

Regulation of Microfilament Reorganization and Invasiveness of Breast Cancer Cells by Kinase Dead p21-activated Kinase-1*

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Liana Adam, Ratna Vadlamudi, Mahitosh Mandal, Jonathan Chernoff‡, and Rakesh Kumar§

From The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030 and the ‡The Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

Stimulation of growth factor signaling has been implicated in the development of invasive phenotype and p21-activated kinase (PAK1) activation in human breast epithelial cancer cells. To further explore the roles of PAK1 in the invasive behavior of breast cancer cells, in the present study we investigated the influence of inhibition of PAK1 activity on the reorganization of cytoskeleton components that control motility and invasiveness of cells, using a highly invasive breast cancer MDA-MB435 as a model system. Our results demonstrate that overexpression of a kinase dead K299R PAK1 mutant leads to suppression of motile phenotypes as well as invasiveness of cells both in the absence or presence of exogenous heregulin- β 1. In addition, these phenotypic changes were accompanied by a blockade of disassembly of focal adhesion points, stabilization of stress fibers, and enhanced cell spreading and were dependent on the presence of the kinase dead domain but independent of the presence of the Rac/cdc42 intact (Cdc42/Rac interactive binding) domain of PAK1. We also demonstrated that in K299R PAK1-expressing cells, F-actin filaments were stabilized by persistent co-localization with the actin-binding proteins tropomyosin and caldesmon. Extension of these studies to invasive breast cancer MDA-MB231 cells illustrated that conditional expression of kinase-defective K299R PAK1 was also accompanied by persistent cell spreading, multiple focal adhesion points, and reduced invasiveness. Furthermore, inhibition of PAK1 activity in breast cancer cells was associated with a reduction in c-Jun N-terminal kinase activity, inhibition of DNA binding activity of transcription factor AP-1, and suppression of *in vivo* transcription driven by AP-1 promoter (known to be involved in breast cancer invasion). These findings suggest that PAK1 downstream pathways have a role in the development and maintenance of invasive phenotypes in breast cancer cells.

Breast cancer is one of the most common malignancies in the United States, affecting one in nine women. Localized breast cancer prior to metastasis can be cured by surgery. The high mortality rate associated with breast cancer, however, is related to its ability to metastasize beyond the mammary gland

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§ To whom correspondence should be addressed: Cell Growth Regulation Laboratory, The M. D. Anderson Cancer Center (Box 36), Rm. Y4.6032, 1515 Holcombe Blvd., Houston, TX 77030. Tel.: 713-745-3558; Fax: 713-745-3792; E-mail: rkumar@notes.mdacc.tmc.edu.

and invade distant sites while the primary tumor is small and undetected. Thus, tumor cell migration/invasion is an important factor in formation of solid tumors and is necessary for their spread to distinct organs. The process of malignancy requires, among other steps, changes in growth factor pathways and increased migration. The exposure of cells to growth factors has been shown to cause cytoskeleton reorganization, formation of lamellipodia, membrane ruffling, and altered cell morphology, and accordingly, such exposure is implicated in stimulating cell migration and invasion (1). Most eukaryotic cells possess the capacity to move over or through a substrate, and cell migration plays a key role in both normal physiology and in a disease setting, including invasion and metastasis (2). In many tissues, the motility function of cells is normally repressed but can be activated by appropriate stimuli, oncogenic transformation, or both. In fact, one of the earliest responses of cells to many extracellular growth factors is rapid reorganization of their cytoskeleton and cell shape. The leading edge of a motile cell is composed of thin protrusions of membrane that continuously extend and retract mediating the initial stage of cell movement and determining the direction of advance. The underlying cytoskeleton of a leading edge is composed of actin-filament bundles (in filopodia) or meshworks (in lamellipodia) oriented toward the membrane (3). Cell migration also involves changes in cytoskeleton actin stress fibers that end in focal adhesions, which are places where the plasma membrane is attached to the substratum. Focal adhesions are specialized sites of adhesion for cells in culture associated with well defined actin stress fibers, and they represent the link between the outside extracellular matrix components and the cytoplasmic integrins coupled with cytoskeletal proteins. Numerous proteins involved in signal transduction are known to be concentrated at these sites. According to a recent model, intracellular components of the focal adhesion complex and actin filaments associate with integrins upon integrin engagement with the extracellular matrix. In the presence of active Rho, these complexes and actin cluster together by actin-myosin contraction, which leads to focal adhesion and stress fiber formation. The focal adhesion points play an important role in the regulation of cell motility, which involves cyclic formation of cell adhesion and disassembly of actin filaments and focal adhesion complexes (1).

The small GTPases, including Cdc42, Rac1, and RhoA, have been implicated in the regulation of morphology, the formation of filopodia/lamellopodia, membrane ruffles, and stress fibers, and motility of mammalian cells (1). More specifically, Rac1 induces cortical actin polymerization seen as the process of membrane ruffling and lamellipodia (4). RhoA induces stress fiber formation possibly by catalyzing the formation of focal adhesion (5), and cdc42 induces the formation of peripheral actin microspikes and filopodia (6). With regard to the mechanism by which the small GTPases initiate and regulate the

formation of cytoskeletal structures, recently a family of serine/theonine kinases known as p21-activated kinases (PAKs)¹ have been identified as GTPase targets (7). Activation of PAK1 has been shown to result in phenotypic changes reminiscent of those produced by GTPases. If activated either by overexpression of dominant active PAK1 mutants or by stimulation of cells with growth factors, PAK1 causes the accumulation of F-actin and the formation of lamellipodia and filopodia. Furthermore, activated PAK1 has been also shown to be co-localized with F-actin at the leading edge of the cells (7, 8). Activation of PAK1 has been shown to be accompanied by the disassembly of stress fibers and focal adhesion complexes (9), both of which are widely believed to be required for increased motility. Because PAK1 is a downstream target of cdc42 and Rac1, which are known to activate extracellular signal-regulated kinase, JNK, p38 kinases, and the transcription factor nuclear factor- κ B (10–12), it has been proposed that PAK1 or a related kinase may mediate the actions of cdc42 and Rac1. Indeed, PAK1 has been shown to activate JNK and extracellular signal-regulated kinase kinases (13, 14). Invasion of T-lymphocytes and fibroblast has been shown to involve activated Rac1 and Tiam-1 (15), a Rac1 exchange factor. In addition to the PAK1-JNK pathway, other effectors for Rac1 such as POR1 have also been implicated in actin rearrangements (16, 17). Using well differentiated mammary breast cancer cells Keely *et al.* (17) showed that PAK1 as well as JNK1 activities are not required to induce motility and invasion in cells that are overexpressing active mutants of Rac1. These studies dissociate Cdc42 and Rac1-induced actin polymerization (ruffles formation) from PAK and JNK activation, raising the possibility of existence of a divergent pathway, which involves another Rac1 effector, POR1. It was shown that Rac1 mutants that are capable of binding PAK1 but not POR1 (and thus activate JNK) are also able to induce a motile phenotype in these cells (16). However, the same Rac mutants behave in an opposite manner when used in different cell systems (17).

It is increasingly accepted that alterations in the reorganization and stability of actin filaments may contribute to increased cell migration and to cell transformation, anchorage-independent growth, and invasiveness. Tropomyosin (TM) and caldesmon (CaD) are two essential proteins required for the reorganization of the actin cytoskeleton mediated by stabilization of microfilaments (18, 19). Although TM was initially discovered as a helical protein that binds to actin grooves in muscle, it is now clear that Ca²⁺-dependent contractility in nonmuscle cells is also controlled by TM in conjunction with CaD (20). CaD is an actin- and calmodulin-binding protein that is distributed at intervals along stress fibers (18, 21). Recent studies have shown relationships between 1) overexpression of CaD and the stabilization of actin microfilament bundles by enhancing the half-life of TM (22); 2) suppression of TM1 and the anchorage-independent growth of hamster embryo cells (23); 3) modulation of the TM isoforms and tumorigenic transformation (24, 25); and 4) CaD/v-erbB interaction and actin stress fiber disassembly (26). Despite the widely acknowledged roles of TM and CaD on one hand and PAK1 on the other hand in actin stabilization, the potential relationship between these cytoskeleton regulators and invasiveness remains elusive.

Recently, we showed that stimulation of a noninvasive human breast cancer cell by heregulin leads to the activation of

PAK1 kinases, redistribution of PAK1 at the leading edges of cells, development of lamellipodia/filopodia and stress fibers, and increased invasiveness of breast cancer cells (27). To further explore the role of PAK1 in the invasive behavior of breast cancer cells, in this study we investigated the influence of suppression of the PAK1 activity on the reorganization of cytoskeleton components that control motility and *in vitro* invasiveness of cells using the highly invasive MDA-MB435 and MDA-MB231 breast cancer cell model systems. Our results demonstrated that overexpression of catalytically defective K299R PAK1 leads to biogenesis of stable focal adhesions and stress fibers with matrix-independent enhanced cell attachment and spreading properties. All these biological effects were dependent on the presence of the kinase defective site and independent on the presence of an intact Rac/cdc42 binding domain. In addition, these phenotypic changes were accompanied by persistent suppression of JNK activity, DNA binding activity of transcription factor AP-1, and *in vivo* transcription driven by collagenase promoter. These findings suggest that the PAK1-dependent pathway have a role in the maintenance of invasive behavior of breast cancer cells.

MATERIALS AND METHODS

Cell Cultures and DNA Transfection—MDA-MB435 and MDA-MB231 cells and their PAK1 clones were maintained in DMEM/F12 (1:1) supplemented with 10% tetracycline-free FCS (CLONTECH). To generate stable MDA-MB435 clones overexpressing K299R PAK-1 mutant, the cells were trypsinized and resuspended at 2×10^7 cells/ml in DMEM/F-12 supplemented with 10% FCS, and 20 μ g of each of the plasmids (CMV, pCMV6myc-hPAK1^{K299R}) were introduced by electroporation (7, 28). Positive clones were selected by adding 400 μ g/ml of Hygromycin for a period of 2–3 months. To generate MDA-MB231 cells overexpressing tetracycline-regulated hPAK-1^{K299R}, cells were sequentially transfected with pTet-tTak + pNeomycin plasmid (pNeo) and pTet-Splice-HA-PAK1 (K299R) plus Hygromycin plasmid. Forty-eight hours posttransfection, the cells were selected in medium containing 800 μ g of neomycin (to retain the tetracycline-VP16 transactivator), 300 μ g/ml hygromycin (to select for the K299R PAK1-regulated expression vector), and 1 μ g/ml tetracycline (to repress transgene expression during selection). Clonal cell lines were isolated and expanded and at least 24 clonal lines were examined for inducible transgene expression by anti-HA immunoblot. These clones express N-terminal HA-tagged PAK1 K299R when cells are grown in the absence of tetracycline or its derivative doxycyclin. For transient transfection assays, MDA-435 cells were plated on coverslips in 6-well plates using DMEM/F12 supplemented with 10% FCS and allowed to attach for 12 h. Transient transfections were performed using Fugene 6 kit according to the manufacturer's instruction (Roche Molecular Biochemicals). The ratio DNA/Fugene-6 used was 3 μ g/6 μ l, and each PAK1 DNA (1 μ g) construct used was co-transfected with an equal amount of the green fluorescence protein (GFP) DNA. The following additional constructs were used: CRIB domain mutant pCMV-cMyc-(L83, L86), CRIB and kinase domain mutant pCMV-cMyc-(L83, L86, K299R), kinase active mutant pCMV-cMyc-(T423E), and PAK kinase autoinhibitory domain mutant pCMV-cMyc(PAK 83–149) (7). Transfected cells were identified by green fluorescence and immunostained with an anti-Myc-monoclonal antibody.

Antibodies—Anti-HA antibody and anti-Myc monoclonal antibody were obtained from the Medical Biomedical Laboratories and Neomarkers, respectively. Antibodies against activated JNK, against total JNK, and PAK-1 were purchased from Santa Cruz Biotechnology. Monoclonal antibodies against vinculin, caldesmon, and tropomyosin were from Sigma. Fluorochrome-conjugated goat anti-mouse and anti-rabbit antibodies were purchased from Molecular Probes.

Reporter Gene Assay—Luciferase fusion plasmids, –70 collagenase promoter containing a functional AP-1 binding site (–70 Col-luc) and –60 collagenase promoter containing deletion of AP-1 binding site (–60 Col-luc) were generously provided by Dr. F. X. Claret (29). Cells were transiently transfected using the FUGENE-6 transfection reagent according the manufacturer's protocol (Roche Molecular Biochemicals). Transfections were carried out in 6-well plates using 2 μ g of DNA and 10^5 cells. Transfection efficiency was monitored by co-transfection of β -galactosidase. After 36 h, cells were lysed using a Luciferase assay kit (Promega), and luciferase activity was measured using a Lumant Luminometer.

Cell Extracts, Immunoblotting, and Immunoprecipitation—Cells

¹ The abbreviations used are: PAK, p21-activated kinase; JNK, c-Jun N-terminal kinase; TM, tropomyosin; CaD, caldesmon; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HA, hemagglutinin; GFP, green fluorescence protein; CRIB, Cdc42/Rac interactive binding; HRG, heregulin β 1; CMV, cytomegalovirus; AP, activation protein.

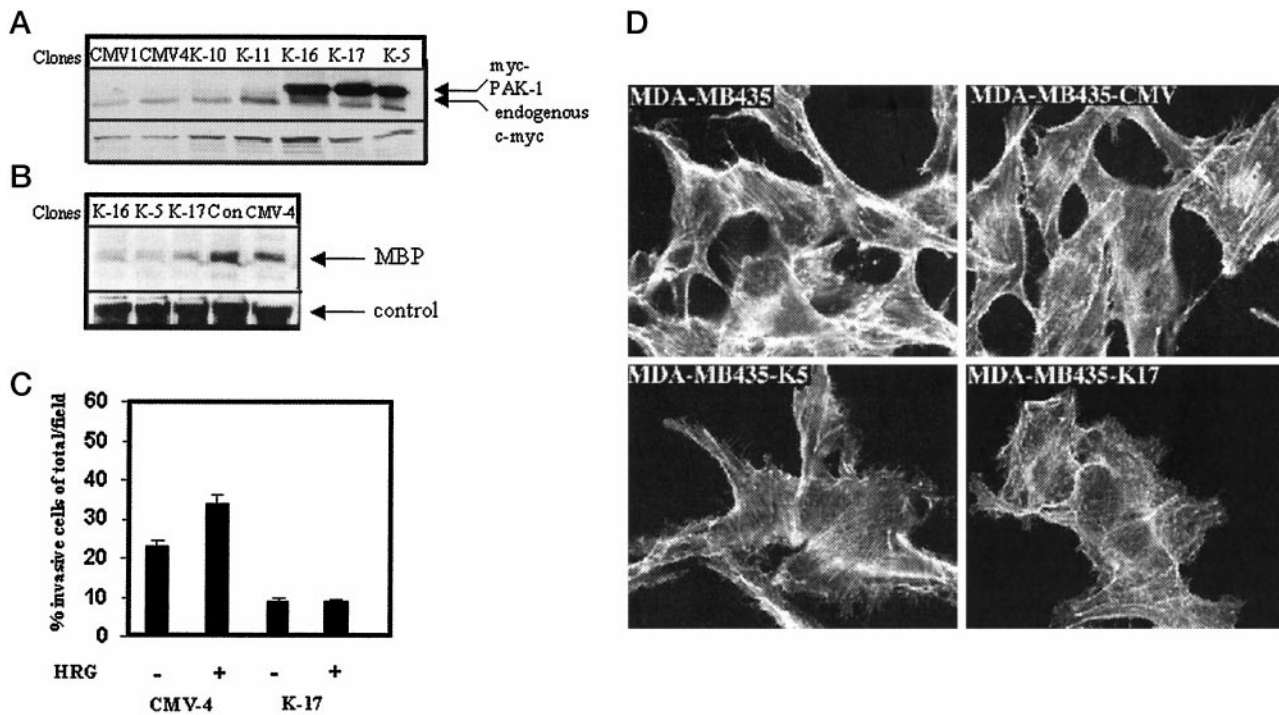


FIG. 1. Characterization of MDA-MB435 cells overexpressing K299R PAK1 mutant. *A*, expression of Myc-tagged PAK1 mutant. Parental MDA-MB435 and vector-transfected MDA-MB435-CMV cells, MDA-MB435-K5, MDA-MB435-K16, and MDA-MB435-K17, represent three prototype clones expressing the c-Myc-tagged PAK1 protein. *B*, PAK1 immunoprecipitates from total cell lysates were assayed for kinase activity using myelin basic protein as a substrate. *C*, cell migration potential was assayed using matrigel-coated Boyden diffusion chambers. Results represent cell counts of 10 different microscopic fields of three different wells, as detailed under "Materials and Methods." *D*, cells grown on matrigel-coated coverslips were cultured in serum-deprived DMEM/F12 and fixed after 12 h. Representative examples of the distribution of F-actin-specific Alexa 564-phalloidin staining followed by confocal microscopy analysis are shown.

were lysed in Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 50 mM NaF, 10 mM β -glycerol phosphate) containing 1 mM sodium vanadate, 1 mM phenylmethylsulphonyl fluoride, and 10 μ g/ml apoptinin. Cell extracts were separated on a 10% SDS-polyacrylamide gel electrophoresis and transferred to Immobilon membranes. Membranes were blocked with bovine serum albumin or fat-free milk and probed with the appropriate antibodies using ECL or alkaline phosphatase-based color methods (27).

Protein Kinase Assays—For PAK1 kinase assay, PAK1 was immunoprecipitated from total lysates using PAK1 antibody (Santa Cruz Biotechnology), and an *in vitro* kinase reaction was performed using myelin basic protein as a substrate as described earlier (27). The JNK kinase activity was determined using a JNK kinase assay kit from New England Biolabs according to the manufacturer's protocol. Reaction products were resolved on a 12% SDS-polyacrylamide gel electrophoresis, autoradiographed, and developed using a phosphorimager.

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared as described (30). AP-1 consensus oligonucleotide (5'-cgcttgat-gactcagccggaa-3') was purchased from Santa Cruz Biotechnology and end-labeled with 32 P using DNA labeling kit (Roche Molecular Biochemicals). An equal amount (10 μ g) of nuclear protein was incubated with labeled AP-1 probe, and DNA binding activity was determined by electrophoretic mobility shift assay.

Immunofluorescence—Cells cultured on uncoated, matrigel-, collagen-, polylysine- or fibronectin-coated 8-well chamber slides (Falcon) were fixed with 3.7% paraformaldehyde followed by incubation with chilled cold acetone for 2 min, washed with phosphate-buffered saline, blocked, and incubated with the respective primary antibodies for 1 h. For filamentous actin staining, 0.1 μ M Alexa 568-conjugated phalloidin was included during incubation with the secondary antibody. Slides were mounted using the Slow Fade Antifade kit (Molecular Probes). Cells were viewed with an inverted Zeiss Axioplan fluorescence microscope with a charged-coupled device camera using the IP-Lab Spectrum software, and in some experiments slides were analyzed by confocal microscopy. In the transient transfection assays the cells that integrated the PAK1 construct were identified by their expression of GFP *in vivo* as well as after fixation in paraformaldehyde/acetone and co-stained for vinculin, F-actin, or Myc-tag followed, where it was the case, by their detection using the goat anti-mouse Alexa 546-labeled secondary antibody.

Migration Assays—To measure the migration potential of MDA-MB231 cells with the K299R PAK1 mutant under the Tet-off system (MDA-MB-231/T3 cells), PAK1 expression was induced by culturing in doxycyclin-free DMEM/F-12 medium plus 10% FCS for 24 h. Cells grown in the presence of doxycyclin were used as control. After a short treatment with trypsin, the cells were washed in the presence of soybean trypsin inhibitor, resuspended in DMEM/F12 plus 0.1% bovine serum albumin in the presence or absence of doxycyclin, and loaded on the upper well of a Boyden chamber at a concentration of 20,000 cells/well. The lower side of the separating filter was coated with a thick layer of 1/2 diluted Matrigel (Life Technologies, Inc.) in serum-free DMEM/F-12. The number of cells that successfully migrated through the filter and invaded the 2-mm Matrigel layer in order to spread, as well as those that remained on the upper side of the filter, were counted by confocal microscopy after staining with propidium iodide (Sigma). MDA-MB435 clones were assayed for their invasive potential in the absence or presence of HRG using same thick layer Matrigel-coating method. Results were expressed as percentages of migrated cells and compared with the total number of cells (cells present in all Z-sections). Immunofluorescence and Laser Scanning Confocal Microscopy were essentially performed as described (27).

RESULTS

Characterization of MDA-MB435 Clones Expressing PAK1 Mutants—Because PAK1 activation has been shown to be closely linked with the heregulin-induced invasiveness of well differentiated estrogen receptor positive epithelial breast cancer cells (27), we asked the question if the PAK1 activity is also required for the maintenance of the invasive phenotype in cell lines with a different background such as MDA-MB435 and MDA-MB231 (estrogen receptor negative and vimentin positive).

MDA-MB435 cells were stably transfected with kinase dead K299R PAK1 or with control CMV vector. We isolated three transfectants expressing 6–10 times higher levels of PAK1 than the levels seen in vector-transfected or in parental cells, as judged by the differences in the expression of Myc-tagged K299R PAK1 mutant and endogenous PAK1 (Fig. 1A). To see

whether or not the introduced K299R PAK1 gene product had a significant impact on the kinase activity of the total PAK1 expressed by the transfectants, PAK1 was immunoprecipitated and subjected to *in vitro* kinase assay using the myelin basic protein as a substrate (Fig. 1B). All three kinase dead PAK1 clones (k-clones) exhibited a significant reduction in the level of PAK1 kinase activity, as compared with the control cells (Fig. 1B, last two lanes).

Effect of Kinase Dead K299R PAK1 on the Invasive Potential of Breast Cancer Cells—To explore the effect of K299R PAK1 overexpression on the invasiveness of MDA-MB435 cells, we evaluated the ability of k-PAK1 clones to transmigrate through a porous filter containing 8- μ m pores and coated with Matrigel. In this Boyden chamber invasion assay, cells that invaded the Matrigel and reattached to the lower side of the membrane were quantitated by confocal scanning microscopy. As a control, the same number of parental MDA-MB435 or mock-transfected cells were plated and counted in the same way. As illustrated in Fig. 1C, overexpression of kinase dead PAK1 resulted in about a 3-fold reduction in the ability of the cells to invade through the Matrigel compared with control cells. This was true for all three K299R PAK1 clones. HRG did not have an enhancing effect on the invasiveness of K299R PAK1 clones. As a positive control, we used HRG-stimulated control cells (Fig. 1C). Although expression of kinase dead PAK1 suppressed invasiveness of three separate clones, we used the K17 clone in our subsequent experiments.

To define the effect of the K299R PAK1 mutant on the motility of MDA-MB435 cells, next we examined the status of ruffle formation, an essential component of invasive phenotype, using confocal scanning microscopy. All three K299R PAK1 expressing clones exhibited similar morphological alterations when plated on plastic plates. K299R PAK1 transfectants develop elongated membrane protrusions without any cell polarity in contrast to parental cells that showed a typical bipolar morphology similar to fibroblasts (data not shown). Despite the development of membrane extensions, the K299R PAK1 transfectants acquired a flatter morphology and occupied roughly twice as much area as parental cells. A flatter morphology was also evident when transfectants were allowed to attach on a Matrigel layer. Representative fields of two K299R PAK1 transfectants (K5 and K17) were plated in these conditions and stained with phalloidin; they showed reduced thickening of the cell border corresponding to F-actin-containing structures as compared with parental or mock-transfected cells (Fig. 1D).

Focal Complex Localization of Kinase Dead K299R PAK1 Protein—To understand the impact of PAK1 inactivation on the potential reorganization of the actin cytoskeleton, we examined the distribution of F-actin by phalloidin staining and also confirmed the expression of Myc-tagged PAK1. Overexpression of kinase dead PAK1 led to the development of stress fibers that contain thick F-actin cables connecting the ventral part of the cells to the substrate (Fig. 2, B, D, and F). The F-actin cables in the parental cells were thin and disposed mostly along the longer axis of the cells (Fig. 2, A, C, and E). A second morphological alteration caused by kinase dead PAK1 expression was the formation of mature focal adhesion contacts independent of the type of extracellular matrix used such as: diffusible components from 10% FCS (Fig. 2B), collagen I (Fig. 2F), or fibronectin (Fig. 2D). Surprisingly, these well defined focal adhesion complexes also contained exogenously expressed K299R PAK1 protein (Fig. 3B, arrows). Parental cells showed exclusively cytoplasmic localization for the c-Myc-tag antibody because of endogenous c-Myc immunoreactivity and represents the background staining (Fig. 3D) and no co-localization with focal points (Fig. 3E, arrowheads). The cellular distribution of

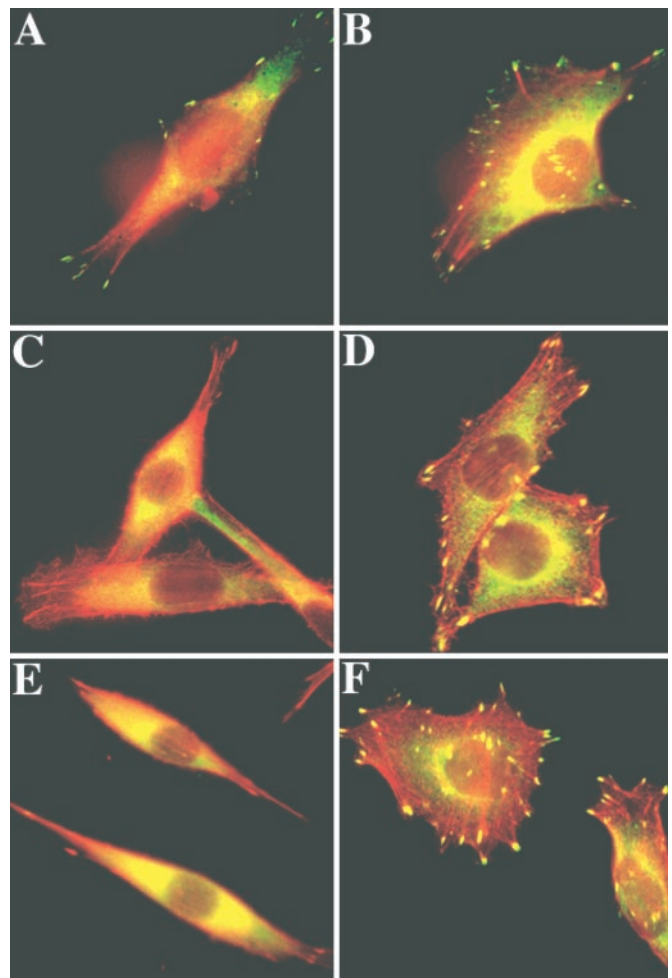


FIG. 2. MDA-MB435 cells overexpressing K299R PAK1 form organized focal contacts and stress fibers in different extracellular matrices. Cells cultured on uncoated (A and B), fibronectin- (C and D), or collagen- (E and F) coated chamber slides were fixed after 6 h and co-stained by indirect immunofluorescence for vinculin and F-actin using Alexa 564-phalloidin. Representative examples of vinculin and F-actin distributions in MDA-MB435-K17 (B, D, and F) or in MDA-MB435-CMV (A, C, and E) are shown.

the K299R PAK1 was maintained even when cells were plated on other components of the extracellular matrix such as fibronectin or collagen I (not shown).

Cell Spreading, Enhanced Focal Adhesion Point Numbers, and Stress Fiber Formations Are Not because of Rac1/cdc42 Sequestration by the CRIB Domain of the K299R PAK1—Blocking the functions of Rac-1 or cdc42 or both could mimic the observed K299R PAK1-associated cellular modifications described above. Because the K299R PAK1 construct has an intact CRIB domain, it is possible that the observed changes in K299R PAK1 expressing cells could be because of sequestration of Rac/cdc42. To address this possibility, we have used the following additional PAK1 mutants: PAK1 double mutant (H83, 86L, K299R), which has mutations in the GTP binding and PAK kinase domains; PAK autoinhibitory domain (PAK1-83-149), which binds and inhibits the endogenous PAK; a constitutively active (T423E) PAK1 kinase; and GTP binding defective (H83, 86L) but kinase active PAK1 (Fig. 4A). To visualize the effects of these PAK1 mutants, cells were cotransfected with each one of the PAK1 constructs and an equal amount of GFP vector. The green fluorescent positive live cells were monitored for their spreading at different time intervals after transfection. In Fig. 4A representative microscopic fields after 6 h posttransfection are presented. Cells transfected with

control vector or kinase active T423E and PAK1 H83, 86L PAK1 mutants exhibited a bipolar shape (Fig. 4B). In contrast, cells transfected with kinase defective PAK1 mutants with or without intact CRIB domain were nonpolar and well spread out (Fig. 4B). Similar morphological alterations, but to a lesser degree, were also recorded in cells transfected with PAK83–149, a known inhibitor of endogenous PAK1 (31, 32). The influence of different PAK1 mutants on the motility of MDA-MB-435 cells was evaluated by the Boyden diffusion chamber assay. Cells were transiently co-transfected with GFP vector and PAK1 construct(s) and seeded in the upper chamber, and GFP positive cells were counted on both sides of the porous filter. As shown in Fig. 4C, transfection of kinase dead K299R and H83, 86L-K299R PAK1 constructs significantly suppressed

the migration of MDA-MB-435 cells as compared with vector or T423E PAK1-transfected cells.

In addition to morphological changes, we have also examined the influence of PAK1 mutants on cell spreading and F-actin distribution. Cells transfected with K299R or H83, 86L-K299R PAK1 exhibited increased cell spreading and the appearance of thick F-actin cables (Fig. 5) and enhanced the number of focal points (data not shown). Interestingly, cells expressing kinase active T423E or H83, 86L PAK1 showed significantly less F-actin polymerization and the absence of stress fibers (Fig. 5). Staining with c-Myc antibody confirmed that the GFP positive cells also overexpressed c-Myc (data not shown). Taken together, these results suggested that the inhibition of PAK1 kinase activity rather than sequestration of the small GTPase may be the cause of the phenotypic changes shown in the present study, including increased actin polymerization and formation of focal points.

HRG Stimulation Failed to Promote Focal Adhesion Dissolution in Kinase Dead PAK1 Clones—To see whether the observed anti-invasive effect of the K299R PAK1 mutant was responsible for the formation of more stable focal adhesion complexes, we tested the effect of HRG on the dynamics of F-actin and focal adhesion complexes. As shown in Fig. 6, actin-containing stress fibers and vinculin-containing focal complexes were not modified by 1 h treatment with HRG in cells bearing the kinase dead mutant K299R PAK1 (Fig. 6, A and C). In contrast, control parental cells exhibited a dramatic change in the redistribution of F-actin by forming thin polar protrusions at the cell periphery corresponding to filopodia associated with a significant dissolution of the stress fibers (Fig. 6, B and D, arrowheads). These results suggested that PAK1 activity is required for focal point dissolution induced by HRG.

Effect of K299R PAK1 on the Reorganization of Actin-stabilizing Proteins—Because PAK1 inactivation was accompanied by increased persistence of stress fiber cables, we examined the possibility of the association of actin filaments with actin-stabilizing proteins such as TM and CaD (21, 33). The K299R PAK1 transfectants were spread out, contained focal complexes with thick actin cables, and exhibited co-localization of TM (Fig. 7, A and A'), whereas the control cells had a fibroblast-like shape and a diffuse TM distribution. (Fig. 7, B and B'). Another actin-binding protein, CaD, showed also a fibrillar pattern being deposited on the stress fibers as evidenced by the appearance of the yellow color because of co-localization of F-actin and CaD

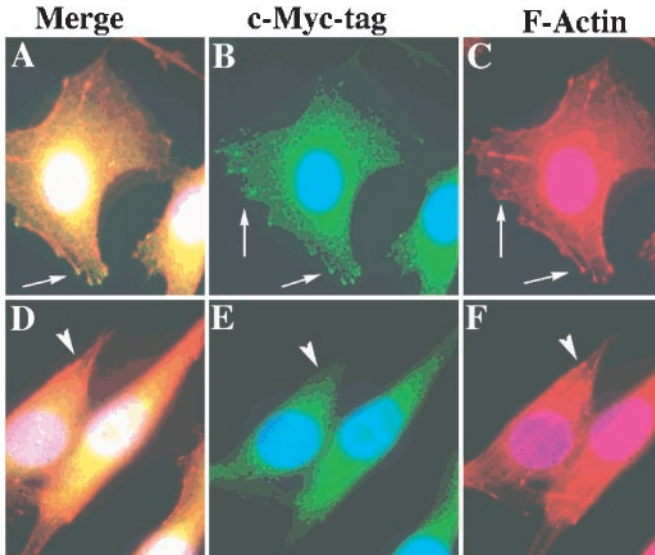
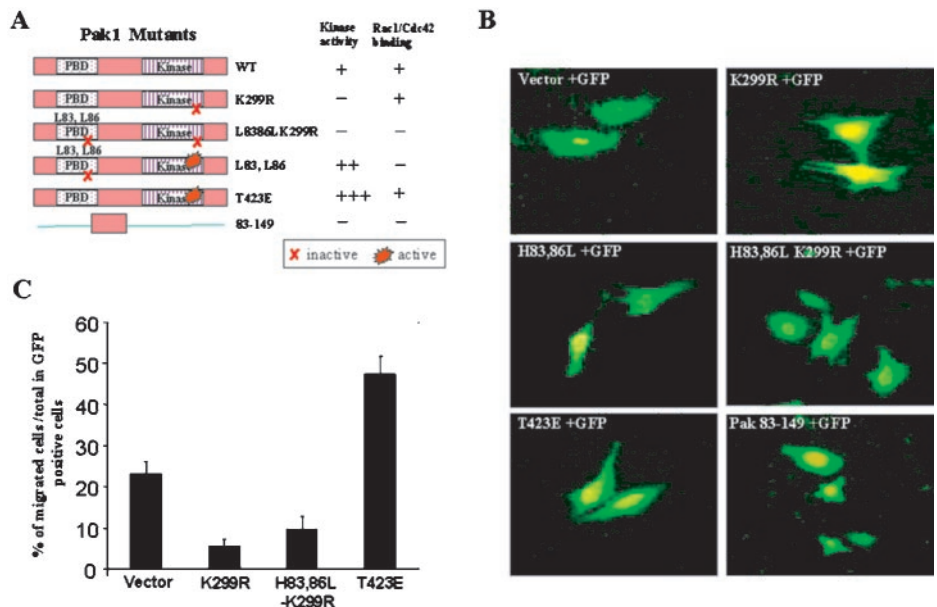


FIG. 3. Effect of K299R PAK1 on focal adhesion complexes distribution. MDA-MB435-K17 cells cultured in chamber slides were kept in DMEM/F-12 supplemented with 10% FCS, fixed after 12 h, and co-stained for c-Myc tag and F-actin. Arrows (A and B) show fluorescein isothiocyanate-immunostaining representative examples of focal contacts distribution of c-Myc-tagged PAK1. Co-localization of c-Myc tag with F-actin is also shown by appearance of the yellow color (A), which corresponds to the focal points (C, arrows). Diffuse cytoplasmic immunoreactivity with anti-Myc antibody corresponding to the endogenous c-Myc in control cells (D–F) and no co-localization with F-actin in the focal points was detected in these cells (compare D with A, arrowheads).

FIG. 4. Effect of PAK1 mutants on the cell shape and migration. A, schematic representation of the constructs used in the study. B, MDA-MB435 cells co-transfected with various PAK-1 mutants and/or a GFP vector were photographed using a computer-based imaging system (Olympus 1 × 70) set for green fluorescence light. C, cells were co-transfected with vector or one of three PAK1 mutants and a GFP-containing vector and plated on the upper side of the filter membrane and allowed to migrate for 24 h toward the lower side of the filter and to invade the matrigel. The number of GFP positive cells that invaded the filter was expressed as a percentage of the total GFP positive cells in the same microscopic field. At least 10 fields/membrane were counted. Each experiment was done in triplicate. PBD, protein binding domain.



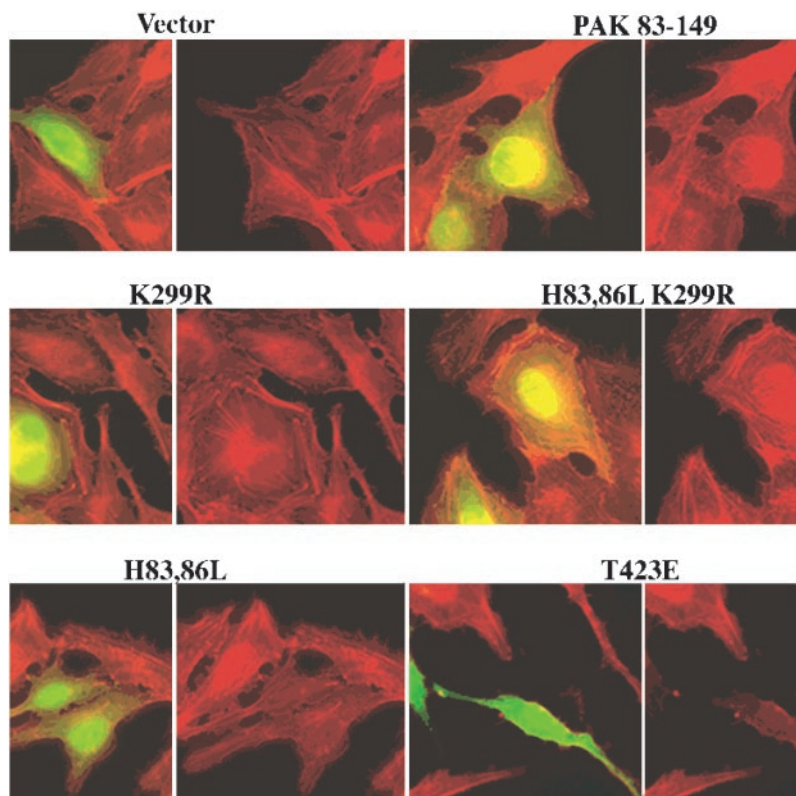


FIG. 5. Kinase-deficient PAK1 mutants increase actin polymerization. MDA-MB435 cells plated on coverslips were co-transfected with various PAK1 mutants and a GFP-containing vector, fixed, and stained for F-actin after 24 h by using Alexa 546-labeled phalloidin. Cells were visualized by using an inverted Zeiss microscope coupled with a charge-coupled device camera for both GFP (green) and F-actin (red) reactivity.

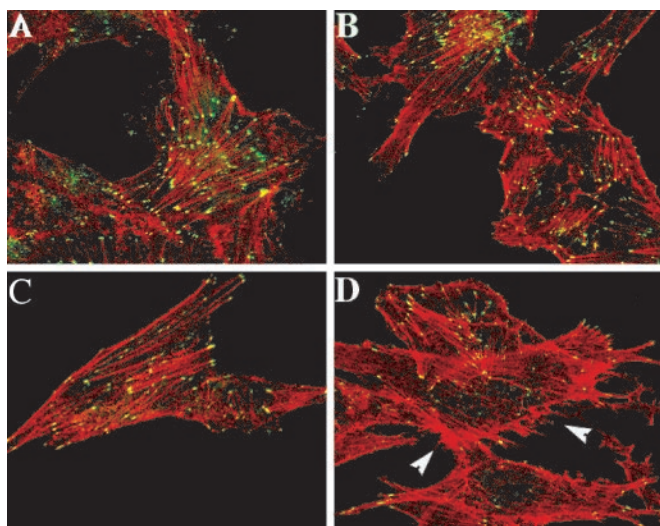


FIG. 6. Expression of K299R PAK1 mutant blocks HRG induction of actin-containing dynamic structures. Cells cultured on chamber slides were serum-deprived for 24 h, stimulated with 20 ng/ml HRG (B and D), and fixed after 1 h. Representative examples of the distribution of F-actin and vinculin in MDA-MB435 K17 (A and B) or MDA-MB435-CMV (C and D) are shown. Note the appearance of filopodia (shown by arrowheads) as well as a significant dissolution of the focal points and stress fibers exclusively in HRG-treated MDA-MB435-CMV cultures (D).

(Fig. 7, C and C'). By contrast, CaD staining in mock-transfected cells showed diffuse cytoplasmic distribution (Fig. 7, D and D'). Interestingly, a closer examination of the focal adhesion structures in some K299R PAK1-expressing cells showed that there was a loop-like disposition of actin-bound CaD in the vicinity of the well defined focal points, and these structures (Fig. 7G, arrowheads) were completely missing in control cells (Fig. 7H).

Regulation of Breast Cancer Invasion by Conditional Expression of K299R PAK1—To investigate if the morphological

changes found in stable transfected cell lines were not because of the long term adaptation during cell culturing, thus related to an indirect effect of transfected K299R PAK1 gene, we generated another model, MDA-MB231, in which the expression of HA-tagged K299R PAK1 protein could be conditionally regulated via a Tet-off regulatory system. The induction of K299R PAK1 (Fig. 8A) with reduction of PAK1 kinase activity in MDA-MB231 (Fig. 8C) cells was associated with a reduction of *in vitro* invasive potential (Fig. 8, panel B) and with changes in cellular shape from a spindle shape to a flatter polygonal shape (Fig. 8D, -Dox). These morphological changes were clearly evident after 24 h of kinase dead PAK1 expression and became more intense at 48 h after induction (Fig. 8D, -Dox). An important feature of this system was its reversibility (Fig. 8D, +Dox).

Similar to our findings presented before in the MDA-MB435 model, a short time induction for 48 h of K299R PAK1 in MDA-MB231 cells also resulted in two major changes that concerned (a) the actin conformation and (b) PAK-1 localization. First, these cells displayed more stress fibers and a higher number of mature focal points. Changes in F-actin conformation were accompanied by co-localization of HA staining to these cables as well as at the cell periphery (not shown). Second, when cells were plated on fibronectin, expression of K299R PAK1 induced formation of peripheral focal points and bundled actin fibers with subsequent increased cell spreading (Fig. 9, A, G, and H). This was very different from the control noninduced cells, which exhibited persistent ruffle formation (Fig. 9, D, I, and J, arrows) located close to the less mature, dynamic focal points (Fig. 9, D, E, and J, arrowheads). These dynamic actin-containing structures present in the uninduced cells (that do not express the kinase-defective PAK1) were immunoreactive to anti-PAK1 antibody. However, no detection of the PAK1 that might correspond to the focal points, could be seen in these cells. On the contrary, PAK1 immunoreactivity was changed when cells were induced to express K299R PAK1, showing a punctuate distribution at the cell periphery (Fig. 9G, arrows). Interestingly, this distribution of PAK1 on the peripheral thick

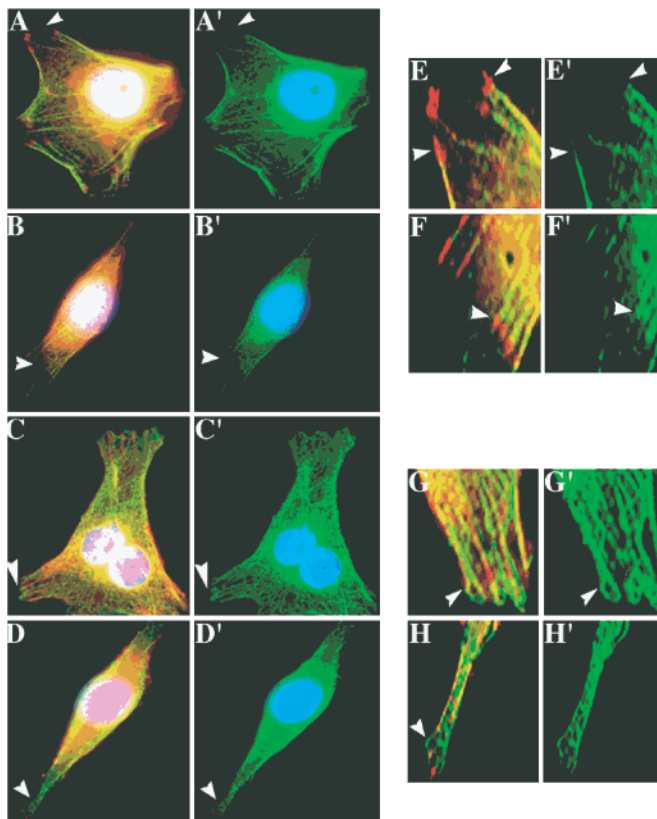


FIG. 7. K299R PAK1 mutant promotes persistent F-actin polymerization with the presence of actin-bound TM and CaD. Cells cultured on chamber slides were fixed after 12 h and co-stained for F-actin and TM (A and B) or F-actin and CaD (C and D). A separation of the two channels allows a better distinction of TM distribution (A' and B') or of CaD (C' and D'). A closer view of the region containing the focal complexes (marked by arrowheads) is shown on the right panels for TM (E, E' and F, F') or for CaD (G, G' and H, H'). Note the presence of thick actin-containing cables that are co-stained with TM or CaD in K17 clones (A and C) compared with the diffuse staining seen in vector-transfected clones (B and D) and loop-like structures around focal complexes in K17 transfectants (G, G').

actin cables was very similar to the distribution of the vinculin-containing focal points (Fig. 9H, arrowheads). Taken together, these morphological changes were very similar in both cellular systems, indicating that the increased cell spreading, changes in the actin conformation, as well as stabilization of the focal adhesion complexes may be closely linked with the inactivation of PAK1 and not with indirect cell culture adaptations.

Effect of K299R PAK1 Expression on Gene Transcription—The upstream regulators of PAK1, Rac, and Cdc42, have been shown to stimulate kinase cascades leading to activation of the JNK/stress-activated protein kinase (11). In addition, a constitutively activated form of PAK1 showed a stimulation of JNK1 activity similar to that obtained with constitutively activated RacV12 (13). Because stimulation of the AP-1 transcription factor (a downstream target of JNK) has been shown to be associated with breast cancer progression (34), we investigated the influence of a kinase dead PAK1 mutant on the JNK/AP-1 pathway. The low invasive potential of K299R PAK1 transfectants was maintained after >25 passages (Fig. 10A). As shown in Fig. 10B, overexpression of K299R in MDA-MB435 cells (K17 clone) was accompanied by a significant suppression of JNK activation as determined by subcellular localization of the phosphorylated JNK using a specific antibody that recognizes only the phosphorylated forms of JNK-1 or JNK-2. More than 70% of MDA-MB435-K17 cells displayed a low antibody immunoreactivity that was predominantly in the cytoplasmic com-

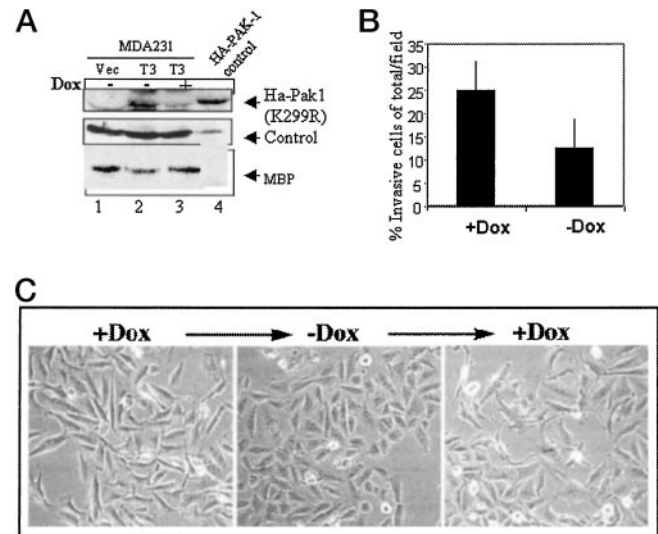


FIG. 8. The influence of K299R PAK1 expression in MDA-MB231 model system. A, expression of HA-tagged PAK1 was assayed using total cell lysates of cells grown in the absence (lane 2) or presence (lane 3) of doxycyclin. Control lysates containing the HA-tagged PAK1 protein represent the positive control (lane 4). Untransfected parental MDA-MB231 cells served as a negative control (lane 1). Corresponding *in vitro* kinase activity of PAK1 immunoprecipitates from A using myelin basic protein as a substrate. B, cells were cultured for 24 h in the absence of doxycyclin before plating them in a Boyden chamber. Cells with suppressed K299R PAK1 were plated in the same way. Results represent cell counts after 24 h in 10 different random microscopic fields of three different wells. Each experiment was repeated at least three times. C, cell morphological appearance by using low magnification phase contrast is shown in uninduced cells (left panel) followed by 48 h of K299R PAK1-induced expression (middle panel) and finally followed by another 48 h of blocked expression (right panel).

partment, compared with the control MDA-MB435-CMV cells, which displayed a strong nuclear and cytoplasmic localization of phosphorylated JNK antibody reactivity. The observed inhibition of JNK activation in K17 cells was confirmed by the inhibition of JNK kinase activity (Fig. 10C) and by the DNA binding activity of the AP-1 transcription factor, a downstream target of JNK (Fig. 10D, lane 4). This was true for the MDA-MB435-K5 clone but to a lesser extent (Fig. 10D, lane 3). To understand the function of AP-1 in the action of PAK1, we next determined whether the K299R PAK1 mutant could modulate the AP-1 site-driven transcription using a collagenase-luciferase reporter system. As illustrated in Fig. 10E, overexpression of kinase dead PAK1 resulted in a substantial reduction of luciferase activity compared with the levels present in control cells. The observed inhibition of transcription in K17 cells was because of the lack of AP-1 activation, because deletion of the AP-1 site resulted in a comparable reduction of luciferase activity. PAK1 kinase has been also implicated in the regulation of the mitogen-activated protein kinase pathway (35), the effect of K299R PAK1 on the mitogen-activated protein kinase pathway using immunoblotting with phospho-p42/44 antibody was examined. We failed to observe any difference in the activation of mitogen-activated protein kinase in K299R PAK1 expressing cells over the level of vector-transfected cells (data not shown).

DISCUSSION

Breast cancer is one of the most common malignancies in western countries. The high mortality rate associated with breast cancer, however, is related to its ability to metastasize beyond the mammary gland and invade distant sites, whereas the primary tumor is small and undetected. Thus, tumor cell migration/invasion is an important factor in the formation of

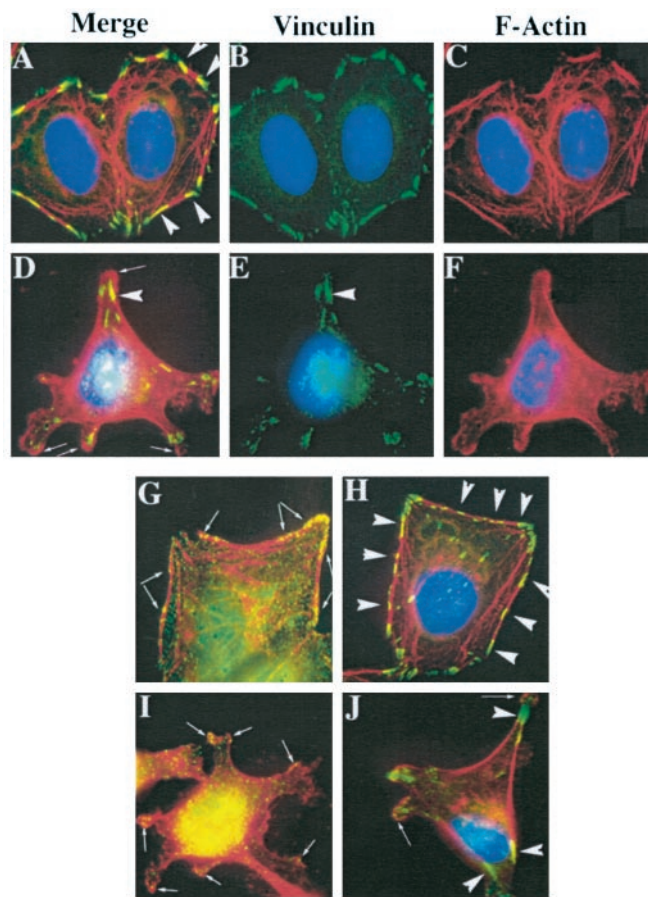


FIG. 9. K299R PAK1 expression induces focal adhesion complexes and F-actin thick cables formation and affects PAK1 distribution. Cells cultured on fibronectin-coated chamber slides were fixed after 12 h and co-stained for F-actin and vinculin (A, D, H, and J) and for F-actin and PAK1 (G and J). Representative examples of vinculin distribution in K299R-induced expression (A and H) or PAK1 (G) as well as in uninduced cells (D and J) and of PAK1 (I) are shown. Blue color, when showed, corresponds to the DAPI-stained nuclei.

solid tumors, and it is necessary for their spread to distinct organs. The progression of breast cancer cells to a more invasive phenotype is believed to be influenced by the migration of cells from the primary site of tumor, incorporating the ability of cancer cells to invade through basement membrane and reestablish themselves at distant sites. Because a successful invasion and metastasis of breast tumor is conditional for cell migration from the primary tumor, cell motility must play a pivotal role in the development and maintenance of the invasive phenotype of breast cancer cells. Therefore, understanding cell motility is likely to delineate pathways that may be an integral part of metastasis. Recently, we showed that PAK1 had a role in cell motility and invasiveness of human breast cancer cells stimulated by heregulin- β 1 (27). To better understand the role of PAK1 in the reorganization of cytoskeleton components that control motility and *in vitro* invasiveness of cells, here we investigated the influence of inhibition of PAK1 activity on the modulation of invasive phenotypes in breast cancer cells. We utilized two distinct highly invasive breast cancer cell model systems, one involving stable overexpression of kinase dead K299R PAK1 in MDA-MB435 cells and another where there is a conditional regulation of the expression of K299R PAK1 in MDA-MB231 cells.

The results presented here indicate that overexpression of kinase dead PAK1 in invasive breast cancer cells leads to significant morphological changes and reduction of *in vitro*

invasiveness. Our conclusion that suppression of PAK1 activity modulated the invasion of breast cancer cells was supported by the following lines of evidence: (i) expression of K299R PAK1 mutant blocked the transmigration of cells across a porous membrane, (ii) K299R PAK1 mutant blocked the formation of ruffles and filopodia induced by other stimuli, (iii) inhibition of PAK1 by K299R was accompanied by persistent cell spreading and an increase in the number and maturity of the focal complexes, (iv) K299R expression resulted in the appearance of thicker actin cables with prolonged co-localization of tropomyosin or caldesmon, and (v) the K299R PAK1-mediated blockade of ruffles and filopodia formation and of migration could not be reversed by HRG. Taken together, these data provide evidence that PAK1-regulated reorganization of cytoskeleton structures may be required to maintain the invasive phenotype of breast cancer cells.

Among the various pathways leading to actin reorganization, the Rho subfamily of the small GTPases, cdc42 and Rac1, has been implicated in the regulation of morphology and motility of mammalian cells. Recent studies have identified the PAKs as a target of activated GTPases, as activation of PAK1 has been shown to result in the phenotypic changes reminiscent of those produced by Rac and/or cdc42 (36). Furthermore, a role of PAK in promoting cell migration has been also suggested by the physical interaction between PAK1 and actin in response to upstream stimuli (7, 27). The propensity of malignant breast cancer cells to invade must depend on their ability to migrate from the primary tumor site. Our observation that kinase dead PAK1 predominantly localizes to the fairly stable focal points supports a role for PAK1 in promoting cell motility, probably by its known function to dissolve the focal adhesions (31). However, because the K299R kinase dead PAK1 mutant still contains binding sites for Rac/cdc42, which are known to promote phenotypic changes similar to those caused by PAK1, it is possible that the observed effects could have been reflections of ineffective transduction of Rac/cdc42 signals. However, transfection of double PAK1 mutant H83, 86L, K299R (which has mutation in CRIB site and PAK1 kinase domain) also demonstrated increased actin bundling, cell spreading, and stabilization of focal points. Furthermore, expression of the PAK1 autoinhibitory domain (PAK 83–149) also exhibited changes in cell shape (to a lesser degree) similar to K299R PAK1. Taken together, these results indicated that PAK1 kinase activity is involved in the cell shape changes, increased focal points and F-actin bundling in MDA-MB-435 cells. These findings suggest a role of PAK1 kinase activity in the motility of human epithelial cancer cells.

Recently, kinase active PAK1 has been shown to cause excessive dissolution of focal adhesions and stress fibers (9, 31) and directional haptotaxis in fibroblasts (8). Another notable finding in this study was the essential role of PAK1 in actin dynamics, as catalytically inactive PAK1 may impair the formation of dynamic actin-containing structures necessary for cell migration and the formation of the focal points. Some of the possible mechanisms for the observed decreased migration potential may include a change in F-actin conformation induced by increasing the actin bundling or stability or blocking the F-actin capping. Caldesmon and tropomyosin are two major actin-binding proteins that can modulate the stability of actin filament (32). It has been shown that caldesmon coupled with tropomyosin not only blocks the binding of human fascin to actin (37) but also dissociates fascin from actin (38), implying that caldesmon together with tropomyosin regulates the assembly of actin bundles. There is also an increasing body of evidence showing that caldesmon stabilizes microfilaments in nonmuscle cells (33), probably by increasing actin binding of

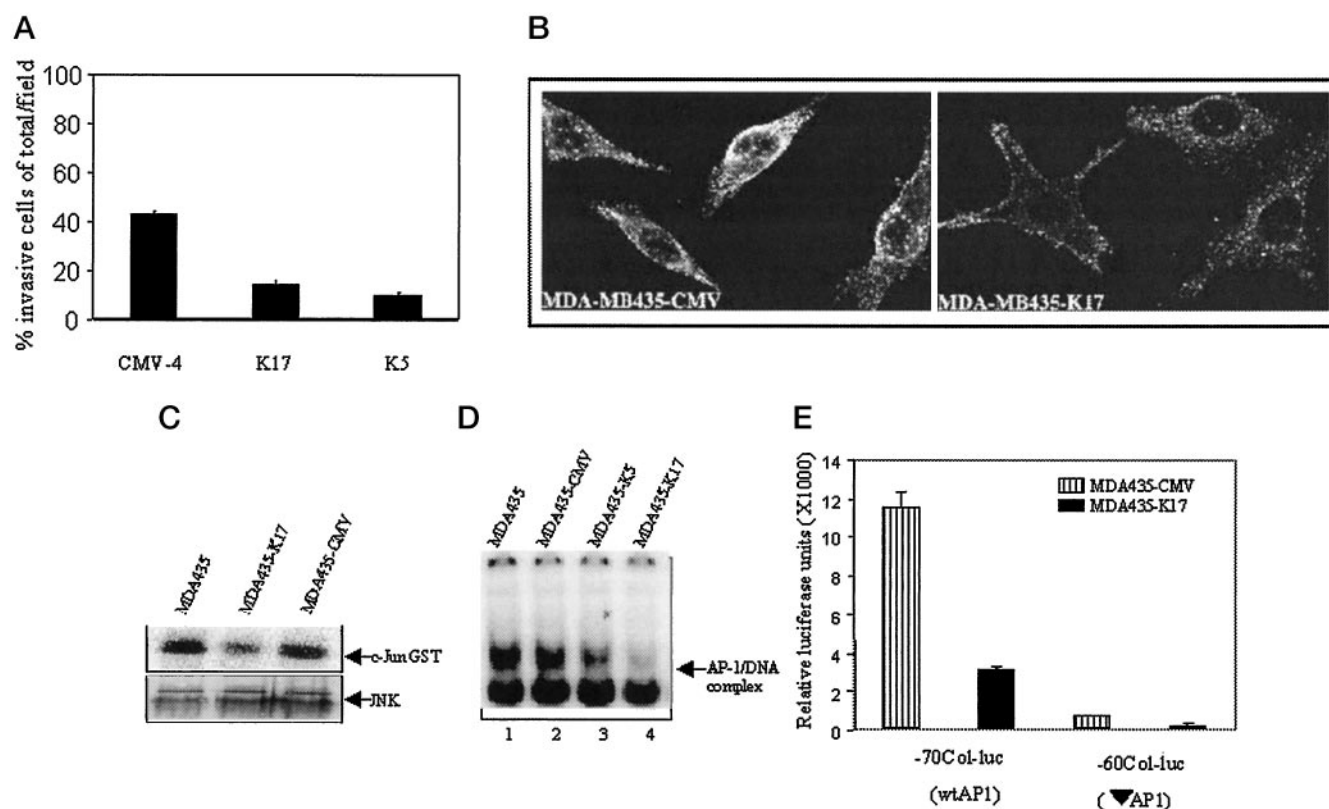


FIG. 10. Effect of K299R PAK1 expression on JNK/AP-1 pathway. *A*, invasive potential of two K299R PAK1-expressing clones (K17 and K5) compared with mock-transfected cells (CMV-4). *B*, MDA-MB435 expressing the K299R PAK1 or mock-transfected cells were plated in chamber slides, stained for the phosphorylated form of JNK-1 and -2, and analyzed by confocal microscopy. Representative intracytoplasmic and nuclear distribution for MDA-MB435-CMV (*left panel*) or faint intracytoplasmic for MDA-MB435-K17 (*right panel*) is shown. *C*, JNK immunoprecipitates from total cell lysates were assayed for the JNK kinase activity by using c-Jun-glutathione *S*-transferase as a substrate. *D*, cell nuclear extracts from two controls (MDAMB435 and MDAMB-CMV) or two K299R PAK1-expressing cell lines (K5 and K17) were incubated with ³²P-labeled AP-1 DNA probe, and the AP-1 binding activity was measured by electrophoretic mobility shift assay. *E*, MDA-MB435-CMV and MDA-MB435-K17 were transiently transfected with a -70 collagenase promoter containing a functional AP-1 binding site (*wtAP-1*) and a -60 collagenase promoter containing one deletion of the AP-1 binding site (Δ AP-1) and assayed for the luciferase activity. The results, corrected for the transfection efficiency and for protein levels, represent the relative luciferase units.

tropomyosin (39, 40). In nonmuscle cells, caldesmon, an actin-binding protein, may have important regulatory functions in cell motility, analogous to those suggested for the regulation of smooth muscle contraction. Dissociation of caldesmon from microfilaments may destabilize the structure of microfilaments. Something similar may happen during cell motility where there is a dramatic cytoskeleton rearrangement of the stress fibers connecting the cells in the focal points (attachment and spreading) with subsequent dissolution and reformation of more dynamic actin-containing structures such as ruffles and lamellipodia. It remains to be established how PAK1 contributes to the interaction of the actin with tropomyosin and caldesmon. Several substrates have been recently found to be phosphorylated by different members of the PAK family with functional impact on the contraction properties of actin filaments. More contraction of actin filaments (stress fibers) causes less motility in nonmuscle cells (41). Recent studies have shown that PAK3 is able to phosphorylate caldesmon independently of the induction of muscle contraction (42). Evidence also suggests that myosin light chain kinase binds to cellular actin filaments and is localized to actinomyosin-containing stress fibers in nonmuscle cells. In a very elegant study on smooth muscle cells that used GFP-labeled myosin light chain kinase the filament-bound kinase was shown to be sufficient for Ca²⁺-dependent phosphorylation of myosin regulatory light chain and contraction of stress fiber (43). One of the recently identified PAK1 substrates is indeed myosin light chain kinase, and its phosphorylation by PAK1 *in vitro* and *in*

in vivo was shown to decrease myosin light chain activity, leading to a decrease in cell spreading and thus less actin-myosin contraction (44). Our finding that the kinase dead mutant K299R PAK1 increased the cell spreading and the appearance of stress fibers suggests a possible role of PAK1 in microfilaments remodeling via the myosin light chain kinase pathway. However our results do not distinguish if these effects are because of the increase in actin-myosin contraction, stabilization of actin by actin-binding proteins, or both. Future studies will be needed to determine the precise nature of interplay between Rac, cdc42, and RhoA effectors in these processes.

The evidence that overexpression of the kinase dead K299R PAK1 mutant in breast cancer cells has the capacity to inhibit the DNA binding activity and of AP-1-dependent transcription *in vivo* is of special interest, as it strongly suggests that PAK1-induced signaling may modulate the expression of genes, such as matrix metalloproteinases (45), which are relevant for invasion via the AP-1-dependent pathway. This hypothesis is further supported by our preliminary studies showing a reduction of gelatinolytic activity in the conditioned medium that had K299R PAK1-overexpressing MDA-MB435 cells (data not shown). Because PAK1 is considered to be an activator of JNK and p38 MAPK (46), it is possible that the JNK/p38 downstream events influence the expression of gene products with functions in motility/invasion of breast cancer cells. Taken together, our findings of regulation of actin reorganization, cell migration and invasion, and gene regulation functions of breast cancer cells by PAK1 open a new avenue of investigation closely

linking PAK1, cytoskeleton-signaling, transcription of genes involved in metastasis, and breast cancer invasiveness. In addition, our present finding may lead to strategies targeting PAK1 as a potential inhibitor of invasion and metastasis of breast cancer cells.

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**Regulation of Microfilament Reorganization and Invasiveness of Breast Cancer Cells
by Kinase Dead p21-activated Kinase-1**

Liana Adam, Ratna Vadlamudi, Mahitosh Mandal, Jonathan Chernoff and Rakesh Kumar

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