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Yong-Bae Kim
George Washington University

Yong Jae Shin

Adhiraj Roy
George Washington University

Jeong-Ho Kim
George Washington University

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The Role of the Pleckstrin Homology Domain-Containing Protein CKIP-1 in Activation of p21-activated Kinase 1 (PAK1)

Yong-Bae Kim[1], Yong Jae Shin[2], Adhiraj Roy[1] and Jeong-Ho Kim[1*]

[1]From the Department of Biochemistry and Molecular Medicine, The George Washington University Medical Center, 2300 Eye Street, Washington, DC 20037, USA and [2]From the Department of Stem Cell Biology and Regenerative Medicine, Lerner Research Institute, Cleveland Clinic, Cleveland, OH 44195, USA

Running Title: PI3K Regulation of PAK1

*To whom correspondence should be addressed: Jeong-Ho Kim, Department of Biochemistry and Molecular Medicine, The George Washington University Medical center, 2300 Eye Street, Washington, DC 20037, USA, Tel.: (202) 994-9937; Fax: (202) 994-8974; E-mail: jh_kim@gwu.edu

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Background: PAK1 is phosphorylated and activated by the protein kinase CK2 in response to EGF.
Results: Upon EGF stimulation, CKIP-1 recruits CK2 to PAK1 in membrane ruffles in a PI3K-dependent manner.
Conclusion: PI3K activates PAK1 at the plasma membrane by promoting CK2 phosphorylation of PAK1 via CKIP-1.
Significance: This is the first evidence of spatial regulation of PAK1 activity.

ABSTRACT

Upon growth factor stimulation, PAK1 is recruited to the plasma membrane and activated by a mechanism that requires its phosphorylation at S223 by the protein kinase CK2. However, the upstream signaling molecules that regulate this phosphorylation event are not clearly defined. Here, we demonstrate a major role of the CK2α-interacting protein CKIP-1 in activation of PAK1. CK2α, CKIP-1 and PAK1 are translocated to membrane ruffles in response to the epidermal growth factor (EGF), where CKIP-1 mediates the interaction between CK2α and PAK1 in a PI3K-dependent manner. Consistently, we observe that PAK1 mediates phosphorylation and modulation of the activity of p41-Arc, one of its plasma membrane substrate, in a fashion that requires PI3K and CKIP-1. Moreover, CKIP-1 knockdown or PI3K inhibition suppresses PAK1-mediated cell migration and invasion, demonstrating the physiological significance of the PI3K-CKIP-1-CK2α-PAK1 signaling pathway. Taken together, these findings identify a novel mechanism for the activation of PAK1 at the plasma membrane, which is critical for cell migration and invasion.

PAK1, a major downstream effector of the Rho-family GTPases Cdc42 and Rac1, plays a critical role in the regulation of cell morphology and motility (1). In its inactive state, PAK1 forms a *trans*-inhibited dimer, where the N-terminal autoinhibitory domain (AID) on one PAK1 molecule binds to and blocks the C-terminal catalytic domain of its counterpart (2,3). This autoinhibition is relieved through binding of GTPases to the PBD (p21-binding domain)/CRIB (Cdc42/Rac-interacting) domain that partially overlaps with AID, leading to autophosphorylation

at specific sites, including Thr-423 within the activation loop, and consequent activation of PAK1 (4). PAK1 is recruited to the plasma membrane via the SH3-containing proteins Nck and Grb2 upon stimulation by growth factors (5-9), leading to efficient activation of PAK1.

CK2 (Casein Kinase 2) is a serine/threonine kinase that regulates a broad range of biological processes, including cell growth and survival, gene expression, and actin cytoskeleton organization (10,11). CK2 is a tetramer composed of two α catalytic subunits and two β regulatory subunits, but the precise mechanism of its regulation is not fully understood. Emerging evidence shows that, in cancer cells, CK2 is frequently elevated and translocated into the nucleus to regulate gene expression (12,13) and also targeted to the plasma membrane to regulate actin cytoskeleton organization by acting on proteins such as WASP (14), CRN2 (15), myosin-9 (16) and WAVE2 (17). Therefore, the regulated translocation of CK2 to different cellular compartments may be critical for its functional regulation (11).

The Pleckstrin homology (PH) domain-containing protein CKIP-1, identified as a CK2α interacting protein (18), recruits CK2α to the plasma membrane (19). Accumulating evidence indicates that CKIP-1 is involved in various cellular processes including cell proliferation, differentiation, morphology and migration in various human cell lines including human osteosarcoma U2-OS and macrophage (20-22). CKIP-1 binds to multiple proteins (e.g., CK2α, CPα, Akt, C-Jun ATM, IFP/Nmi and Smurf1) via its PH domain, implicating its role as a scaffold protein in various signaling pathways (23). Of note, CKIP-1 appears to associate with the plasma membrane through binding to phosphoinositides and to regulate muscle differentiation in mouse myoblast C2C12 cells (24) as well as myoblast fusion in mouse and zebrafish, in PI3K-dependent fashion (25). These observations have raised the possibility that CKIP-1 may serve as a key controller of cellular processes regulated by PI3K.

We have recently demonstrated that CK2 catalyzes phosphorylation of PAK1 at S223, which is critical for the activation of the kinase (26). Here, we show evidence that CKIP-1 appears to recruit CK2α to PAK1 in response to EGF in a PI3K-dependent manner. Consequently, inhibition of either CKIP-1 or PI3K activity blocks PAK1-mediated actin cytoskeleton dynamics and cell migration. These results demonstrate the role of CKIP-1 in EGF-induced activation of PAK1, providing a novel regulatory mechanism for PAK1 signaling.

EXPERIMENTAL PROCEDURES

Cell cultures—The human prostate cancer PC3 cells were maintained in RPMI 1640 containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a humidified incubator (37°C and 5% CO_2). The benign prostate epithelial RWPE-1 cells were cultured in keratinocyte serum-free medium (K-SFM) containing 5 ng/ml of epidermal growth factor (EGF) and 50 μg/ml of bovine pituitary extract (BPE). CKIP-1 knockdown PC3 and RWPE-1 cells were grown in suitable medium containing 2 μg/ml of puromycin.

Lentiviral infection—Standard lentiviral transduction was performed as described previously (27). In brief, lentiviral transduction was used for CKIP-1 knockdown and GFP-$PAK1^{WT}$ overexpression in prostate cancer cells (PC3 and RWPE-1). Lentiviral short hairpin RNAs (shRNAs) specific for CKIP-1 were purchased from Sigma-Aldrich (sh1, TRCN00001-65913; sh2, TRCN0000165914). For viral production, 293T cells were cotransfected with pLKO.1-shNT or pLKO.1-CKIP-1 specific shRNAs, and packaging and envelope vectors psPAX2 and pCMV-VSV-G using the Lipofectamine™ LTX transfection reagent (Invitrogen). Transfection was performed as described previously (27).

Western blot analysis—Whole cell extracts were prepared in RIPA buffer (50 mM Tris pH 7.4, 15 mM Nacl, 1% NP-40, 0.5% Sodium

deoxycholate, 0.1% SDS) with protease inhibitors (28). For immunoprecipitation, cells were lysed lysed in IP lysis buffer (25 mM HEPES pH7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100) and mixed with protein G-Sepharose beads (GE healthcare) prebound to mouse normal IgG and anti-GFP (Santa Cruz) for 4 h with rotation at 4°C. Pull-down beads were washed four times with IP wash buffer (25 mM HEPES pH7.4, 1 M NaCl, 1 mM EDTA, 0.5% Triton X-100). Plasma membrane fractions were prepared using Mem-PER™ Eukaryotic Membrane Protein Extraction Reagent Kit (Thermo Scientific) following the manufacture`s protocols. Protein concentration was quantified using Bradford to calculate the ratio of each sample volume. The protein samples were resolved on a 10% SDS–PAGE gel and transferred to a nitrocellulose membrane. Antibodies used in this study include anti-phospho-PAK1^{S223} (26), anti-phospho-PAK1^{S144}, anti-PAK1, anti-Na/K-ATPase (Cell Signaling), anti-CK2α, anti-GFP (Santa Cruz), anti-α-Tubulin (Neo Markers), anti-phospho-p41-Arc^{T21} (ECM Biosciences) and anti-CKIP-1 (Sigma-Aldrich) antibodies.

Immunofluorescence staining—Cells were grown on glass coverslips and transfected with either control (NT) or CKIP-1 specific shRNA constructs. After 24 h, cells were washed twice in cold PBS before fixation with 4% PFA for 10 min at room temperature (RT). After fixation, the cells were washed three times with PBS for 5 min each and incubated with the blocking buffer containing 5% BSA and 5% normal goat serum. Coverslips were incubated with primary antibodies overnight at 4°C or with Rhodamine-labeled Phalloidin in PBS for 30 min at room temperature. After washing three times with PBS, cells were treated with secondary antibodies for 1 h. DNA was stained with 0.1 ug/ml DAPI (4', 6-diamidino-2-phenylindole) in PBS for 1 min. Cells were mounted in a mounting medium (KPL) and imaged on an Olympus IX71 inverted microscope (Olympus Imaging America Inc).

Cell migration and invasion assays—For the wound-healing migration assay, PC3 cells were seeded at 70% confluence into 6-well culture dishes and, 24 h later, infected with CKIP-1-specific or control shRNA lentivirus. When the cells grew to confluence, scratch wounds were made on the monolayers using sterile 200 µl pipette tips. After 24 h incubation, the wounded monolayers were washed gently twice with 1X PBS to remove the detached cells and replenished with fresh media. The cells were fixed with 4% paraformaldehye for 30 minutes and then photographed. Wound areas were measured using ImageJ software. Cell invasion assay was performed using the cell invasion kit (Transwell Boyden's chamber with Transwell™ Permeable Support Inserts Coated with Cultrex® BME (basement membrane extract, Corning Costar) according to the manufacture's instruction. Briefly, serum-starved PC3 and RWPE-1 (2×10^5 cells/well) cells were added to the upper chamber, whereas suitable media containing 10% FBS was added to the lower chamber and cultured in an incubator for 24 h. Nonmigrating cells were gently removed from the upper surface of the filter with a cotton swab; migrating cells which were attached to the lower surface of the filter were fixed and stained with hematoxylin for counting.

Soft agar colony forming assay—Anchorage-independent growth was determined by examining cell growth in soft agar. RWPE-1 cells (3×10^4) were seeded in 0.3% agar on top of a base layer of 0.6% agar containing K-SFM (keratinocyte serum-free medium) and 1 µg/ml of puromycin. The cells were placed in a 37°C, 5% CO_2 incubator. After 2 weeks, colonies were stained with crystal violet (0.05%) and counted. Colonies were counted under a microscope (Nikon Eclipse Ti; Melville, NY). All assays were performed in triplicate.

RESULTS

PAK1, CK2α and CKIP-1 are recruited to membrane ruffles in response to EGF—PAK1

regulates cell polarity and migration in response to growth factors by modulating the actin cytoskeleton (29-31). Immunofluorescence microscopy indicates that EGF induces the formation of membrane ruffles at the leading edge of polarized PC3 prostate cancer cells (Fig. 1*A*, F-actin) by promoting the recruitment of PAK1, CK2α, and CKIP-1 to membrane ruffles (Fig. 1*A*). Increased PAK1 activity is associated with auto-phosphorylation of specific sites including S144 (32). Activated PAK1 phosphorylates T21 of p41-Arc, a subunit of the human Arp2/3 complex, at the plasma membrane (33). Thus, PAK1 activity can be assessed by the phosphorylation state of S144 of PAK1 ($PAK1^{S144}$) and T21 of p41-Arc ($p41\text{-}Arc^{T21}$). EGF treatment does not affect the expression of CK2α, CKIP-1, or PAK1, but does increase phosphorylation of $PAK1^{S144}$ and $p41\text{-}Arc^{T21}$ (Fig. 1*B*). Given that CKIP-1 interacts with CK2α (19), we addressed the role of CKIP-1 in CK2 activation of PAK1. CKIP-1 does not regulate the expression of CK2α or PAK1 (Fig. 1*C*); instead, it mediates the EGF-induced recruitment of CK2α to membrane ruffles (Fig. 1*D*). It is of note, however, that PAK1 localization to the plasma membrane is not regulated by CKIP-1 (Fig. 1*D*). Thus, CKIP-1 seems to play a key role in EGF-induced PAK1 activation by recruiting CK2α to the plasma membrane.

CKIP-1 mediates EGF-induced PAK1 interaction with CK2α—To gain more insights into the role of CKIP-1 in EGF-induced PAK1 activation, we assessed the effect of CKIP-1 knockdown on the interaction between CK2α and PAK1. Upon EGF treatment in PC3 cells, PAK1 and CK2α are colocalized and interact with each other at the plasma membrane (Figs. 2*A* and 2*C*). However, this interaction is greatly reduced by CKIP-1 knockdown (ShCKIP-1 in Figs. 2*A* and 2*C*). This is perhaps because CKIP-1 silencing results in decreased localization of CK2α to the plasma membrane (Fig. 2*B*), which renders PAK1 inactive ($PAK1^{S144}$) and unable to phosphorylate its substrate p41-Arc at T21 ($p41\text{-}Arc^{T21}$) (Fig. 2*D*). Consequently, CKIP-1 silencing decreases the colocalization of CK2α with an active form of PAK1 (phospho-$PAK1^{S144}$) (Fig. 2*E*) and that of F-actin with either phospho-$PAK1^{S144}$ (Fig. 2*F*) or phospho-$p41\text{-}Arc^{T21}$ (Fig. 2*G*) to the cell periphery. Collectively, these results indicate that CKIP-1 functions to promote CK2-dependent phosphorylation and activation of PAK1 by recruiting CK2α to PAK1 to the plasma membrane.

Expression of a constitutively active PAK1 ($PAK1^{S233E}$) bypasses the requirement for CKIP-1 in PAK1-induced cell polarity and migration—Our recent work shows that CK2 phosphorylates S223 of PAK1, and that a phosphomimetic mutation at S223 (S to E) generates a constitutively active PAK1 (26). We find that, upon EGF treatment, PAK1 is recruited to the plasma membrane (Fig. 1A) and phosphorylated at S223 (Figs. 3*A* and 3*B*). To understand further CKIP-1 regulation of PAK1, we infected CKIP-1 knockdown PC3 cells with a lentivirus expressing GFP-PAK1 and examined the activity and localization of PAK1 using anti-phospho-specific PAK1 (S223) antibody. Western blot analysis shows that CKIP-1 silencing decreases S223 phosphorylation of PAK1 (Fig. 3*C*). In addition, fluorescence microscopy analysis shows that CKIP-1 knockdown results in decreased phosphorylation at S223 (Fig. 3*D*, middle) and, consequently, in loss of cell polarity (Fig. 3*D*, CKIP-1), and that the polarization defect of CKIP-1 knockdown cells (Fig. 2, shCKIP-1) is partially rescued by overexpression of GFP-PAK1 (Fig. 3*E*, shCKIP-1).

To examine the physiological significance of CKIP-1 regulation of PAK1 activity, we investigated whether PAK1-induced cell migration and invasion are affected by CKIP-1 knockdown. Wound healing assays show that CKIP-1 silencing inhibits migration of PC3 cells expressing wild

type GFP-PAK1 by 70%, but this inhibitory effect is not observed in PC3 cells expressing GFP-PAK1^{S223E} (Fig. 3*F*). Similarly, cell invasion assays using Transwell Boyden's chamber system indicate that invasion of PC3 cells expressing GFP-PAK1 is inhibited 70 – 80% by CKIP-1 knockdown, and that, however, CKIP-1 inhibition does not significantly reduce the invasiveness of PC3 cells expressing GFP-PAK1^{S223E} (Fig. 3*G*). These results indicate that CKIP-1 knockdown suppresses PAK1-mediated cell migration and invasion.

The CKIP-1-mediated interaction between CK2α and PAK1 is regulated by PI3K—CKIP-1, as a Pleckstrin Homology domain-containing protein, is recruited by phosphatidylinositol 3-phosphate (PI3P) to the plasma membrane in a PI3K (phosphatidylinositol 3-kinase)-dependent manner (24). This notion is further supported by the finding that the inhibition of PI3K signaling reduces plasma membrane localization of CKIP-1 (34). An early study showed that PAK1 regulates the invasiveness of breast cancer cells via PI3K signaling (35). These observations suggest that PI3K regulates PAK1 activity through CKIP-1. To address this idea, we treated PC3 cells with the PI3K inhibitors LY294002 and wortmannin and examined the subcellular localization of CKIP-1, CK2α and PAK1. Both LY294002 and wortmannin inhibit the EGF-induced membrane localization of CKIP-1 (Fig. 4*A*), CK2α and PAK1 (Figs. 4*D* and 4*E*), and this may be associated with a decrease in the formation of membrane ruffles (Figs. 4*A*, 4*D* and 4*E*). In agreement, Western blot analysis demonstrates low levels of CKIP-1 and CK2α at the plasma membrane (Fig. 4*B*) and decreased PAK1 activity, as measured by the phosphorylation of PAK1^{S144} and p41-Arc^{T21} (Fig. 4*C*), in PC3 cells treated with LY294002 or wortmannin.

Consistently, the PI3K inhibitors LY294002 (Fig. 5) and wortmannin (data not shown) disrupt the interaction of PAK1 with CK2α and CKIP-1 (Fig. 5*A*) and inhibit the colocalization of CKIP-1 with either CK2α (Fig. 5*B*) or PAK1 (Fig. 5*C*) and that of PAK1 with CK2α (Fig. 5*D*). Thus, PI3K may regulate the CKIP-1-mediated interaction between CK2α and PAK1 by controlling the plasma membrane localization of CKIP-1.

Expression of a constitutively active PAK1 (PAK1^{S233E}) bypasses the requirement for PI3K in the EGF-induced activation of PAK1—Given that PI3K acts as an activator of PAK1 (Fig. 4 and 5), we examined the effects of PI3K inhibition on the CK2-mediated phosphorylation and activation of PAK1. Akt is phosphorylated at S473 by PI3K, thus this phosphorylation is used as a biochemical marker for PI3K activation (36). Our results show that LY294002 inhibits the phosphorylation of Akt^{S473}, PAK1^{S223} and p41-Arc^{T21} (Fig. 6*A*) and the EGF-induced colocalization of phospho-PAK1^{S223} and phospho-p41-Arc^{T21} to the membrane ruffles (Fig. 6*B*). However, expression of the constitutively active PAK1 (PAK1^{S223E}) induces the phosphorylation of p41-Arc^{T21} (Fig. 6*C*) and PAK^{S144} (Fig. 6*D*) even in LY294002-treated PC3 cells. Furthermore, the migration defect of these cells is rescued by the expression of GFP-PAK1^{S223E} (Fig. 6*E*). Thus, these observations indicate that PI3K is required for PAK1-mediated cell migration.

CKIP-1 is required for PAK1-mediated malignant transformation of the benign prostate RWPE-1 cells— Overexpression of PAK1 induces malignant transformation of the benign prostate RWPE-1 cells *in vitro* and *in vivo* (26). Given that CKIP-1 regulates PAK1 activity (Figs. 2 and 3), we examined the effects of CKIP-1 silencing on the PAK1-mediated migration and invasion of RWPE-1 cells. Overexpression of PAK1 induces the formation of membrane ruffles, which, however, is inhibited by CKIP-1 knockdown (Fig. 7*A*). As observed in PC3 cells (Fig. 3), CKIP-1 knockdown decreases phosphorylation of PAK1 (Fig. 7*B*) and suppresses PAK1-induced migration

(Fig. 7*C*) and proliferation (Fig. 7*D*) of RWPE-1 cells. These results suggest that CKIP-1 regulates prostate cancer migration and proliferation by modulating PAK1 activity.

DISCUSSION

PAK1 is a pluripotent kinase that regulates numerous cellular processes, including cell growth and proliferation, actin cytoskeleton organization, cell cycle and apoptosis (37-40). It remains unknown, however, how PAK1 can perform such diverse functions remains unknown. It has been suggested that the fine control of localization and activation of PAK1 may be the mechanism used by the cell to activate the right PAK1-dependent pathway according to the cell cycle status or in response to extracellular stimuli (39). An early study showed that PI3K activation induces PAK1 kinase activity in a Cdc42/Rac1 or an Akt-independent mechanism (41). However, the underlying mechanism remains unknown. In this study, we establish the role of CKIP-1 in the PI3K activation of PAK1 in response to EGF. CKIP-1 was originally identified as a CK2α-interacting protein. However, it has not been demonstrated whether CKIP-1 is involved in phosphorylation of CK2 substrates (18,19,23). Our data show for the first time that CKIP-1 delivers CK2α to PAK1 in response to EGF, and that this process is regulated by PI3K, demonstrating a mechanistic link between CK2 and PI3K signaling.

Emerging evidence indicates that PI3K mediates plasma membrane association of CKIP-1 (23). CKIP-1 exhibits a very broad spectrum of binding to phosphoinositols through its PH domain (19). In myoblast C2C12 cells, CKIP-1 binds to PI3P (phosphoinositol-3-phosphate) at the plasma membrane in response to insulin and regulates muscle differentiation; however, PI3K inhibition or coexpression of PI3K (Δp85), a dominant negative mutant of PI3K, leads to nuclear accumulation of CKIP-1 (24). CKIP-1 bound to phosphoinositides via its PH domain regulates cell morphology and lamellipodia formation by recruiting the Arp2/3 complex to the plasma membrane (25). Subcellular localization of CKIP-1 is dependent on the cell type. CKIP-1 localizes in the nucleus in osteosarcoma Saos-2, mouse preosteoblastic MC-3T3 and rodent fibroblast Rat-1 cells, but is also found at the plasma membrane in monkey kidney COS-7 cells (18). Therefore, it has been suggested that CKIP-1, as a scaffolding protein, may shuttle between the nucleus and the plasma membrane and redistributes signaling complexes between these two compartments in a PI3K-dependent manner (24).

An intriguing finding in the present study may be that PI3K tightly controls PAK1 activity by regulating EGF-induced localization of CK2α and PAK1 to the plasma membrane. We show that CKIP-1, CK2α and PAK1 are recruited to the leading edge of the cells in response to EGF (Fig. 1), and that this process is regulated by PI3K (Fig. 4). Accordingly, PAK1 phosphorylation of one of its plasma membrane substrates p41-Arc^{T21} occurs in PI3K-, CKIP-1- and CK2α–dependent manners. These findings reveal a novel signaling pathway by which PI3K activates PAK1 in response to EGF (PI3K→ CKIP-1→CK2→ PAK1). Our data provide evidence that PI3K regulates localization of PAK1 to the membrane ruffles (Figs. 4*D* and 4*E*), but the underlying mechanism is unknown. PAK1, like CKIP-1, associates with the plasma membrane through phosphoinositides (42), and this process may be regulated by Nck and Grb2 (8,9). PI3K/Akt also appears to regulate plasma membrane localization of PAK1; a mutation of the Akt phosphorylation site in PAK1 blocks PAK1 activation and inhibits PAK1 redistribution in response to chemoattractant stimulation, but LY294002 treatment results in a rapid loss of cell polarity and PAK1 redistribution (43). Thus, these observations are consistent with our results that PI3K regulates localization of PAK1 to the plasma membrane.

PAK1 acts as a convergence point of many signaling pathways that are frequently

dysregulated in human cancers and thus is activated by various signaling factors, including p21 GTPases, thrombin, growth factors and hormones (38,40). Phosphorylation of PAK1 at T423 in the kinase activation loop is crucial for enzymatic activity. Binding of GTPases and lipids, such as sphingosines, to PAK1 results in exposure of the activation loop of the kinase domain (44), making it accessible for T423 phosphorylation by 3-phosphoinositide-dependent kinase 1 (PDK1) (45). It should be noted that PDK1 phosphorylation of PAK1 is not blocked by pretreatment with PI3K inhibitor wortmannin or when PDK1 was mutated to prevent phosphatidylinositol binding, indicating that this process is independent of PI3K activity (46). Therefore, T423 phosphorylation by PDK1 may be necessary but not sufficient for the activation of PAK1. Our results have demonstrated that CK2 phosphorylation of S223 is crucial for activation of PAK1 (26), and that this process is regulated by PI3K (this study). The kinase activity of a constitutively active PAK1 ($PAK1^{H83,86L}$), postulated to mimic GTPase-induced structural changes (47), requires phosphorylation of S223, suggesting that S223 may become available for phosphorylation by CK2 after the conformational change (26). Therefore, PI3K-dependent, CKIP-1-mediated recruitment of CK2α to the plasma membrane is essentially required for the activation of PAK1.

PAK1 is the major isoform of PAKs expressed in most cancers (38,48) and its expression is sufficient to promote prostate tumor growth (49). Given that S223 of PAK1 is not conserved in other PAK family members, CK2-mediated S223 phosphorylation is considered a unique regulatory mechanism for PAK1 activation (26). Activation of oncogenic kinases by elevated expression is a common feature of human primary tumors. However, there is no significant difference in PAK1 expression between prostate normal and malignant tissues (GeneCards, www.genecards.org). Instead, blocking S223 phosphorylation leads to growth inhibition of prostate cancer PC3 cells (26). Therefore, PI3K-, CKIP-1-, and CK2-dependent phosphorylation of PAK1 plays a key role in malignant transformation of prostate cells. This concept is also supported by the observation that the silencing of CKIP-1 by shRNA inhibits PAK1-mediated migration and invasion of benign prostate epithelial RWPE-1 cells (Fig. 7). Aberrant activation of growth factor receptor signaling is associated with human cancer (38), and the EGF receptor is overexpressed in PC3 cells (50). These observations strengthen the view that CKIP-1-mediated CK2 phosphorylation of PAK1, which is triggered by PI3K activation, may be the principal mechanism for the activation of PAK1 in prostate cancer cells.

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CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS
YBK and YJS designed, performed experiments and wrote the first draft of the manuscript. AR provided technical assistance and contributed to the preparation of the figures. JHK conceived and coordinated the study and wrote the paper.

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FOOTNOTES

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[1]To whom correspondence should be addressed: Jeong-Ho Kim, Department of Biochemistry and Molecular Medicine, The George Washington University Medical center, 2300 Eye Street, Washington, DC 20037, USA, Tel.: (202) 994-9937; Fax: (202) 994-8974; E-mail: jh_kim@gwu.edu

[2]The abbreviations used are: LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; DAPI, 4'-6-diamidino-2-phenylindole.

FIGURE LEGENDS

FIGURE 1. PAK1, CK2α and CKIP-1 are recruited to membrane ruffles in response to EGF. PC3 cells were serum-starved for 24 h and then incubated in the absence (-) or the presence (+) of EGF (100 ng/ml) for 1 h. *A,* Fluorescence microscopy images of PC3 cells stained with rhodamine-labeled phalloidin (red) or an antibody to PAK1, CK2α or CKIP-1 (green) (top) and quantification of plasma membrane (PM) localization of PAK1, CK2α and CKIP-1 (bottom). *B,* Whole-cell lysates of PC3 cells were analyzed by Western blotting using antibodies to the indicated proteins. *C,* Total lysates of PC3 cells

with (Sh) or without (NT) CKIP-1 knockdown were analyzed by Western blotting using anti-CK2α or anti-PAK1 antibody. *D,* Fluorescence microscopy images of CKIP-1 knockdown PC3 cells stained with anti-CK2α or anti-PAK1 antibody (left) and quantification of PM localization of PAK1 and CK2α (right). The quantitative evaluation of the immuno-staining patterns was performed on at least 1000 cells per sample, in at least three independent experiments, and the results were expressed as the percentages of cells with PM localization of the respective proteins (*A* and *D*). ***P <0.001.

FIGURE 2. CKIP-1 recruits CK2α to PAK1 to membrane ruffles in response to EGF. PC3 cells with (Sh) or without (NT) CKIP-1 knockdown were serum-starved for 24 h and then treated with EGF (100 ng/ml) for 1 h. *A,* PC3 cells were costained with anti-CK2α (green) and anti-PAK1 (red) antibodies. *B,* The plasma membrane-enriched fraction of PC3 cells was analyzed by Western blotting using antibodies to CKIP-1, CK2α or PAK1. The plasma membrane Na^+/K^+-ATPase was used as a loading control. *C,* GFP-PAK1 expressed in PC3 cells (a ShNT and two Sh-CKIP-1s) was immunoprecipitated using anti-GFP antibody (IP), and blots were probed with antibodies to the indicated proteins. *D,* Whole-cell lysates of PC3 cells with (Sh) or without (NT) CKIP-1 knockdown were blotted with antibodies to the indicated proteins. *E,* Colocalization of CK2α (green) with an activated form of PAK1, $PAK1^{S144}$ (pS144, red) in PC3 cells. *F* and *G*, PC3 cells were costained with rhodamine-labeled phalloidin (red) and either anti-phospho-$PAK1^{S144}$ antibody (green) (*F*) or anti-phospho-p41-Arc^{T21} antibody (green) (*G*). DNA was stained with DAPI (blue) in *A, E, F* and *G*.

FIGURE 3. Expression of a constitutively active PAK1 ($PAK1^{S233E}$) bypasses the requirement for CKIP-1 in EGF-induced cell polarity and migration. PC3 cells were serum-starved for 24 h and then stimulated by EGF (100 ng/ml) for 1 h. *A,* PC3 cells were stained with anti-phospho-$PAK1^{S144}$ antibody (red). *B,* Quantification of cells stained with anti-phospho-$PAK1^{S223}$ antibody. Cells with plasma membrane (PM) localization of phospho-$PAK1^{S144}$ were quantified as described above (Fig. 1). ***P <0.001. *C,* Western blot analysis of S223 phosphorylation of PAK1. Whole cell lysates of CKIP-1 knockdown (CKIP-1) and control (NT) PC3 cells expressing GFP-PAK1 were blotted with antibodies to the indicated proteins. *D,* PC3 cells stimulated by EGF were stained with anti-phospho-$PAK1^{S223}$ antibody (green) and rhodamine-labeled phalloidin (red). *E,* Immunofluorescence analysis of the localization of GFP-PAK1 and phospho-$PAK1^{S223}$. PC3 cells expressing GFP-PAK1 were stained with anti-phospho-$PAK1^{S223}$ antibody (middle). DNA was stained with DAPI (blue) in *D* and *E*. *F,* Wound healing assay of PC3 cells expressing GFP-PAK1 or GFP-$PAK1^{S223E}$ (left). The results were expressed as the percentages of the remaining area as determined by normalizing the area of wound after 24 h (black bars) to the initial wound area at 0 h (white bars, set to 100%) (left). Each bar represents the mean ± s.e of five fields counted (***, P<0.001) (right). *G.* Invasion assay of PC3 cells expressing GFP-$PAK1^{Wt}$ or GFP-$PAK1^{S223E}$. Cell invasiveness was assessed using a Transwell Boyden chamber assay (top). The invasion rate was determined by counting the number of cells having migrated through the membrane into the lower chamber, and the results were expressed as a percentage relative to control cells (shNT cells expressing GFP-PAK1, set to 100%). Each bar represents the mean ± s.e of five fields counted (***, P<0.001) (bottom).

FIGURE 4. PI3K regulates the localization of CKIP-1 to the plasma membrane. PC3 cells were serum-starved 24 h, stimulated by EGF (100 ng/ml) for 1 h, and then treated with LY294002 (25 μM) for 4 h or wortmannin (WT, 100 nM) for 1 hr. *A,* Fluorescence microscopy images of PC3 cells stained with

anti-CKIP-1 antibody (top) and quantification of plasma membrane (PM) localization of CKIP-1 as described in Fig. 1 (bottom). *B,* The plasma membrane-enriched fraction of PC3 cells was analyzed by Western blotting using antibodies to CKIP-1, CK2α and PAK1. Na^+/K^+-ATPase was used as loading control. *D,* PAK1 activity in PC3 cells treated with (+) or without (-) LY294002 or wortmannin. *C.* Western blot analysis of the phosphorylation of $PAK1^{S144}$ and p41-Arc^{T21}. Whole-cell lysates were blotted with antibodies to the indicated proteins. *D* and *E,* PC3 cells were costained with rhodamine-labeled Phalloidin (red) and either anti-CKIP-1, CK2α or PAK1 antibody (green). DNA was stained with DAPI in *D* and *E.*

FIGURE 5. PI3K regulates the interaction of PAK1 with CK2α and CKIP-1. PC3 cells were incubated and treated with EGF and LY294002 as described above (Fig. 4). *A,* Western blot analysis of the interaction of PAK1 with CK2α and CKIP-1. GFP-PAK1 expressed in PC3 cells (- LY and + LY) was immunoprecipitated using anti-GFP antibody (IP) and blotted with anti-GFP, anti-CK2α or anti-CKIP-1 antibody (IB). *B,* PC3 cells were costained with anti-CKIP-1 (green) and anti-CK2α (red) antibodies. *C* and *D,* PC3 cells were costained with anti-PAK1 antibody (red) and either anti-CKIP-1 antibody (green) (*C*) or anti-CK2α antibody (green) (*D*). DNA was stained with DAPI in *B,* C and *D.*

FIGURE 6. Expression of a constitutively active PAK1 ($PAK1^{S233E}$) bypasses the requirement for PI3K in EGF-induced PAK1 activation. PC3 cells were incubated and treated with EGF and LY294002 as described above (Fig. 4). *A,* Whole-cell lysates of PC3 cells were blotted with antibodies to the indicated proteins. AKT phosphorylation at S473 (AKT^{S473}) was used as an indicator of PI3K activation. *B,* Colocalization of phospho-$PAK1^{S223}$ and phospho-p41-Arc^{T21} in PC3 cells. *C,* PC3 cells expressing GFP-PAK1 or GFP-$PAK1^{S223E}$ were costained with anti-phospho-p41-Arc^{T21} and anti-phospho-$PAK1^{S223}$antibodies. *D,* Whole-cell lysates of PC3 cells expressing GFP-PAK1 or GFP-$PAK1^{S223E}$ were blotted with antibodies against the indicated proteins. *E,* Wound healing assay of PC3 cells expressing PAK1 or GFP-$PAK1^{S223E}$. The results were expressed as the percentages of the remaining area as determined by normalizing the area of wound after 24 h (black bars) to the initial wound area at 0 h (white bars, set to 100%) (left). Each bar represents the mean ± s.e of five fields counted (***, P<0.001) (right). DNA was stained with DAPI in *B* and *C.*

FIGURE 7. CKIP-1 is required for PAK1-mediated malignant transformation of the benign prostate RWPE-1 cells. *A,* RWPE-1 cells (shNT and shCKIP-1) expressing GFP-PAK1 were incubated in serum-containing medium and stained with rhodamine-labeled phalloidin. DNA was stained with DAPI (blue). *B,* Whole-cell lysates of RWPE-1 cells expressing GFP-PAK1 were blotted with antibodies to the indicated proteins. *C,* Invasion assay of RWPE-1 cells expressing GFP-PAK1. Cell invasiveness was assessed using a Transwell Boyden chamber assay (top), and the invasion rate was determined as described in Fig. 3*G* (bottom). *D,* Anchorage-independent growth assay of RWPE-1 cells (shNT and shCKIP-1) expressing GFP-PAK1. GFP-PAK1-induced shNT or shCKIP-1 cells were visualized by phase-contrast microscopy (top). Quantitative analysis of colony numbers (mean ± SD) in week 3 is shown (n = 4) (bottom). **, $p < 0.05$ (as compared with control).

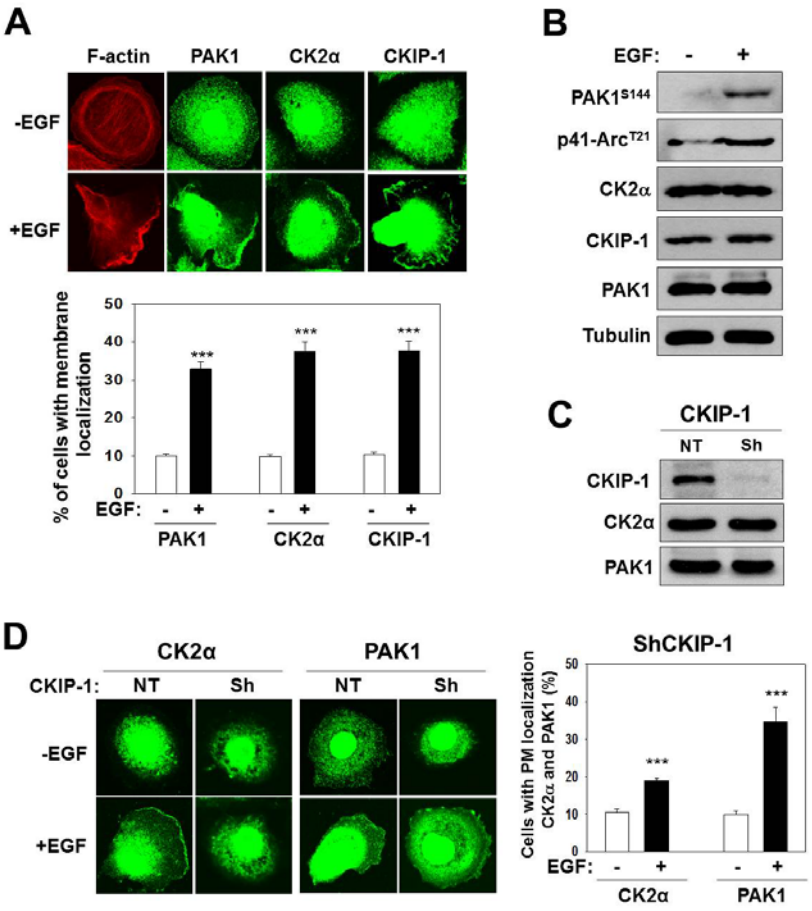


FIGURE 1

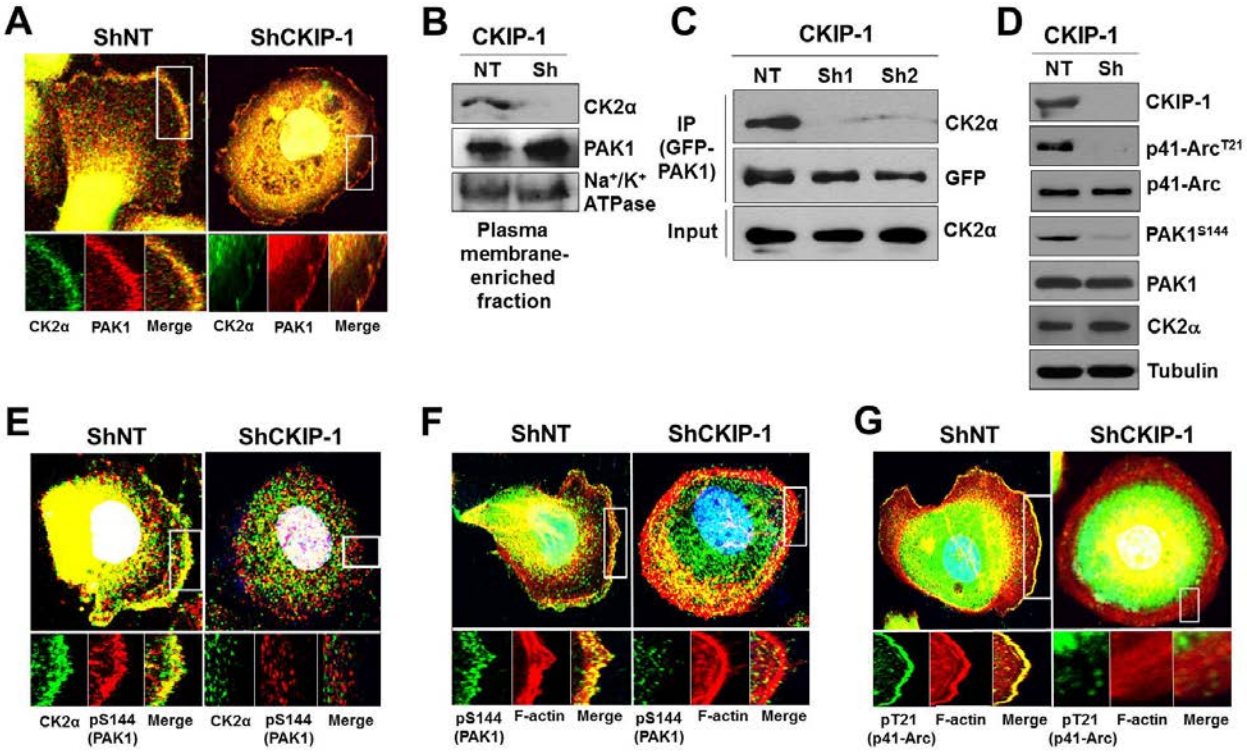


FIGURE 2

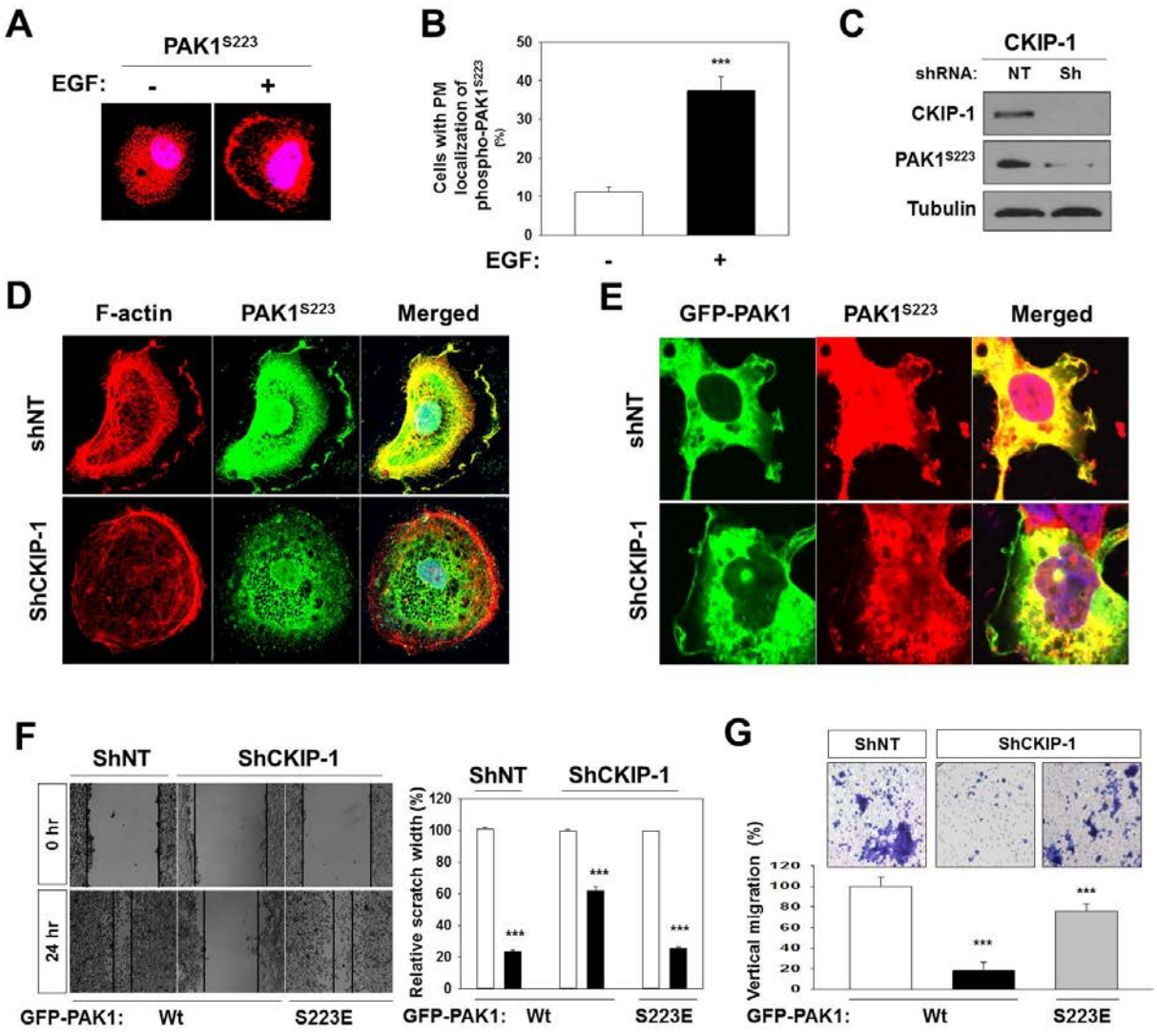


FIGURE 3

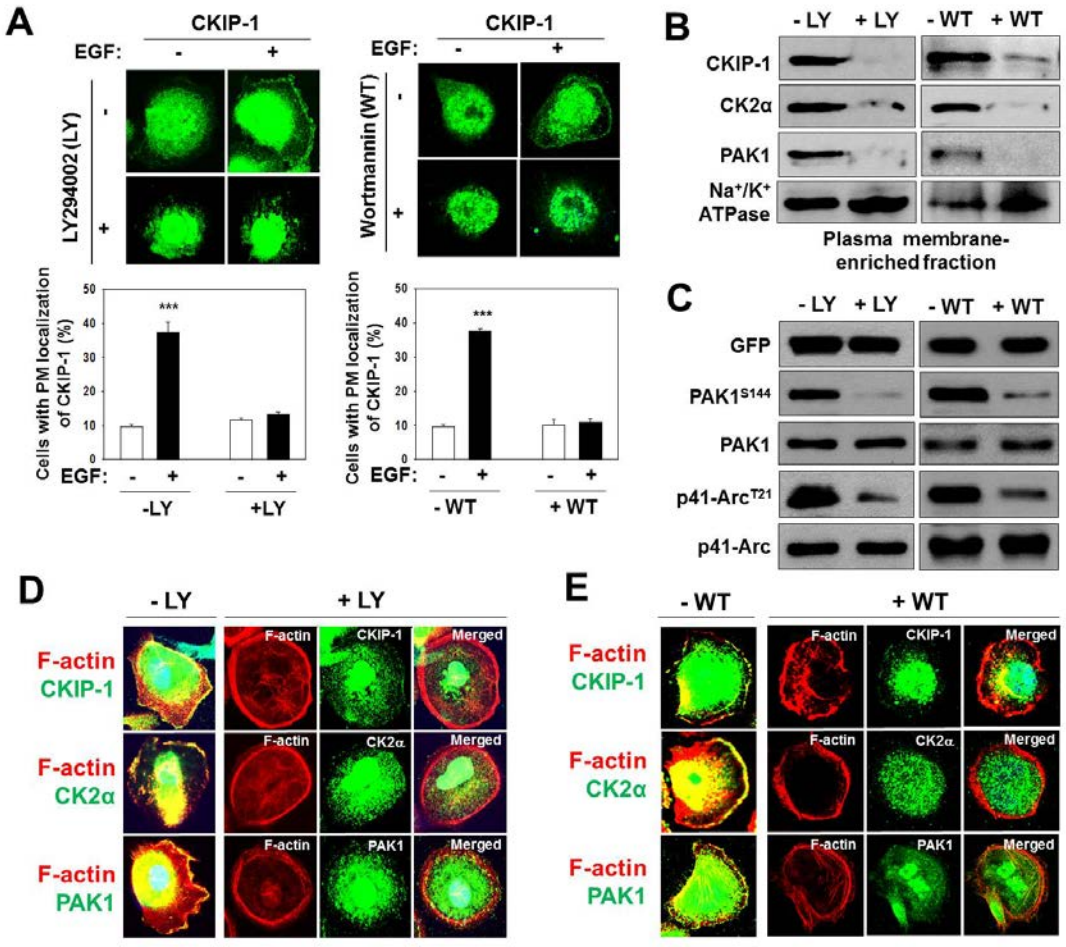


FIGURE 4

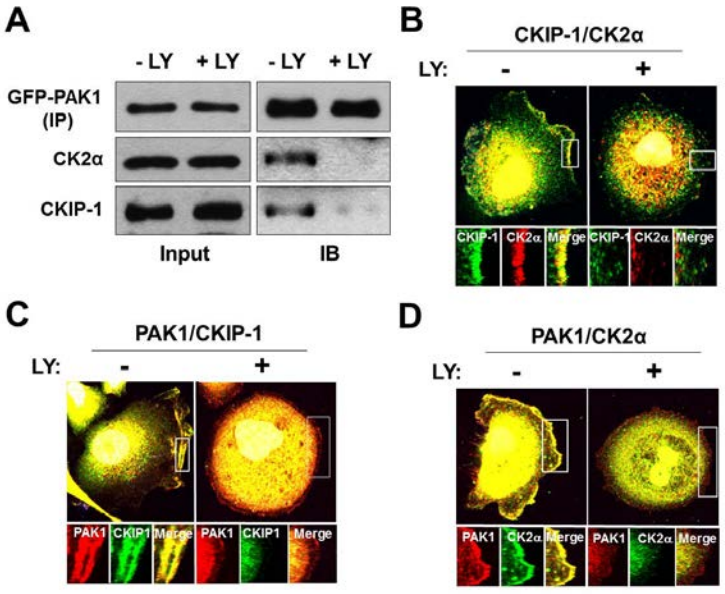


FIGURE 5

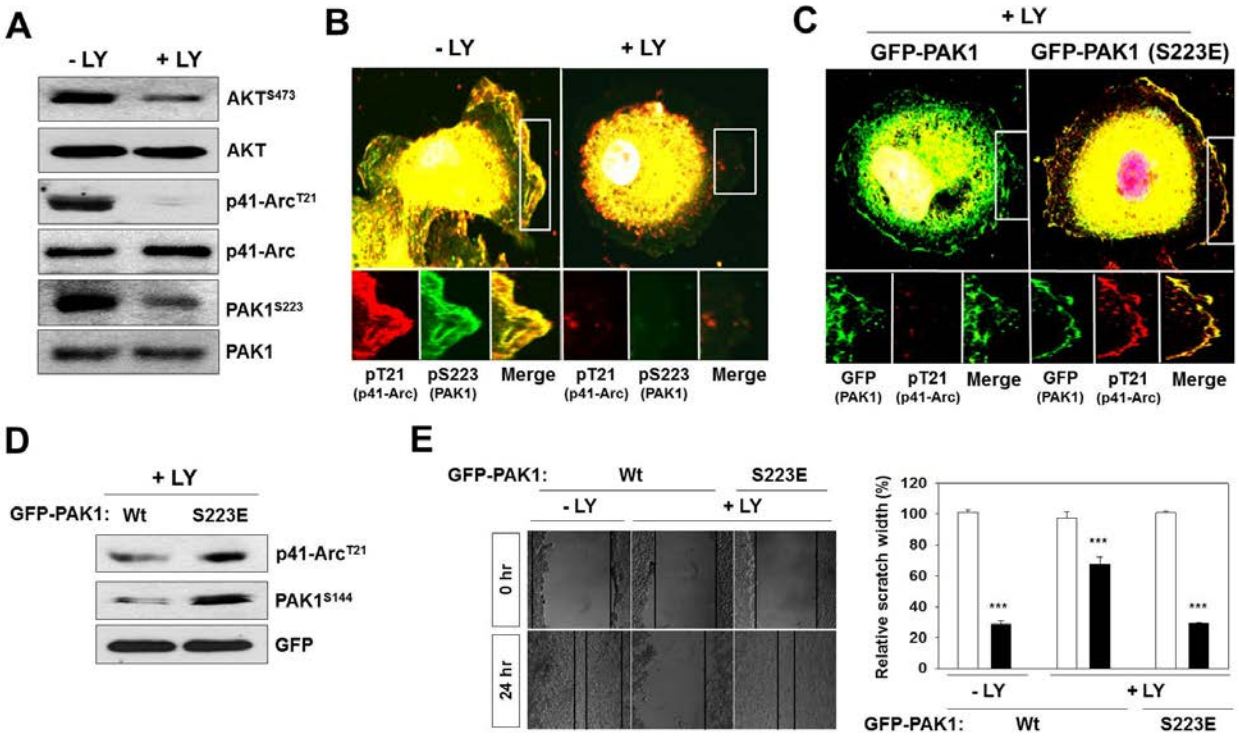


FIGURE 6

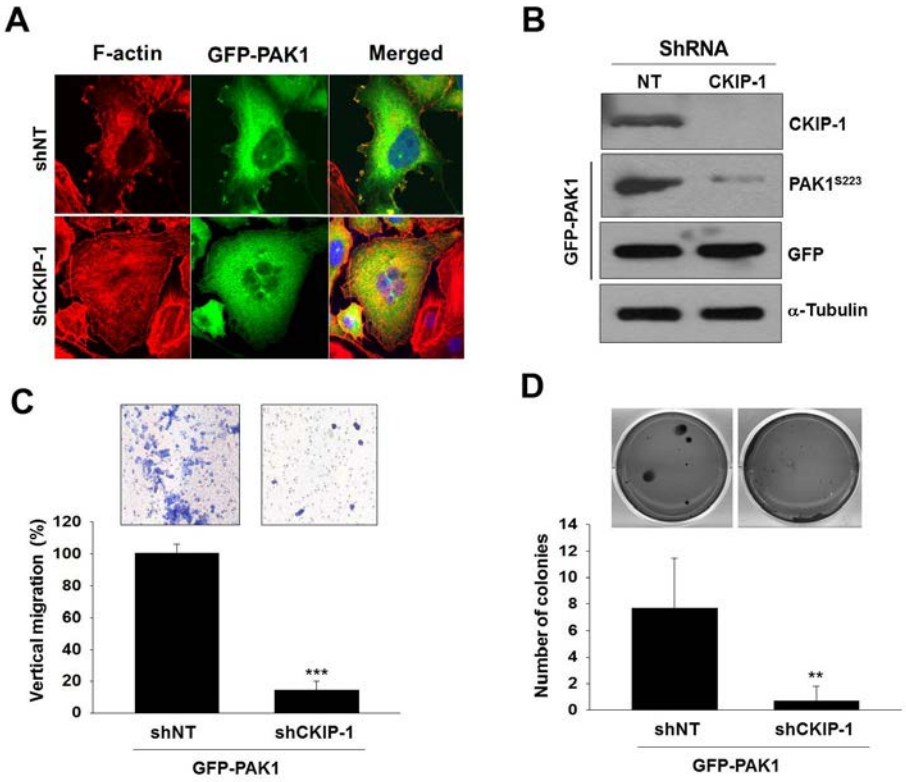
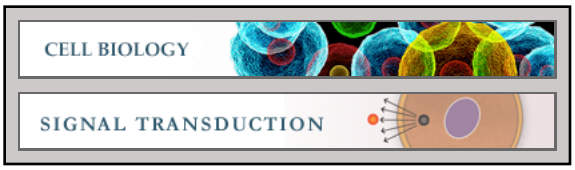


FIGURE 7

Cell Biology:

The Role of the Pleckstrin Homology Domain-Containing Protein CKIP-1 in Activation of p21-activated Kinase 1 (PAK1)



Yong Bae Kim, Yong Jae Shin, Adhiraj Roy and Jeong-Ho Kim

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