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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% redistributes 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.

1. 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 it different intracellular localization. PAK1 is a cytoplasmic kinase that shuttles between the plasma membrane, adhesion sites, cell–cell junction and nucleus (reviewed in Refs. [1–4]). 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 re-localization in almost 40% of cells [6]. Three nuclear localization signals (NLS) were identified in the PAK1 N-terminal domain and the one spanning residues 243–246 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 (ERα), and Snail ([8–11]; reviewed in Refs. [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 ([14–16]; reviewed in Ref. [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

Abbreviations: E2, 17β-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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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 (ERα) 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.

2. Material and methods

2.1. Antibodies and reagents

Primary antibodies (Ab) used in this study were monoclonal αHA from Covance, polyclonal αpPAK1(Thr423)/PAK2(Thr402) from Cell Signaling, polyclonal αRARα and polyclonal αpaxillin from Santa-Cruz Biotechnology, Inc. Prolactin was purchased from Dr. Parlow (National Hormone and Peptide Program, NIDDK), 17β-estradiol (E2) from Sigma–Aldrich, [γ-^{32}P]ATP from MP Biomedical and histone H4 from New England Biolabs.

2.2. Cell cultures

MCF-7 clones stably overexpressing vector, HA-tagged PAK1 WT and PAK1 Y3F (described in Ref. [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).

2.3. 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 $MgSO_4$, 33 mM $KC_2H_3O_3$, and 0.02% NaN_3). 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 αpPAK1(Thr423) Ab followed by goat-αrabbit-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 $n \geq 100$ cells quantified for each condition.

2.4. 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 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/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 μg/ml aprotinin, and 10 μg/ml leupeptin and incubated for 10 min at 4 °C. The suspension was centrifuged at 13,000 rpm at 4 °C for 30 s. 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 μg/ml aprotinin, and 10 μg/ml leupeptin and incubated for 10 min 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.

2.5. PAK1 in vitro kinase assay in cellular fractions

Cells were treated with vehicle, PRL (200 ng/ml, 20min), E2 (1 nM, 30 min) 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 [γ-^{32}P] 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.

2.6. 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 ± SE. When individual experiments were analyzed, the results were indistinguishable from those obtained from the pooled data.

3. 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 αphospho-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%,