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Regulatable Expression of p21-activated Kinase-1 Promotes Anchorage-independent Growth and Abnormal Organization of Mitotic Spindles in Human Epithelial Breast Cancer Cells*

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Stimulation of growth factor signaling has been implicated in the development of invasive phenotypes and the activation of p21-activated kinase (Pak1) in human breast cancer cells (Adam, L., Vadlamudi, R., Kondapaka, S. B., Chernoff, J., Mendelsohn, J., and Kumar, R. (1998) *J. Biol. Chem.* 273, 28238–28246; Adam, L., Vadlamudi, R., Mandal, M., Chernoff, J., and Kumar, R. (2000) *J. Biol. Chem.* 275, 12041–12050). To study the role of Pak1 in the regulation of motility and growth of breast epithelial cells, we developed human epithelial MCF-7 clones that overexpressed the kinase-active T423E Pak1 mutant under an inducible tetracycline promoter or that stably expressed the kinase-active H83L,H86L Pak1 mutant, which is deficient in small GTPase binding sites. The expression of both T423E and H83L,H86L Pak1 mutants in breast epithelial cells was accompanied by increased cell motility without any apparent effect on the growth rate of cells. The T423E Pak1 mutant was primarily localized to filopodia, and the H83L,H86L Pak1 mutant was primarily localized to ruffles. Cells expressing T423E Pak1 exhibited a regulatable stimulation of mitogen-activated protein kinase and Jun N-terminal kinase activities. The expression of kinase-active Pak1 mutants significantly stimulated anchorage-independent growth of cells in soft agar in a preferential mitogen-activated protein kinase-sensitive manner. In addition, regulatable expression of kinase-active Pak1 resulted in an abnormal organization of mitotic spindles characterized by appearance of multiple spindle orientations. We also provide evidence to suggest a close correlation between the status of Pak1 kinase activity and base-line invasiveness of human breast cancer cells and breast tumor grades. This study is the first demonstration of Pak1 regulation of anchorage-independent growth, potential Pak1 regulation of invasiveness, and abnormal organization of mitotic spindles of human epithelial breast cancer cells.

Breast cancer is one of the most common malignancies in the United States, affecting one in nine women. Overexpression of the human epidermal growth factor receptor-2 (HER 2,[1] also known as c-ErbB2) is associated with increased progression and metastasis, an aggressive clinical course, and decreased disease-free survival in human breast cancer patients (1). Accumulating evidence suggests that in addition to HER 2 overexpression, the heregulin (HRG; a combinatorial ligand for HER 3, HER 4) pathway is involved in the progression of breast cancer cells to a more invasive phenotype (2–5). Exposure of cells to growth factors induces cytoskeleton reorganization, lamellipodia formation, and membrane ruffling; such changes contribute to increased cell migration and invasion (3, 6). Members of Rho family of the small GTPases Rac1, Cdc42, and RhoA have been implicated in the regulation of cytoskeletal rearrangements; RhoA is involved in the maintenance of actin stress-fibers and focal adhesion points; Rac1 in the formation of lamellipodia and membrane ruffles (7); and Cdc42 in the formation of peripheral actin microspikes and filopodia (8). The small GTPases also regulate gene expression: Cdc42 and Rac1 activate Jun N-terminal kinase and p38 mitogen-activated protein kinase (p38 MAPK) pathways (9). Thus GTPases Rac1, Cdc42, and Rho are implicated in cellular transformation (10, 11). Cdc42 and Rac1 family members also activate extracellular signal-regulated protein kinases and ternary-complex factor (12). The signaling pathway by which the small GTPases regulate their diverse cellular functions is an evolving area of investigation.

In mammalian cells, p21-activated kinases (Paks) are identified as one target of the small GTPases Cdc42 and Rac1, and binding of GTP-bound GTPases to Pak1 stimulates its kinase activity via autophosphorylation (13). Expression of the activated T423E Pak1 mutant triggers the formation of lamellipodia, dissolution of stress fibers, and dissolution of focal adhesion complexes in fibroblast cells (14, 15). Expression of another kinase-active Pak1 mutant with a mutation in GTPase binding sites, H83L,H86L, supports the formation of actin ruffles (16).

The Pak1 contains five potential SH3 domains (16, 17). In addition to kinase activity, SH3 domains of Pak1 have been implicated in cytoskeletal changes and Pak1 localization, probably via their interaction with adapter molecules such as Nck, guanine nucleotide factor PIX, and paxillin (18–20). Pak1 kinase activity is essential for the formation of polarized lamellipodia at the leading edge (21) and for actin-myosin-mediated

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[1] The abbreviations used are: HER, human *e*pidermal growth factor *r*eceptor; HRG, heregulin β-1; Pak, p21-activated kinase; MAPK, mitogen-activated protein kinase; DMEM, Dulbecco's modified Eagle's medium; Dox, doxycycline; HA, hemagglutinin.

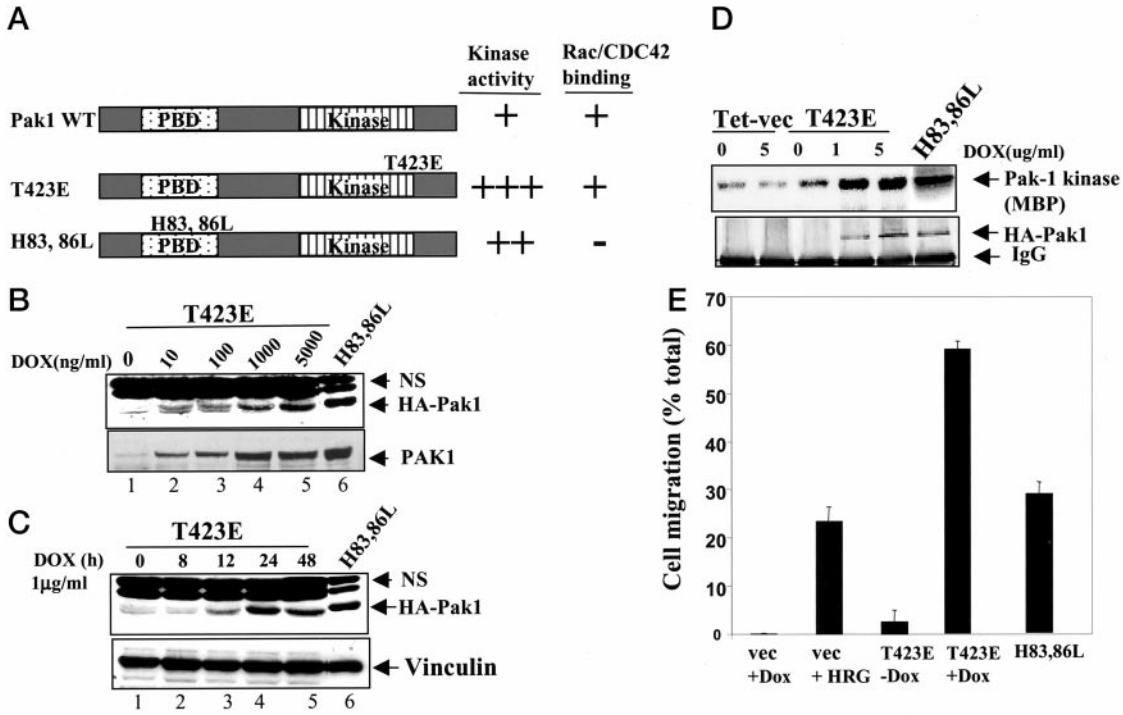

FIG. 1. **Characterization of kinase-active Pak1-expressing cell lines.** *A*, schematic representation of Pak1 mutants (T423E and H83L,H86L) used in the study. *PBD*, p21 GTPase binding domain. *B*, T423E-expressing MCF-7 cells were treated with increasing concentrations of Dox, and induction of T423E Pak1 was analyzed by immunoblotting with HA mAb. Lysates from stable clones expressing HA-H83L,H86L Pak1 were loaded in the *last lane*. A duplicate set of blots was immunoblotted with a Pak1 antibody. *C*, kinetics of HA-Pak1 expression was analyzed by treating cells at various time points with 1 μg/ml of Dox and analyzed by Western blot using a mouse anti-HA mAb. The blot was reprobed with an anti-vinculin antibody as a loading control. *D*, an equal amount of protein from serum-starved cells was immunoprecipitated with an anti-HA antibody, and kinase activity of exogenously expressed Pak1 mutants was analyzed by *in vitro* kinase assay using myelin basic protein as a substrate. *E*, migration of T423E- and H83L,H86L-expressing clones was analyzed using a modified Boyden chamber as described under "Materials and Methods." Results shown are representative of three separate experiments.

cytoskeletal changes involving myosin light chain phosphorylation (22). Pak1 also activates a number of signaling pathways, including p38 MAPK, extracellular signal-regulated protein kinase, Jun N-terminal kinase, and NF-κB (12, 23). Expression of the kinase-dead K299R Pak1 mutant blocks the transformation of Rat1 fibroblast by Ras (24) and cooperative extracellular signal-regulated protein kinase activation by Ras, Rho, and Rac1 GTPases, suggesting that Pak1 plays a role in the cell transformation and extracellular signal-regulated protein kinase activation (25).

Treatment of noninvasive human breast epithelial MCF-7 cells with HRG induces Pak1 kinase activity and motility (4). To further understand the role of Pak1 in the regulation of motility and growth of breast epithelial cells, we established stable breast cancer cell lines expressing T423E and H83L,H86L Pak1 mutants. The expression of kinase-active Pak1 mutants was accompanied by a significant stimulation of motility, invasiveness, and anchorage-independent growth of epithelial cells. The Pak1-linked enhanced ability of cells to grow in soft agar was preferentially sensitive to a specific MAPK inhibitor compared with p38 MAPK inhibitor, implying that Pak1 regulates anchorage-independent growth of epithelial cells via a MAPK kinase pathway.

MATERIALS AND METHODS

Cell Cultures and Reagents—MCF-7 human breast cancer cells (4) were maintained in DMEM/F-12 (1:1) supplemented with 10% fetal calf serum. Antibodies were purchased from the following companies: Pak1 from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); Vinculin from Sigma; anti-HA from Roche Molecular Biochemicals; phospho-MEK1, phospho-p42/44 MAPK, and phospho-p38 MAPK from New England Biolabs. Inhibitors SB203580 and PD980599 were purchased from Calbiochem.

Constructs and Production of Stable Cell Lines—MCF-7 cells were sequentially transfected with pTak (Teton vector, CLONTECH) plus pNeomycin plasmid (pNeo) and pTet-Splice-HA-Pak1 (T423E) (21) and hygromycin plasmid (pHyg, CLONTECH) using the calcium phosphate method. Forty-eight hours post transfection, cells were selected in medium containing 1000 μg/ml G418 (to retain the tetracycline-VP16 transactivator) and 200 μg/ml hygromycin (to select for the T423E Pak1-regulated expression vector). Stable cells expressing H83L,H86L Pak1 were transfected and selected with hygromycin. Expression of Pak1 mutants was verified by immunoblotting using anti-HA mAb.

Cell Migration and Immunofluorescence Assays—The cell migration assays were performed using the Boyden chambers using a confocal microscope (4). T423E cells were induced for 24 h with doxycycline (1 μg/ml) in DMEM containing 10% serum. Cells grown in the absence of doxycycline were used as control. After a short treatment with trypsin, the cells were washed, resuspended in DMEM/F-12 plus 0.1% bovine serum albumin in the presence or absence of doxycycline, 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 chemoattractant (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). The percentage of migrating cells compared with the total number of cells was recorded and represents the mean ± S.E. of triplicate wells from three separate experiments. For co-staining of filamentous actin and HA-tagged Pak1, 0.1 μM Alexa 546-conjugated phalloidin was included during incubation with the fluorescein isothiocyanate-goat anti-mouse secondary antibody. Slides were mounted and analyzed by a Zeiss-LSM inverted microscope or by confocal microscopy.

Cell Proliferation and Soft Agar Assays—Cell proliferation assays were performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye method as described (4). Colony growth assays were performed as described previously (26). Briefly 1 ml of solution of 0.6% Difco agar in DMEM supplemented with 10% FBS with insulin was layered onto 60 × 15-mm tissue culture plates. MCF-7 cell (10,000 cells) were mixed with 1 ml of 0.36% Bactoagar solution in

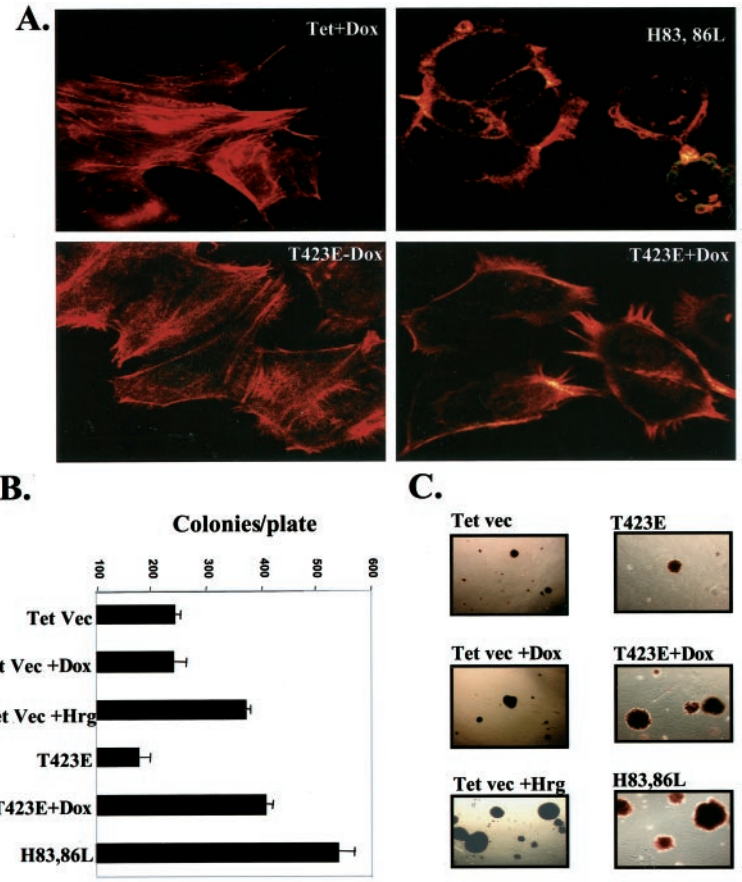

FIG. 2. *A*, Localization of kinase-active Pak1 mutants. Vector-transfected or active Pak1-expressing cells were co-stained with Alexa 546-phalloidin to visualize F-actin-containing structures and with an anti-HA mAb to visualize the localization of active HA-tagged Pak1 mutants. *Yellow* indicates colocalization between the F-actin and Ha-tagged Pak1 protein (*arrows*). T423E cells were treated with or without doxycycline for 24 h to induce the expression of Pak1 mutant. T423E Pak1 cells exhibited numerous F-actin-containing filopodia (*T423E + Dox*), and H83L,H86L Pak1-expressing cells exhibited extensive membrane ruffling as compared with control Tet vector-transfected cells. The *arrows* indicate co-localization of HA-tagged Pak1 with F-actin. Similar results were obtained in three separate experiments. *B*, effect of kinase-active Pak1 expression on anchorage-independent growth of MCF-7 cells. Anchorage-independent growth potential of the active Pak1-expressing clones was measured by their ability to form colonies on soft agar. HRG treatment was used as a positive control. Similar results were seen on four independent experiments. *Error bars* represent S.E. *C*, representative photographs of soft agar colonies. Results shown are representative of four independent experiments.

DMEM prepared in a similar manner and layered on top of the 0.6% Bactoagar layer. Plates were incubated at 37 °C in 5% CO_2 for 21 days. For cells expressing T423 Pak1, doxycycline (1 μg/ml) was added where indicated. As a positive control, HRG 10 ng/ml was also included in the medium. When indicated, some cultures were treated with MEK1/2 inhibitor PD980599 (10 μM) and p38 MAPK inhibitor SB203580 (10 μM).

Biochemical Assays—We have prepared the cell extracts and performed immunoprecipitation, immunoblotting, kinase reactions, and DNA-binding gel shift assays following the methods described by us earlier (3–6).

Immunohistochemistry—Nine cases of 10% formalin-fixed, paraffin-embedded human breast carcinoma samples was processed for peroxidase anti-peroxidase immunohistochemical staining to reveal the Pak-1 expression. Briefly, the sections were deparaffinized with xylene and rehydrated through graded ethanol. The sections were then incubated with rabbit-anti-Pak-1 (1:50 dilution; Santa Cruz Biotechnology) for 2 h, goat anti-rabbit IgG (1:100 dilution, Sigma) for 1 h, and rabbit peroxidase anti-peroxidase (1:200 dilution, Sigma) for 1 h at room temperature. The sections were washed three times with PBS after each incubation. Finally, the staining was visualized with diaminobenzidine-H_2O_2 and counterstained with hematoxylin. For specificity control, the sections were stained with the same concentration of normal rabbit IgG in place of the primary antibody.

RESULTS AND DISCUSSION

Characterization of Inducible Expression of Kinase-active T423E Pak1 in Epithelial Cancer Cells—To study the influence of Pak1 kinase on the biology of epithelial breast cancer cells, we established stable cell lines with inducible expression of the activated form of Pak1 under the control of the tetracycline-regulated promoter. We used the T423E Pak1 mutant, which behaves as activated Pak1 kinase but retains its ability to bind Cdc42 and Rac1 (16, 21). We also used another stable clone expressing the H83L,H86L Pak1 mutant, which lacks the ability to bind Cdc42/Rac1 but behaves as an activated Pak1 kinase (16, 21). These Pak1 mutants allowed us to differentiate the effects that were caused by sequestering of Cdc42/Rac1, Pak1 kinase activity, or both.

To induce the expression of T423E Pak1, MCF-7 cells stable transfected with Tet-T423E plasmid were treated with doxycycline for various time intervals or with different doses of doxycycline (Dox), and T423E Pak1 expression was analyzed by Western blotting analysis using an anti-HA tag monoclonal antibody. Doxycycline induced expression of T423E Pak1 protein in a dose- and time-dependent manner (Figs. 1, *B* and *C*). The identity of the induced band as T423E Pak1 was also confirmed by immunoblotting with an anti-Pak1 antibody. The levels of induced expression of T423E Pak1 were 3–5 times those of endogenous Pak1. To determine if the expressed protein was functional and retained its kinase activity, HA-tagged T423E Pak1 was immunoprecipitated from cells grown in serum-free conditions with an anti-HA mAb, and exogenously expressed Pak1 kinase activity was measured by *in vitro* kinase assay. Myelin basic protein was used as a substrate, and signal was quantitated by a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). As shown in Fig. 1*D*, Pak1 kinase activity was also induced by doxycycline in a dose-dependent manner. For subsequent studies, we used a 1 μg/ml dose of doxycycline, which could effectively induce about 3-fold induction of T423E Pak1 protein and its kinase activity as compared with endogenous Pak1 levels. The level of expression of H83L,H86L Pak1 in stable clones was 5–6-fold that of vector-transfected control cells. The kinase activity of H83L,H86L Pak1, however, was lower than that of T423E Pak1 cells, taking into account the 3-fold lower expression of T423E Pak1 (Fig. 1*D*, compare *lanes 4* and *6*).

We next analyzed the influence of Pak1 expression on the migration of MCF-7 cells using Boyden chamber assay. Vector-transfected cells showed low motility (Fig. 1*E*). In contrast,

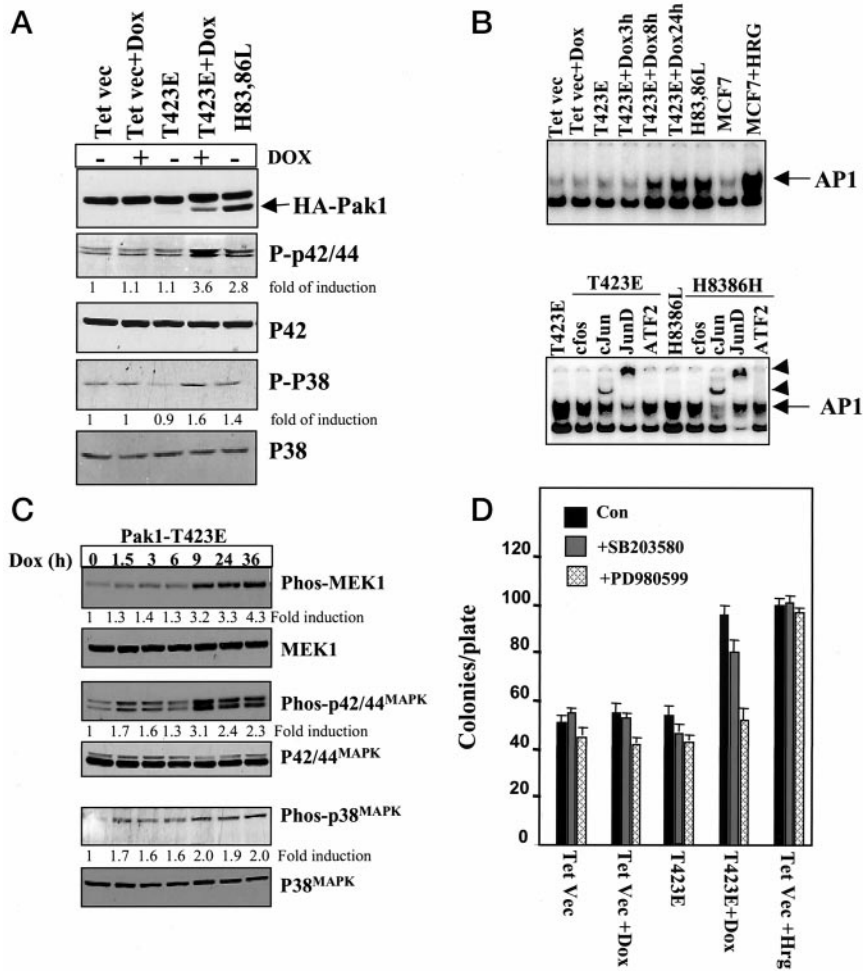

FIG. 3. **Signaling pathways activated by T423E and H83L,H86L Pak1 mutants.** MCF-7 cells expressing either vector or Pak1 mutants were serum-starved for 24 h and induced to express T423E Pak1 by treating cells with doxycycline (1 μg/ml) for 24 h. *A*, activation of signaling molecules was measured by Western blotting using phosphospecific antibodies. *B*, AP-1 binding activity was measured as a measure of activation of JNK pathway by electrophoretic mobility shift assay (*upper panel*) and Supershift (*arrowheads*) assays with various antibodies (*bottom panel*). *C*, kinetics of activation of MEK1 and 42/44 MAPK kinases by induction of T423E. Results shown are representative of three separate experiments with similar findings. -Fold induction over control was calculated using the Sigma Scan program. *D*, MEK1 inhibitor PD98059 significantly reduced active Pak1-mediated anchorage-independent growth. Soft agar colony assays were performed in the presence of p42/44 MAPK pathway inhibitor (PD98059) and p38 MAPK kinase pathway inhibitor (SB203580). Treatment with HRG was used as a positive control. Results shown are representative of three independent experiments.

induction of T423E Pak1 with doxycycline resulted in a significant increase in cell motility. HRG treatment was used as a positive control. H83L,H86L Pak1 mutant also induced motility of epithelial cells to a level similar to that induced with HRG treatment. Recently, it was shown that kinase activity affects directional cell movement (21). Although both active mutants (T423E Pak1 and H83L,H86L Pak1) stimulated cell migration, the higher activity of T423E suggests that kinase activity and its localization and interaction with other proteins also play a role in increasing the migratory potential of MCF-7 cells.

Localization of T423E Pak1 and H83L,H86L Pak1—The subcellular localization of active Pak1 mutants was visualized by immunofluorescence staining for tagged HA and actin. MCF-7 cells expressing T423E and H83L,H86L Pak1 exhibited distinct actin localization. Cells expressing T423E Pak1 showed the presence of filopodia, and HA-Pak1 was predominantly localized at the cell periphery that corresponds to the filopodia structures (Fig. 2*A*). Cells expressing T423E Pak1 showed very few stress fiber compared with vector-transfected cells (Fig. 2*A*) and exhibited scattered phenotype. Thirty to forty percent of cells expressing T423E Pak1 generated a leading edge reminiscent of motile phenotype. In contrast, cells expressing H83L,H86L Pak1 showed lower levels of filopodia or stress fibers but exhibited extensive membrane ruffling to which HA-Pak1 staining was localized. Formation and induction of actin-containing structures, including filopodia, ruffles, and leading edge, and dissolution of stress fibers by active kinase mutants, which in turn loosen their contacts with the substratum, may provide an advantage for the cells and may have contributed to the increased migration observed in Boyden chamber assays (Fig. 2*A*).

Increased Anchorage-independent Growth of MCF-7 Cells Expressing Kinase-active Pak1 Mutants—Kinase-deficient Pak1 can suppress transformation that is mediated by Ras, Rho, and Rac1 (24, 25) in Rat1 cells but not in NIH3T3 cells, suggesting that the transformation-blocking function of Pak1 depends on the cellular context. To examine the potential influence of regulatable expression of kinase-active Pak1 on the growth characteristics of breast epithelial cancer cells, we measured the growth rate and ability of cells to grow in an anchorage-independent manner. Expression of kinase-active Pak1 mutants had very little or no significant effect on the growth rate of MCF-7 cells on plastic (data not shown). Kinase-active Pak1 mutants did, however, significantly enhance the ability of MCF-7 cells to form colonies on soft agar (Fig. 2*B*). There was no difference in the number or size of colonies between vector-transfected control cells and uninduced T423E-expressing cells. Doxycycline-mediated increase in the level of expression of T423E Pak1 was accompanied by a significant reproducible enhancement of the ability of cells to form colonies in soft agar comparable with that of the cells treated with HRG as a positive control. Cells expressing H83L,H86L Pak1 also exhibited a profound increase in ability to grow in an anchorage-independent manner (Fig. 2*B*). This finding contradicts an earlier observation (24) showing the inability of H83L,H86L Pak1 in Rat1 cells to form colonies in soft agar. It is possible that these different results reflect the use of fibroblast *versus* epithelial cells in these two studies.

Regulation of MAPK Signaling Pathways by T423E Pak1—To study the biochemical basis of the increased ability of MCF-7 cells to form colonies in soft agar, we analyzed the signaling pathways activated in cells expressing T423E Pak1.

We used doxycycline to induce the expression of T423E Pak1 and analyzed the activation status of the signaling components by blotting with phosphospecific antibodies. Both T423E and H83L,H86L Pak1 mutants demonstrated increased p42/44 MAPK activity (2–4-fold) over vector-transfected cells. T423E Pak1 mutant demonstrated a modest increase in the level of p38 MAPK (1.6–2-fold, Fig. 3*A*). Both Pak1 mutants exhibited increased AP-1-DNA binding activity, which was determined by electrophoretic mobility shift assay (Fig. 3*B*, *upper panel*). Results of supershift experiments using specific antibodies that recognize specific components of the AP-1 complex suggested that the increased AP-1 DNA binding activity was caused primarily by c-Jun and JunD transcription factors (Fig. 3*B*, *lower panel*). The exhibition of similar patterns of super shifts of AP-1 DNA complex by both Pak1 mutants suggested the possibility of regulation of c-Jun and JunD transcription factors by the Pak1 pathway. To further characterize the regulation of the p42/44 MAPK pathway by Pak1, we used doxycycline to induce T423E Pak1 expression in MCF-7 as a function of time. Consistent with the earlier results, p42/44 MAPK and its upstream kinase MEK1/2 were activated in a time-dependent manner by the induction of Pak1 (Fig. 3*C*). When vector-transfected cells were exposed to doxycycline, the p42/44 MAPK pathway was not induced (data not shown).

To address the possibility that activation of p42/44 MAPK contributed to the anchorage-independent growth by active Pak1 mutants, the soft agar experiment was repeated in the presence or absence of PD98059, a specific MAPK inhibitor, or SB203580, a specific p38 MAPK inhibitor. As illustrated in Fig. 3*D*, inclusion of a nontoxic dose of PD98059 blocked the T423E Pak1-mediated increase in anchorage-independent growth; inclusion of SB203580 had a small inhibitory effect compared with PD98059. Nonspecific inhibitory effects of PD98059 did not cause the observed blockade effect of PD98059 on anchorage-independent growth, because it failed to suppress the anchorage-independent growth supported by HRG treatment (Fig. 3*D*). These results suggest that Pak1 plays a role in activating p42/44 MAPK. Although it is well accepted that Pak1 activates Jun N-terminal kinase and p38 MAPK pathways, there are contradictory reports on whether Pak1 activates p42/44 MAPK (12, 23, 27). The use of different cell systems or the use of transient and inducible systems could have caused the variation between the earlier reports and the results presented in this study. These results suggest that p42/44 MAPK may have a possible preferential role of supporting the anchorage-independent growth of cells expressing kinase-active T423E Pak1.

Active Pak1 Expression Leads to an Abnormal Organization of Mitotic Spindles—Earlier studies with Pak homologues in *Saccharomyces cervisiae* (Ste20) showed that Pak homologues have a role in cytokinesis and in mitosis (28, 29). To determine whether extended expression of the mammalian Pak1 homologue affected cell cycle progression, we induced expression of kinase-active Pak1 by treating MCF-7 cells expressing T423E for 72 h with doxycycline. Cell cycle progression was analyzed by fluorescence-activated cell sorting analysis, and there were no significant differences between the cell cycle progression of these treated cells and that of the control vector-transfected cells (data not shown). We next examined whether prolonged activation of Pak1 could influence the organization of mitotic spindles and thus possibly contribute to anchorage-independent growth via modifying the status of DNA ploidy and genomic instability. To explore this possibility, MCF-7 cells expressing Tet vector or T423E Pak1 were treated with doxycycline for 72 h to induce the expression of active Pak1. Cells were costained with monoclonal antibody against β-tubulin (*green*) to mark spindles and polyclonal antibody against the BTAK protein (*red*) to localize the centromere. The cells were analyzed by confocal microscopy. As shown in Fig. 4, cells expressing activated Pak1 exhibited multiple spindles with several orientations and centromere spots (*arrows*); vector-transfected cells did not show multiple spindles. For quantitation, we scored 40 mitotic cells per field in eight different fields (× 20) and counted cells exhibiting multiple spindles by analyzing each of them at a higher magnification. Induction of kinase-active T423E Pak1 for 72 h resulted in the appearance of multiple spindles in 11 ± 1% of mitotic cells compared with <2% in vector-transfected cells or T423E cells without doxycycline. The multiple spindles in T423E Pak1-expressing MCF-7 cells could have been caused by defects in cytokinesis, but we could not differentiate this possibility in the present study. Additional studies are needed to delineate the potential role of Pak1 in mitosis, including the effect of abnormal Pak activation on cellular events responsible for segregation of mitotic spindles.

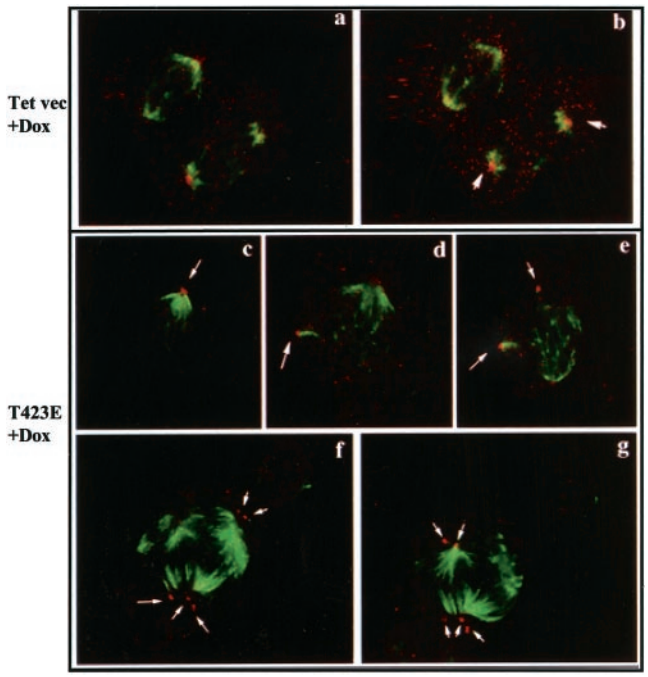

FIG. 4. **Prolonged expression of Pak1 leads to appearance of multiple spindles.** MCF-7 cells expressing Tet vector (*a* and *b*) or Tet-T423E construct (*c–g*) were treated with Dox for 72 h. Cells were co-stained with anti-β1-tubulin mAb (*green*) and anti-BTAK antibody (*red*) and analyzed by confocal microscopy. Two vector-transfected cells are shown: one in metaphase (*upper cell*) and one in anaphase (*lower cell*) sectioned at one 400-nm interval (*a* and *b*). Note the typical localization of BTAK at the level of each spindle pole and a quasiplanar distribution of the symmetric spindles. By contrast, cells expressing the active T423E Pak1 showed three (*c–e*) or multiple (*f* and *g*) spindles that form different angles between them (one antero-superior, one latero-posterior, and one supero-posterior (*d*). Multiple spindles with different orientations are shown in another example of the effect of T423E Pak-1 induction (*f* and *g*).

Pak Expression and Breast Cancer—Since HRG activates Pak1 activity and breast cancer progression (3–6), we hypothesized that Pak1 expression and activity may be closely associated with the invasive phenotypes of breast cancer cells. To explore this possibility, we analyzed the level of Pak1 expression and activity in small numbers of grade II and grade III breast tumor biopsies. As shown in Fig. 5*A*, there was a significant increase in the expression and kinase activity of Pak1 in grade III tumors, as compared with grade II tumors (4). Interestingly, increased expression of another band at 55 kDa was also observed in grade III tumors. Since Pak1 antiserum is also

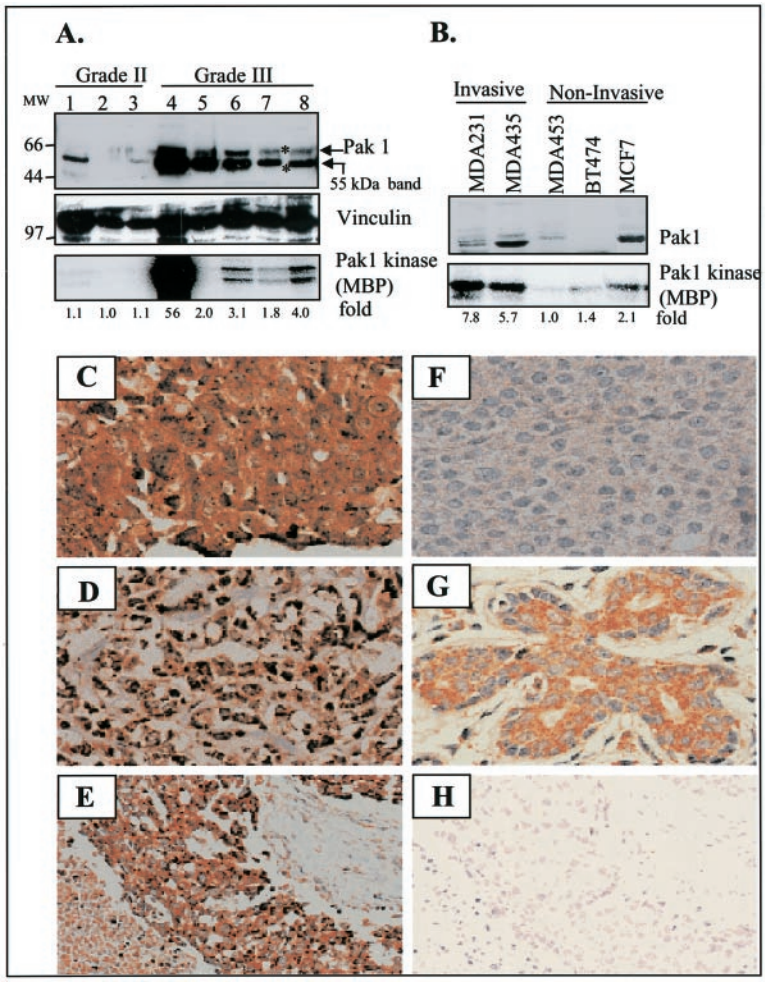

FIG. 5. **Levels of Pak1 expression and activity in human breast cancer cell lines and tumors.** *A*, Pak1 levels and activity in breast tumors. Breast tumor lysates (4) were analyzed by Western blotting for Pak1 expression (*upper panel*) and subsequently reprobed with a vinculin antibody as a loading control (*middle panel*). Tumor lysates were immunoprecipitated with a Pak1 Ab and assayed for *in vitro* Pak1 kinase activity (*bottom panel*). Quantitation of Pak1 kinase activity is shown as -fold change. *B*, status of endogenous Pak1 expression was analyzed by immunoblotting in a panel of breast cancer cells grown in complete medium supplemented with 10% fetal calf serum (*upper panel*). Pak1 was immunoprecipitated, and kinase activity was determined by *in vitro* kinase assay using myelin basic protein as a substrate (*bottom panel*). Quantitation of Pak1 kinase activity is shown as -fold change. *C–H*, immunohistochemical demonstration of Pak1 in breast tissue samples. *C–E*, are invasive poorly differentiated ductal carcinomas of the breast (documented as grade III). All show very strong positively for Pak1. *F*, grade 2-breast carcinoma showing low level staining for PAK1. *E*, ductal epithelial hyperplasia with a low level of Pak1 staining. *H*, negative control of section adjacent to stained with normal rabbit IgG in place of the primary antibody. The sections were stained with the peroxidase anti-peroxidase method and counterstained with hematoxylin.

known to react with the Pak2 isoform, we believe that Pak2 may also be elevated in grade III samples.

We also examined the level of endogenous Pak1 activity in a panel of invasive and noninvasive breast cancer cell lines grown in complete medium supplemented with 10% fetal calf serum. Invasive breast cell lines (MDA-MB435, MDA-MB231) exhibited a significant elevation in the level of Pak activity as compared with noninvasive breast cancer cell lines (MDA-453, BT-474, and MCF-7) (Fig. 5*B*). Although Pak1 expression was observed in MCF-7 cells, the level of Pak1 kinase activity in MCF-7 cells was significantly lower as compared with its levels higher in invasive cells, suggesting that Pak activity rather than the amount Pak1 may be responsible for the phenotype. Similarly, grade III tumors (four out of five) exhibited an elevation in the level of Pak kinase activity compared with grade II tumors. There was no direct correlation between the expression level of Pak1 protein and its activity. It is possible that the Pak1 kinase activity in tumor cells may be stimulated by mutation or signaling pathways or autocrine/paracrine growth factors. Taken together, these observations support the notion that Pak1 activity rather than the expression of Pak1 protein may be closely related with the invasiveness of breast cancer.

We next analyzed the expression of Pak1 in a panel of tumor biopsies by immunohistochemical staining. As shown in Fig. 5 (*C–H*), there were low levels of Pak1 immunoreactivity in low grade tumors (*F*, *G*). In contrast, poorly differentiated ductal carcinomas of the breast (documented as grade III) demonstrated a significant intense Pak1 staining (Figs. 5, *C–E*). Fig. 5*F* displays grade 2 breast carcinoma showing low level staining for Pak1. A larger study is needed to further establish the validity of these new results.

In summary, our findings demonstrated the ability of Pak1 activity to stimulate the growth of breast epithelial cancer cells in an anchorage-independent manner and to promote an abnormal organization of mitotic spindles. We also provide new evidence to suggest a close relationship between the levels of Pak1 expression and activity Pak1 and invasive phenotypes of human breast cancer cell lines and tumor grades.

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Regulatable Expression of p21-activated Kinase-1 Promotes Anchorage-independent Growth and Abnormal Organization of Mitotic Spindles in Human Epithelial Breast Cancer Cells

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