant Pak1 as fusion proteins with a Gal4 DNA-binding domain. The Pak1-fusion plasmids were co-transfected with Gal4-luciferase (Gal4-luc) reporter construct, and the effect on transcription was tested by the luciferase assay. In these studies, we used the activation function 2 (AF2) of ERα fused with Gal4 (Gal4-AF2) (22) as positive control, and Gal4-MTA1 as negative control (23). Results from the Gal4-luc assays showed that both WT Pak1 and T423E-Pak1 reproducibly enhanced the Gal4-luc activity by two to three-fold (Fig. 2B). The activation of Gal4-luc activity with Gal4-AF2 and a corresponding repression by Gal4-MTA1 validated the performance of these assays. The noticed three-fold enhancement of the Gal4-luc activity by Gal4-WT Pak1 suggested that Pak1 could influence gene transcription.

**Search for Pak1 chromatin targets** - Our findings of the Pak1 translocation to the nucleus in growth factor stimulated cells and its association with the active chromatin fraction led us to postulate that Pak1 might interact with specific promoter chromatin. To experimentally explore this possibility, we performed a Pak1-specific antibody based double chromatin immunoprecipitation (ChIP) assay in MCF-7 cells, following the procedure described earlier (23). The DNA fragments precipitated with Pak1-associated chromatin were cloned into pBluescript (Stratagene, La Jolla, CA) vector and sequenced. A BLAST search was done with the sequences to compare these sequences with the human genome database to identify the genes with which Pak1 might be associated. A partial list of representative genes identified in this analysis is provided in Table 1. Since we discovered that Pak1 could influence the Pak1-associated chromatin-driven transcription in both stimulatory and inhibitory manner (see below), we wished to present proof-of-principle evidence in support of such notion using two representative examples.

**Phosphofructokinase-muscle-type isoform (PFK-M), a novel Pak1 chromatin target** - To further characterize the functions of nuclear Pak1, we focused on the PFK-M (muscle type isoform) gene chromatin and validated it as a target of Pak1. PFK is the key regulatory rate-limiting enzyme in glycolysis, and catalyzes the phosphorylation of fructose-6-phosphate to fructose-1, 6-bisphosphate, an important rate-limiting step in glycolysis. The fragment of chromatin DNA pulled down by the Pak1-specific antibody during ChIP analysis was a 700 bp region, a possible enhancer sequence, which was located on chromosome 12 and 6.8 kilo base upstream of the PFK-M promoter (Fig. 3A). ChIP analysis with anti-Pak1 antibody and subsequent PCR analysis found that Pak1 association with the PFK-M chromatin was enhanced upon stimulation of cells with EGF (Fig. 3B). The fact that the region of PFKM chromatin initially identified as Pak1 target was 6.8 kb upstream of PFKM promoter 1 raised the possibility that Pak1 may also be associated with the promoter region. We performed the PCR analysis with primers specifically designed against the promoter region and found that indeed, Pak1 was also associated with the PFKM promoter and such association was distinctly enhanced upon growth factor stimulation (Fig 3C).
Functionality of Pak1 binding to PFKM chromatin - To investigate the possibility that the identified 700 base pair sequence was part of an enhancer sequence; we cloned this sequence into a PGL2 basic vector (Fig. 4A). This vector has a minimal TATA promoter upstream of a luciferase reporter gene and has no intrinsic mammalian promoter or enhancer sequence, making it suitable for testing regulatory sequences. To directly demonstrate an effect of Pak1 signaling upon the PFK-M gene transcription, we examined the effect of Pak1 upon PFK-PGL2 in MCF-7 cells. The levels of reporter gene expression were measured by the luciferase assay, which would demonstrate the effect of Pak1 on gene expression. This could be extrapolated as the effect of Pak1 association with the enhancer in its effect on PFK-M expression. We found a distinct enhancement of luciferase activity in cells stimulated with 10% fetal bovine serum (FBS) and also in cells with Pak1 overexpression (Fig. 4B). A similar increase of luciferase activity was seen upon EGF treatment (Fig. 4C). These findings suggested that growth factor signaling stimulated the nuclear localization and association of Pak1 with the PFK-M chromatin, which eventually contributed towards the elevated expression of PFK-M. To independently validate these findings, in the next set of experiments, we repeated similar experiment using Pak1 NLS mutant with all the three nuclear localizing sequences mutated. We found that such mutant was incapable of stimulating the luciferase activity in comparison to WT Pak1 (Fig. 4D). This was additional evidence that the mutant was incapable of nuclear localization and hence could not affect the expression of luciferase. Since a motif search of Pak1 showed a lack of any DNA-binding domains, we speculate of Pak1 does not associate with the enhancer sequence directly but as a part of the transcription complex.

Pak1 is a mediator of growth factor induced PFK-M expression - As growth factor stimulation promoted the nuclear localization of Pak1, we next examined the effects of EGF signaling on PFK expression (Fig. 5). MCF-7 breast cancer cells were treated with EGF for different lengths of time, and the expression of PFK-M mRNA and protein was examined. Results from Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis showed an increase in PFK expression as early as 1 h after EGF treatment with a considerable additional increase by 4 h of treatment (Fig. 5A). These results were confirmed by the northern blot analysis (Fig. 5B), which showed a sustained increase of PFK-M mRNA after EGF treatment, reaching a maximum at 16 h. Similarly, EGF stimulation of MCF-7 cells was accompanied by increased expression of PFK-M protein, as demonstrated by the Western blot analysis (Fig. 5C). To confirm the involvement of Pak1 in the observed enhancement of PFK-M expression in MCF-7 cells, we knocked down the expression of Pak1 with Pak1-specific RNAi (RNA interference) and examined the ability of EGF to stimulate PFK-M expression (Fig. 5D). As expected, EGF stimulation of cells transfected with the control RNAi was accompanied by a very clear enhancement of PFK-M levels (2.5 fold), whereas in cells with knocked-down Pak1 expression, there was only a marginal induction of PFK-M by EGF. The induction of PFK-M expression with EGF treatment on Pak1 knockdown was 50% - 60% less than that in the control cells, emphasizing the importance of Pak1 in bringing about an increase of PFKM expression in response to EGF treatment. Successful knockdown of Pak1 expression with RNAi was confirmed by western blotting of cell lysate from the control and Pak1 RNAi transfected cells. Levels of Pak1 protein were determined by probing with Pak1 specific antibody (Fig. 5D, lower panel).
NFAT1 chromatin is a target of nuclear Pak1 – After realizing that the association of Pak1 with PFKM chromatin led to its increased transcription, we were prompted to study one more Pak1 chromatin target as an example to investigate its effect on gene expression. One of the targets of Pak1 was the gene chromatin of NFAT1, which belongs to the family of nuclear factor of activated T cell (NFAT) (26). This family is involved in diverse cellular functions ranging from development to cell adaptation. The chromatin fragment pulled down by PAK1 encompasses a part of NFAT1 gene upstream of NFAT1 gene (region A; Figure 6A). We confirmed the binding of PAK1 to NFAT1 chromatin by a ChIP assay with PCR primers amplifying region A (Figure 6B). Association of Pak1 with the NFAT1 chromatin could be brought about by stimulation of the cells with serum. Similar to the studies done with PFK-M gene target, we also cloned region A into PGL2 basic luciferase vector and performed luciferase assay in order to see effect of Pak1 on NFAT1 promoter activity. It was interesting to note that wild type Pak1, but not Pak1 NLS mutant, repressed luciferase activity, suggesting that association of Pak1 with NFAT1 chromatin could lead to downregulation of NFAT1 expression in a manner that is sensitive to NLSs of Pak1 (Figure 6C). The ChIP and luciferase experiments clearly showed NFAT1 chromatin was a bona fide target of nuclear Pak1.

Pak1 represses NFAT1 gene expression - To study Pak1 effect on NFAT1 gene expression; we silenced Pak1 expression by Pak1 specific RNAi and examined the expression of NFAT1 by RT-PCR and western blot analysis. Both the protein and mRNA levels of NFAT1 were increased when Pak1 expression was knocked-down (Figure 7A and 7B). These results suggested that Pak1 represses NFAT1 expression and supported the NFAT1 PGL2 luciferase data (Fig 6C). Western blot of the cell lysate with Pak1 antibody confirmed the successful knocking down of Pak1 by RNAi used. The noted association of Pak1 with the NFAT1 gene and inhibitory effect of Pak1 on NFAT chromatin-driven transcription suggests existence of Pak1-targets that might be inhibited by Pak1.

DISCUSSION

Pak1 pathway is stimulated in response to variety of extracellular signals including, growth factors such as EGF and heregulin (1). Stimulation of mammalian cells with these signals results in autophosphorylation and increased Pak1 kinase activity, leading to cytoskeleton rearrangement, cross-talk with other signaling cascades and other phenotypic changes (1). Many of these Pak1-responsive changes have been primarily cytoplasmic events and the substrates and mechanisms involved have been well characterized and reported (reviewed in 1, 2). In a previous report from our laboratory we showed histone 3.3 to be a substrate of Pak1 and found nuclear localization of Pak1 in 18-24% of interphase cells (7). This prompted us to further investigate the biochemical basis of nuclear localization of Pak1 and possible functions of Pak1 in the nucleus.

Analysis of Pak1 sequence resulted in the identification of three potential nuclear localization sequences (NLS1, 2 and 3). Sequential mutations of these signals, individually and in combination and subsequent study of the nuclear localizing of Pak1 mutants resulted in the identification of NLS2 as the most potent of NLS. NLS1 and NLS3 had weak nuclear localizing potential. We also observed that nuclear entry of Pak1 in response to serum or EGF treatment, suggesting that an activating signal like EGF may be important for Pak1 to localize in the nucleus.

Study of the subcellular localization of Pak1 showed an appreciable amount of Pak1
associated with active chromatin (Fig 1A). Since earlier studies have shown Pak1 binding and phosphorylation of Histone 3.3 (7), here we utilized chromatin immunoprecipitation approach with Pak1 specific antibody and identified several Pak1-associated gene chromatin fragments (Table 1). We further validated and characterized two targets namely PFKM and NFAT1 genes to gain insight into the functions of chromatin associated Pak1 in regulating gene transcription.

PFK is the key regulatory rate-limiting enzyme in glycolysis. The PFK chromatin-fragment associated with Pak1 was about 6.8 kb upstream of the PFKM promoter 1 (Fig 3A). It was identified to be an enhancer element by the PGL2 functional assay studies, which showed a stimulatory effect of EGF upon reporter gene transcription (Fig 4A and 4B). Pak1 mutant-deficient in all three NLS failed to stimulate the PGL2 luciferase (Fig 6C), validating the identification and functionality of these signal motifs in Pak1. ChIP analysis also showed that Pak1 was associated with the promoter region of PFKM on EGF treatment, and that growth factor stimulation of cells increases PFKM expression. Consist with these results, silencing of Pak1 expression considerably decreased the EGF stimulated upregulation of PFKM expression (Fig 5D), suggesting that Pak1 may be intimately involved in the EGF induced upregulation of PFKM expression by associating with regulatory gene chromatin of PFKM. The noted Pak1-mediated EGF induced enhancement of the key rate-limiting glycolytic enzyme PFK might participate in the enhancing glycolysis. In this context, recently Pak1 has been also shown to phosphorylate and stimulate phosphoglucomutase (PGM) activity and thus, may be involved in shifting the metabolism towards more glycolysis and pentose phosphate pathway (28). In addition to this, by demonstrating that Pak1 associates with and enhances the expression of PFKM we have highlighted one of the possible modes by which increased growth factor signaling and deregulated Pak1 activity might cooperate in altering the cellular metabolism and potentially aid in the manifestation of cancerous phenotype in cells.

Another notable feature of our investigation was the identification of chromatin that might be repressed by Pak1 signaling. Our Chip analysis identified the NFAT1 gene chromatin as one of such Pak1 targets. NFAT1 belongs to the NFAT family of transcription factors that play a pivotal role in transcriptional activation of cytokine genes and other genes responsible for the immune response (5, 6). The chromatin fragment pulled down with Pak1 included a part of NFAT1 gene and some region upstream of it (Fig. 6A). ChIP analysis also showed a greater association of Pak1 to this chromatin region in stimulated cells (Fig 6B). Cloning of 1kb region A in PGL2 vector and subsequent luciferase assay found that Pak1 indeed represses the NFAT1 expression. This was further validated by silencing of the Pak1 expression in MCF-7 and MDA-MB-435 cells which resulted in a distinct enhancement in NFAT1 expression (Fig 7).

The effect of Pak1 association with the chromatin of two candidate genes here as examples were opposing, and raises the possibility of Pak1 recruitment to different transcription complexes with distinct functional outcome. These findings also mirror the complexity of the mechanisms with which Pak1 could influence cellular changes.

To summarize, we have demonstrated for the first time the nuclear localization of Pak1 in MCF-7 breast cancer cells upon growth factor stimulation. We have identified and characterized the functionality of NLS sequences responsible for its nuclear localization. EGF is a potent activator of Pak1 activity (29) and our finding that EGF stimulates nuclear translocation of Pak1
highlights potential novel functions of Pak1, which might play an important role in manifesting the effects of growth factor signaling in the cell. Another important novel finding of this study is the observation that nuclear Pak1 interacts with the PFK-M and NFAT1 chromatin and is involved in regulating their gene transcriptions. Our findings of the nuclear localization of Pak1 and identification of its chromatin targets signify potential nuclear functions of Pak1 and open a new avenue of investigation leading to identification of novel substrates, mechanisms and functions of Pak1 in the nucleus.

FOOTNOTES

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The abbreviations used are: Pak1, p21-activated kinase1; EGF, Epidermal growth factor; ChIP, Chromatin immunoprecipitation; NLS, Nuclear localization signal, NFAT- Nuclear Factor of Activated T- cell.

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FIGURE LEGENDS

Fig. 1. Nuclear Localization of Pak1 and identification of nuclear localizing sequence motifs - A. Proteins from different sub cellular locations of MCF-7 cells were isolated by sequential extraction and were analyzed by SDS-polyacrylamide gel electrophoresis followed by western blot analysis with Pak1 specific antibody. Poly (ADP-ribose) polymerase (PARP), Paxillin and Lamin B1 were used as control markers for chromatin, cytoplasm and nuclear matrix, respectively. B, Confocal microscopy pictures of MCF-7 cells show the sub cellular localization of transiently transfected myc-tagged Pak1 WT protein (+- EGF) and different Pak1 NLS mutants treated with EGF. Cells were treated with 100 ng of human EGF/ mL media for 45 min, and the staining was done as described in methods. The best representative picture of each group is shown. The three nuclear localizing signals (NLS1, NLS2 and NLS3) and their positions on Pak1 protein are shown schematically. C, Fifty transiently transfected cells were checked for localization of Pak1 (wildtype [WT] and nuclear localizing signal [NLS] mutants). The percentages of cells with nuclear localization were calculated and are shown as a histogram.

Fig. 2. Effect of Pak1 on gene transcription. In MCF-7 cells WT Pak1 and active (T423E) Pak1 were expressed as fusion proteins with Gal4-DNA binding domain by transiently transfecting Gal4-WT Pak1 and Gal4-T423E Pak1 construct plasmids. After 24h gal4 luciferase assay was done. pSG424 (Gal4 vector) was used as the control whereas Gal4-AF2 and Gal4-MTA1 were used as positive and negative control, respectively. An average fold induction measured from three separate luciferase assays was plotted as a histogram. Schematic representations of each Gal4 construct and Gal4 luciferase are shown above.

Fig. 3. Chromatin Immunoprecipitation assay shows PFKM chromatin as Pak1 target. A, A schematic representation of PFKM gene showing the position of 700bp sequence originally identified as Pak1 target and its position with reference to the two promoters P1 and P2. B, Chromatin immunoprecipitation assay (ChIP) assay was done in MCF-7 cells (with and without EGF treatment) with Pak1-specific antibody. After sonication of the cells and dilution with chromatin-dilution buffer, 1% was set aside as “input”. With the “input” DNA and the DNA eluted after the ChIP procedure, PCR analysis was done with primers to amplify the 700 basepair sequence 6.8kb upstream of PFK-M promoter. C, A similar PCR was done with primers to amplify the promoter 1 sequence of PFKM. PCR products were resolved on 2% agarose gel and visualized with ethidium bromide.
**Fig. 4.** Binding of Pak1 to PFKM chromatin can potentially upregulate transcription.  
*A,* 700 base pair fragment upstream of PFKM promoter, which was identified as Pak1 target in the ChIP screen was cloned into the PGL2 basic luciferase vector and the effect of transcription on association of Pak1 with this sequence was examined by co-transfecting the luciferase plasmid with and without WT Pak1 in MCF-7 cells maintained in DMEM with 10% FBS.  
*B,* PGL2 basic vector with the PFKM 700bp sequence was cotransfected with WT Pak1 and the effect of EGF treatment on the association of Pak1 with the cloned sequence was tested.  
*C,* The Pak1 NLS mutant incapable of nuclear localization did not increase the luciferase activity. Fold induction values plotted were an average of three independent experiments. Schematic representation of the PGL2 luciferase reporter construct is shown above.

**Fig. 5.** EGF stimulation and effect of Pak1 Knockdown on PFK-M expression.  
*A,* RT-PCR analysis for PFK M after EGF treatment. MCF-7 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) without fetal bovine serum (FBS), treated with EGF (100 ng/mL). After 1 h and 4 h RNA was extracted and RTPCR was done with primers designed for PFK-M mRNA. RTPCR was also done with primers for human GAPDH and used as a control. PCR product was resolved on 2% agarose gel.  
*B,* RNA was isolated from MCF-7 cells at different intervals with EGF treatment and separated on a 0.8% agarose RNA gel. RNA was blotched onto the nitrocellulose membrane crosslinked with ultra violet light and probed for PFK mRNA.  
*C,* Cell lysate from MCF-7 cells extracted at different intervals of time after EGF treatment were run on 8% SDS-polyacrylamide gel after which proteins were transferred to supported nitrocellulose membrane and western blot analysis was done with PFK-M specific antibody. The blot was also probed for vinculin, which was used as control. The PFK-M band intensity in RTPCR, northern and western blot analysis, in reference to the control band was calculated using the program ImageQuant (version 5.1) software and the histogram are provided for comparison.  
*D,* Pak1 expression was knocked down in MCF-7 cells by transfecting Pak1 specific RNAi. Similar set of cells, which were transfected with control RNAi, was used as control. 36 hours after transfection the cells were again transfected with Pak1 RNAi to ensure efficient silencing of Pak1 expression. Cells were later cultured in media without serum for 24 h and followed by EGF treatment (100 ng/mL) for 8 h. RNA from the cells (with and without EGF treatment) was extracted and RTPCR was done for PFK-M mRNA with PFK-specific primers and with human GAPDH specific primers, which was used as control. RTPCR products were resolved on 2% agarose gels and the PFK M band was quantitated using GAPDH bands as a control and the histogram is shown below. Western blot analysis with Pak1 antibody was done to show the effective knockdown of Pak1 and is shown below with vinculin as control.

**Fig. 6.** Pak1 binds to NFAT1 gene chromatin.  
*A,* Schematic representation of the NFAT1- gene chromatin fragment pulled down by Pak1 ChIP.  
*B,* MCF-7 cells were grown in medium without serum for 48 hrs and then treated with 10% serum for 8hrs. ChIP was done with Pak1 specific antibody and with the DNA eluted after the ChIP procedure, PCR analysis was done with primers for region A.  
*C,* MCF-7 cells were transfected with ‘region A-PGL2’ reporter along with wild type Pak1, or NLS mutant Pak1, with CMV vector as control. Luciferase assay was done and fold repression (average of two separate experiments) is shown.

**Fig. 7.** Knockdown of Pak1 expression increases NFAT1 mRNA and protein levels  
*A,* Pak1 RNAi were transfected into MCF-7 cells. Similar set of cells transfected with control RNAi was used as control. 48 hours after transfection, RNA was extracted from these cells and RT-PCR was performed for NFAT1 mRNA with NFAT1 specific primers and with human GAPDH specific primers, which was used as control. Western blot analysis with Pak1 antibody was done to show effective knock down of Pak1 with the RNAi. Vinculin protein level was examined by specific antibody to show equal loading of cell lysate.  
*B,* Pak1 was knocked down in both MCF-7m and MDA-MB-435 cells and the NFAT1 protein levels were checked by western blot analysis with NFAT1 specific antibody. Efficient knock down of Pak1 protein along with actin control, in both the cells, is shown below.
Fig. 8. An overall view of the nuclear localization and regulation of gene transcription by Pak1 – In response to signals like EGF, Pak1 is imported to the nucleus by the virtue of its three nuclear localization signals (NLS1, 2 and 3). NLS 2 (shown in black) is the most potent whereas NLS 2 and 3 (shown in grey) were weak signals. Nuclear Pak1 associates with the chromatin, which ultimately leads to activation or repression of gene transcription as seen in the case of PFKM and NFAT1 genes, respectively.
Table I

| Targets identified | Gene Name                                                                 | Localization    | Size of fragment |
|--------------------|---------------------------------------------------------------------------|-----------------|------------------|
| PC1                | Cell adhesion molecule with homology to L1CAM (close homolog of L1)       | 3p26.1          | 950 bp           |
| PC34               | protein phosphatase 4, regulatory subunit 1-like                          | 20q13.32        | 680 bp           |
| PC58               | Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2 | 20q13.2-q13.3   | 1.1 Kb           |
| PCE1               | cytochrome c oxidase subunit Va                                          | 15q25           | 650 bp           |
| PCE18              | Rap guanine nucleotide exchange factor (GEF) 4                           | 2q31-q32        | 1 Kb             |
| PC26               | Phosphofructokinase, muscle isoform                                      | 12q13.3         | 700 bp           |
A

| Total lysate | Soluble | Cytoplasm/nucleoplasm | Chromatin | High salt | Nuclear matrix |
|--------------|---------|------------------------|-----------|-----------|----------------|
| Pak1         |         |                        |           |           |                |
| PARP         |         |                        |           |           |                |
| α-Lamin B1   |         |                        |           |           |                |
| α-Paxillin   |         |                        |           |           |                |

B

| NLS 1 | NLS 2 | NLS 3 |
|-------|-------|-------|
| KKKK  | 51    | 243   |
| KKKP  | 246   | 266   |
| PKKK  | 269   |       |

C

- **Wt Pak1 (-EGF)**
- **Wt Pak1 (+EGF)**
- **Mut NLS (1+2)**
- **Mut NLS (1+3)**
- **Mut NLS (2+3)**
- **Mut NLS (1+2+3)**

**Fig. 1**

% of cells with nuclear Pak1

- Pak1 WT (-EGF)
- Pak1 WT (+EGF)
- Mut NLS (1+2)
- Mut NLS (1+3)
- Mut NLS (2+3)
- Mut NLS (1+2+3)
Fig. 2
Fig. 3
Fig. 4
**Fig. 5**

A. 
- **+ EGF**
- **- EGF**

PFK M  
GAPDH  

Relative change

B. 
- **+ EGF**
- **- EGF**

PFK M  
GAPDH  

Relative change

C. 
- **+ EGF**
- **- EGF**

PFK M  
Vinculin  

Relative change

D. 

Pak1 RNAi  
Con RNAi  
EGF  

PFK M  
GAPDH  

Relative change

Con RNAi  
Pak1 RNAi  

Pak1  
Vinculin
Fig. 6

A

Chromosome 20

NFAT1 gene

Pak1 target region

1st Exon

1st Intron

Region A (1.0kb)

B

serum

-  +

PAK1 Ab

No Ab

Input

C

Fold repression

CMV vector

Pak1 wt

Pak1 mut NLS (1+2+3)

NFAT1 reporter

+  -  -

-  +  -

-  -  +

+  +  +

TATA

NFAT1 gene (region A)

Luc

Fig. 6
Fig. 7
Fig. 8
Nuclear localization and chromatin targets of p21-activated kinase
Rajesh Singh, Chunying Song, Zhibo Yang and Rakesh Kumar

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