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PAK1 translocates into nucleus in response to prolactin but not to estrogen

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Abstract

Tyrosyl phosphorylation of the p21-activated serine-threonine kinase 1 (PAK1) has an essential role in regulating PAK1 functions in breast cancer cells. We previously demonstrated that PAK1 serves as a common node for estrogen (E2)- and prolactin (PRL)-dependent pathways. We hypothesize herein that intracellular localization of PAK1 is affected by PRL and E2 treatments differently. We demonstrate by immunocytochemical analysis that PAK1 nuclear translocation is ligand-dependent: only PRL but not E2 stimulated PAK1 nuclear translocation. Tyrosyl phosphorylation of PAK1 is essential for this nuclear translocation because phospho-tyrosyl-deficient PAK1 Y3F mutant is retained in the cytoplasm in response to PRL. We confirmed these data by Western blot analysis of subcellular fractions. In 30 min of PRL treatment, only 48% of pTyr-PAK1 is retained in the cytoplasm of PAK1 WT clone while 52% re-distributes into the nucleus and pTyr-PAK1 shuttles back to the cytoplasm by 60 min of PRL treatment. In contrast, PAK1 Y3F is retained in the cytoplasm. E2 treatment causes nuclear translocation of neither PAK1 WT nor PAK1 Y3F. Finally, we show by an *in vitro* kinase assay that PRL but not E2 stimulates PAK1 kinase activity in the nuclear fraction. Thus, PAK1 nuclear translocation is ligand-dependent: PRL activates PAK1 and induces translocation of activated pTyr-PAK1 into nucleus while E2 activates pTyr-PAK1 only in the cytoplasm.

Graphical abstract

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Keywords

PAK1; tyrosyl phosphorylation; prolactin; estrogen

Introduction

Serine-threonine kinase PAK1 plays an important role in a range of cellular processes including cell proliferation, survival, motility, epithelial-mesenchymal transition (EMT) and invasion. This functional diversity of PAK1 may rely on its different intracellular localization. PAK1 is a cytoplasmic kinase that shuttles between the plasma membrane, adhesion sites, cell-cell junction and nucleus (reviewed in [1,2,3,4]). Endogenous PAK1 is localized in the nucleus in 20% of interphase cells where it phosphorylates histone H3 [5]. Overexpressed PAK1 WT in serum-free conditions localized exclusively in cytoplasm while EGF treatment led to nuclear relocalization in almost 40% of cells [6]. Three nuclear localization signals (NLS) were identified in the PAK1 N-terminal domain and the one spanning residues 243–246d is the most critical for PAK1 nuclear import [6]. In addition to the NLS, the interaction of PAK1 with the dynein light chain LC8 protein is required for PAK1 nuclear localization [7]. Various evidences supported the notion that PAK1 is involved in promoters and/or transcription regulators as several transcription factors and transcriptional co-regulators have been identified as PAK1-interacting substrates, including the forkhead transcription factor (FKHR), estrogen receptor α (ER α), and Snail ([8,9,10,11]; reviewed in [2,12]). Furthermore, the presence of PAK1 in the nucleus is necessary for zebrafish development [7].

We have previously discovered that PAK1 is a target for prolactin-activated JAK2 and that JAK2 phosphorylates PAK1 on three tyrosines; 153, 201, and 285 [13]. Tyrosyl phosphorylation of PAK1 (pTyr-PAK1) enhances such important PAK1 functions as kinase activity and the ability to form protein/protein interactions that are important for adhesion, motility, and invasion of breast cancer cells in response to PRL ([14,15,16]; reviewed in [17]). We have also previously demonstrated that the three tyrosines on PAK1 molecules and PAK1-Nck interaction play a critical role in PAK1-dependent regulation of cyclin D1 promoter activity in response to PRL [18]. We have proposed that Nck-PAK1 complex

(formation of which does not depend on PAK1 kinase activity) can sequester PAK1 in cytoplasm to prevent PAK1 nuclear shuttling thereby inhibiting PAK1-dependent activation of cyclin D1 promoter [18]. Similarly to PRL treatment, irradiation of lung cancer cells also leads to phosphorylation of tyrosines 153, 201 and 285 by JAK2 resulting in increases in PAK1 stability, PAK1/Snail binding, EMT and radioresistance of lung cancer cells [19].

In attempt to define the role of PAK1 in the synergistic effect of PRL and estrogen (E2) on breast cancer cell proliferation, we have recently demonstrated that PAK1 phosphorylates Ser305 of estrogen receptor α (ER α) in response to PRL while protein kinase A (PKA) phosphorylates the same site in response to E2 [20]. In this study we provide evidence that PRL activates and promotes PAK1 translocation into the nucleus in ligand- and phospho-tyrosyl-dependent manner. Furthermore, PRL-activated nuclear PAK1 is active. In contrast, E2-activated PAK1 is retained in the cytoplasm.

Material and Methods

Antibodies and reagents

Primary antibodies (Ab) used in this study were monoclonal α HA from Covance, polyclonal α PAK1(Thr423)/PAK2(Thr402) from Cell Signaling, polyclonal α RAR α and polyclonal α paxillin from Santa-Cruz Biotechnology, Inc. Prolactin was purchased from Dr. Parlow (National Hormone and Peptide Program, NIDDK), 17 β -estradiol (E2) from Sigma-Aldrich, [γ -³²P]ATP from MP Biomedical and histone H4 from New England Biolabs.

Cell Cultures

MCF-7 clones stably overexpressing vector, HA-tagged PAK1 WT and PAK1 Y3F (described in [20]) were maintained in DMEM (Corning Cellgro) supplemented with 10% FBS (Sigma-Aldrich). Deprivation media consisted of DMEM supplemented with 1% bovine serum albumin (Sigma-Aldrich).

Immunocytochemistry

MCF-7 clones were plated on coverslips, serum deprived for 48 h and treated with vehicle, PRL (200 ng/ml, 20 min), E2 (1 nM, 30 min) or PRL+E2 (25 min). The coverslips were fixed for 15 min at 37 °C in CFA (4% paraformaldehyde, 5% polyethylene glycol 400 in intracellular buffer consisting of 30 mM HEPES, pH 7.4, 10 mM EGTA, 0.5 mM EDTA, 5 mM MgSO₄, 33 mM KC₂H₃O₃, and 0.02% NaN₃). Cells were permeabilized with CFB (1% Triton X-100, 4% paraformaldehyde, 5% polyethylene glycol 400 in intracellular buffer) for 15 min at 37 °C. Cells were blocked with 2% human serum and incubated with α PAK1(Thr423) Ab followed by goat- α rabbit-AlexaFluor 594 (Invitrogen) Ab to localize active PAK1. Staining by secondary antibody reagent alone was negligible (not shown). DAPI (4',6'-diamidino-2-phenylindole; Invitrogen) was used for DNA staining. Confocal imaging was performed with an inverted Leica TCS SP8 laser scanning confocal microscope using a 63X/1.4 numerical aperture (NA) objective lens. All confocal images are maximal-intensity projections. All experiments were repeated at least 3 times with n = 100 cells quantified for each condition.

Subcellular protein extraction

MCF7 clones stably expressed HA-PAK1 WT or HA-PAK1 Y3F were serum deprived for 48 h and treated with 200 ng/ml PRL, 1 nM E2 or PRL+E2 together for the indicated time. The cellular components were sequentially extracted using a widely adopted biochemical fractionation and sequential extraction procedure [6,21,22]. Cells were washed with cold phosphate-buffered saline (PBS) and scraped in 1 ml of PBS supplemented with 1mM Na_3VO_4 , 1 mM PMSF, 10 $\mu\text{g/ml}$ aprotinin, and 10 $\mu\text{g/ml}$ leupeptin. The cells were centrifuged at 2500 rpm at 4°C for 3 min, resuspended in 1 ml of hypotonic buffer (20 mM HEPES (pH7.9), 1 mM EDTA, 1 mM EGTA, and 0.2% Triton X-100) supplemented with 1 mM Na_3VO_4 , 1 mM PMSF, 10 $\mu\text{g/ml}$ aprotinin, and 10 $\mu\text{g/ml}$ leupeptin and incubated for 10 minutes at 4°C. The suspension was centrifuged at 13,000 rpm at 4 °C for 30 sec. The supernatant, corresponding to the cytosolic fraction, was collected. The nuclear pellet was washed 3 times in hypotonic buffer, then resuspended in lysis buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 6 mM EGTA, 150 mM NaCl, and 0.1% Nonidet P-400) supplemented with 1 mM Na_3VO_4 , 1 mM PMSF, 10 $\mu\text{g/ml}$ aprotinin, and 10 $\mu\text{g/ml}$ leupeptin and incubated for 10 minutes at 4°C. The suspension was centrifuged at 13,000 rpm at 4°C for 10 min. The supernatant which contains the soluble nuclear fraction (nucleoplasm) was collected. The insoluble pellet, corresponding to the insoluble nuclear fraction (nuclear matrix and chromatin), was dissolved in Laemmli sample buffer. Equal amount of proteins were resolved by SDS-PAGE followed by immunoblotting with the indicated antibodies. Each experiment was performed at least three times with similar results.

PAK1 *in vitro* kinase assay in cellular fractions

Cells were treated with vehicle, PRL (200 ng/ml, 20min), E2 (1 nM, 30min) or PRL+E2 (25 min) and cell fractionation was performed as described above. HA-PAK1 was immunoprecipitated from the cytosolic fraction and the nuclear fraction (a combination of soluble and insoluble nuclear fractions) with αHA Ab, and subjected to an *in vitro* kinase assay in the presence of 10 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and histone H4 (substrate of PAK1). Relative levels of incorporation of ^{32}P into histone H4, an indicator of phosphorylation, were assessed by autoradiography and estimated by a phosphoimager. The same membrane was blotted with αHA to assess the amount of PAK1 for each condition. Membrane patterns were scanned and the amount of PAK1 was quantified using Multi-Analyst (Bio-Rad) software. Relative PAK1 kinase activity was then normalized by the amount of immunoprecipitated PAK1 for each lane. Each experiment was performed at least three times with similar results.

Statistical Analysis

Data from at least three separate experiments per each condition were pooled and analyzed using one-way ANOVA plus Tukey's HSD test. Differences were considered to be statistically significant at $P < 0.05$. Results are expressed as the mean $\pm$ SE. When individual experiments were analyzed, the results were indistinguishable from those obtained from the pooled data.

Results

Prolactin but not estrogen causes translocation of PAK1 into nucleus

We have previously demonstrated that PRL causes nuclear translocation of endogenous PAK1 [18]. Here we decided to determine PAK1 localization in response to different ligands and a role of PAK1 tyrosyl phosphorylation in this localization. In serum-deprived and vehicle-treated cells, both PAK1 WT and PAK1 Y3F (phospho-tyrosyl-deficient PAK1 Y3F mutant in which 3 JAK2 phosphorylation sites are mutated) were un-activated as assessed by α -phospho-Thr423-PAK1 immunostaining (pThr423-PAK1 is marker of PAK1 activation) and demonstrated background level of nuclear distribution of PAK1 WT and Y3F (12% and 13%, respectively; Fig. 1). After 30 min of PRL treatment, activated PAK1 WT was accumulated in nuclei in the majority of the cells (80%, Fig. 1). In contrast, PAK1 Y3F was activated to a much lesser extent as compared to PAK1 WT and retained in the cytoplasm (13% cells with nuclear PAK1 Y3F, Fig. 1). Although E2 activates PAK1 WT [8,20], this treatment failed to translocate PAK1 WT into nucleus (15% cells demonstrated nuclear PAK1 WT, Fig. 1). As expected, estrogen did not activate PAK1 Y3F as we have shown previously [20] and did not change PAK1 Y3F cellular distribution (18%, Fig. 1). Cytoplasmic and nuclear immunostaining of activated PAK1 was present in PAK1 WT cells when we combined PRL+E2 (69% cells had nuclear PAK1 WT) while only 11% of PAK1 Y3F cells demonstrated nuclear signal. These data suggest that PRL activates PAK1 and induces pTyr-PAK1 translocation into the nucleus while E2 activates PAK1 only in the cytoplasm.

Next we confirmed our immunocytochemical data by assessment of PAK1 localization in cytosolic and different nuclear fractions (“nuclear soluble fraction” and “nuclear insoluble fraction”) performed by sequential extraction procedure. In untreated cells, 75–95% of both PAK1 WT and PAK1 Y3F were present in the cytoplasm (Fig. 2 A–C, 0 min for all treatments, blue bars). In 30 min of PRL treatment, only 48% of pTyr-WT was retained in the cytoplasm of PAK1 WT clone while 52% re-distributed into the nucleus and pTyr-PAK1 shuttled back to the cytoplasm by 60 min of PRL treatment (Fig. 2A, left panel, blue bars in plot). In contrast, PAK1 Y3F retained in the cytoplasm during the entire time-course of PRL treatment (Fig. 2A, right panel). E2 treatment caused nuclear translocation of neither PAK1 WT nor PAK1 Y3F (Fig. 2B). Combination of E2 with PRL induced re-localization of PAK1 WT in the nucleus in 30 min of treatment (66% of PAK1 retained in the cytoplasm, Fig. 2C, left panel, blue bars) and shuttling back to the cytoplasm at 60 min (84% of PAK1 was cytoplasmic) while PAK1 Y3F resided in the cytoplasm during whole course of treatment (Fig. 2C, right panel, blue bars).

Taken together, these results suggest that PAK1 translocates into nucleus in response to PRL but not E2 and the observed nuclear accumulation of PAK1 WT in response to PRL seems to be due to tyrosyl phosphorylation of PAK1 because PAK1 Y3F was unable to translocate into nucleus.

Prolactin but not estrogen stimulates PAK1 kinase activity in nucleus

PAK1 is known to be activated by both PRL [14,15] and E2 ([8,20]. However, nuclear PAK1 kinase activity has not been demonstrated previously. We set out to determine whether PRL and/or E2 altered PAK1 kinase activity in cytosolic and nuclear fractions. MCF-7 cell clones were treated with vehicle, PRL, E2 or PRL+E2 and PAK1 was immunoprecipitated separately from cytosolic and nuclear fractions. PAK1 kinase activity was measured in an *in vitro* kinase assay with H4 histone as a substrate. As expected, PRL activates PAK1 in the cytoplasm of both PAK1 WT and PAK1 Y3F clones (PAK1 Y3F mutant is kinase-active) although, in the presence of PRL, the kinase activity of cytoplasmic PAK1 WT was significantly stronger than PAK1 Y3F (2.3-fold and 1.5 –fold increase, respectively) (Fig. 3A). These data confirm our previously published findings that tyrosyl phosphorylation of PAK1 enhances PAK1 kinase activity [14,15]. Importantly, PRL treatment increased the kinase activity of nuclear PAK1 WT in more than 4-fold as compared with vehicle-treated cells (“N” bars for PAK1 WT in Fig. 3A) while the nuclear activity of PAK1 Y3F was on the background level (“N” bars for PAK1 Y3F in Fig. 3A). We have shown previously that E2 activates PAK1 WT but not PAK1 Y3F [20]. Here we extend these data to demonstrate that E2 caused more than 2-fold activation of cytoplasmic PAK1 WT but not cytoplasmic PAK1 Y3F. In contrast to PRL treatment, E2 was unable to activate nuclear PAK1 in both WT and Y3F clones (Fig. 3B). Combination of PRL+E2 demonstrated the same effect as treatment with PRL only: increased PAK1 activity in the cytoplasm of both PAK1 WT and PAK1 Y3F clones with maximal activity in WT clone and activation of nuclear PAK1 WT but not nuclear PAK1 Y3F (Fig. 3C). These data indicated that PRL but not E2 activates PAK1 in nucleus and suggested that Tyr(s) 153, 201, and 285 were responsible for this activation.

Overall, our data suggest that PRL activates PAK1 and induces translocation of activated pTyr-PAK1 into nucleus while E2 activates pTyr-PAK1 only in the cytoplasm.

Discussion

We have recently introduced PAK1 as a common node for estrogen- and prolactin-dependent pathways in the synergetic effect of both hormones in breast cancer cells proliferation and tumor growth [20]. We have shown that, in response to PRL, pTyr-PAK1 phosphorylates Ser305 on estrogen receptor α (ER α) thereby activating it in an E2-independent manner. In response to E2, the same site of ER α is phosphorylated by PKA. We have also demonstrated that in cells exposed to both PRL and estrogen, Ser305-ER α is phosphorylated by both PKA and pTyr-PAK1 resulting in maximal signal [20]. However, the mechanism to choose which kinase - PAK1 or PKA - phosphorylates Ser305-ER α was undetermined.

In the current paper we extend our previous data to show that, in response to PRL, pTyr-PAK1 is activated and translocates into nucleus where it retains its activity. However, in response to E2, activated pTyr-PAK1 resides in the cytoplasm and fails to translocate into the nucleus. These data allow us to suggest that this different cellular localization of PAK1 may provide a mechanism of ligand-dependent phosphorylation of Ser305-ER α . When cells are exposed to estrogen, nuclear ER α is phosphorylated by PKA but not PAK1 because E2-activated PAK1 is cytoplasmic while nuclear translocation of the PKA catalytic subunit in response to activation was documented in early 90-s [23]. However, when cells are exposed

to PRL, ER α is phosphorylated by nuclear PRL-activated PAK1. When the two hormones are present together, Ser305-ER α might be phosphorylated by both kinases that leads to synergetic phosphorylation of Ser305-ER α , subsequent phosphorylation of Ser118-ER α and increased cell proliferation as we demonstrated previously [20]. We do not have experimental to support this hypothesis, and it will be addressed in detail in our planned future studies.

PAK1 intracellular localization plays an important role in human breast cancer since nuclear localization of PAK1 is associated with tamoxifen resistance in a subset of ER-positive tumors: patients who had nuclear PAK1 expression showed no response to tamoxifen [24]. Tamoxifen is an estrogen competitor, commonly used as adjuvant therapy in ER-positive breast cancer. The binding of tamoxifen to ER prevents the recruitment of coactivators and impedes ER-mediated transcription. Nonetheless, many tumors relapse due to either de novo or acquired tamoxifen resistance. Tamoxifen response depends on ER α modifications, including phosphorylation of the ER α and its coactivators. PAK1 phosphorylates ER α at Ser305 and promotes its transactivation, thereby leading to up-regulation of cyclin D1, promotion of hormone independence and tamoxifen resistance [9,10,25,26]. Clinically, localization of both PAK1 and pSer305-ER α in the nucleus indicated reduced response to tamoxifen in breast cancer patients [24,27,28,29]. Our current data, combined with recently published, shed some light on a possible mechanism underlying the tamoxifen resistance: in response to E2, PAK1 retains in the cytoplasm and PKA phosphorylates Ser305-ER α . However, breast cells constantly expose to both hormone and breast cancer cells have increased amount of PRL receptors [30,31]. Thus, PRL-activated PAK1 may translocate into nucleus where it is kinase-active as we have shown herein. Nuclear PAK1 can phosphorylate Ser305-ER α causing enhanced phosphorylation of S118-ER α and development of tamoxifen resistance.

We have shown here that PAK1 nuclear translocation is ligand-dependent: only PRL and PRL+E2 but not E2 alone stimulated PAK1 nuclear translocation. These data underlie a high degree of specificity of PAK1 intracellular localization that is important because it does not rely exclusively on the PAK1 activation status only. We have also implicated three tyrosines – Tyr153, 201 and 285 - of PAK1 in its nuclear translocation because PAK1 Y3F mutant failed to translocate into the nucleus. We have previously proposed a Nck-dependent mechanism of this translocation [18]. PAK1 has been recently shown to get phosphorylated by JAK2 in response to irradiation of non-small cell lung cancer cells [19]. This irradiation-induced tyrosyl phosphorylation is required to activate PAK1 and maintains its stability and translocation into the nucleus [19].

The exact molecular mechanism underlying pTyr-PAK1 nuclear translocation has been still largely elusive. However, taken together, our findings and data from the literature indicate that tyrosine phosphorylation events are critical for PAK1 functions. In this study, we demonstrated that, in response to PRL but not to E2, pTyr-PAK1 translocates into the nucleus where it keeps its an activated state. These findings offer an opportunity for the discovery of new nuclear PAK1 functions, including possible roles in tamoxifen resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

E2	17 β -estradiol
EGF	epidermal growth factor
JAK2	Janus kinase 2
NLS	nuclear localization signal
PAK	p21-activated serine-threonine kinase
PRL	prolactin
pTyr	phosphorylated on tyrosines
Tyr	tyrosine

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Highlights

- Prolactin but not estrogen causes translocation of PAK1 into nucleus
- Tyrosyl phosphorylation of PAK1 is required for nuclear localization
- Prolactin but not estrogen stimulates PAK1 kinase activity in nucleus

Figure 1. Nuclear localization of PAK1 in response to different ligands

MCF-7 cell lines stably overexpressing either HA-PAK1 WT or HA-PAK1 Y3F were treated with vehicle, PRL, E2 or PRL+E2. Activated PAK1 (red) was subjected to confocal immunofluorescence with α pPAK1(Thr423) antibody. Nuclei were stained with DAPI (blue). The percentages of cells with nuclear localization were calculated and are shown in the plot (At least 100 cells were calculated for each condition). Each experiment was repeated 3 times. Scale bar, 25 μ m.

Figure 2. PRL but not E2 causes translocation of pTyr-PAK1 into nucleus

MCF-7 cell lines stably overexpressing either HA-PAK1 WT or HA-PAK1 Y3F were treated with PRL (A), E2 (B) or PRL+E2(C) during the time course. Cytosolic (C), nuclear soluble (NS) and nuclear insoluble (NI) fractions were separated by SDS-PAGE, transferred to membrane, and immunoblotted with α HA for HA-PAK1, α paxillin as a cytosolic marker, and α RAR α as a nuclear marker. The graphs represent densitometric analysis of the bands obtained for PAK1 in nuclear insoluble (green), nuclear soluble (brown) and cytosolic (blue) fractions and normalized by the amount of immunoprecipitated PAK1 for each lane. Statistical analysis was performed for the cytoplasmic PAK1 (in cytosolic fraction, blue bars). Bars represent mean $\pm$ SE; *, $P < 0.05$, $n = 4$.

Figure 3. PRL but not E2 activates nuclear pTyr-PAK1

PAK1 WT or PAK1 Y3F clones of MCF-7 cells were treated with PRL (A), E2 (B) or PRL +E2 (C). PAK1 was immunoprecipitated from the cytosolic (C) and the nuclear fraction (N), subjected to an *in vitro* kinase assay with H4 histone as a substrate and probed with αHA for HA-PAK1. Relative PAK1 kinase activity was normalized by immunoprecipitated PAK1 for each lane and plotted. *, $p < 0.05$, $n = 3$.