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Pak1 and Pak2 Mediate Tumor Cell Invasion through Distinct Signaling Mechanisms[▽]

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Pak kinases are thought to play critical roles in cell migration and invasion. Here, we analyze the roles of Pak1 and Pak2 in breast carcinoma cell invasion using the transient transfection of small interfering RNA. We find that although both Pak1 and Pak2 contribute to breast carcinoma invasion stimulated by heregulin, these roles are mediated by distinct signaling mechanisms. Thus, whereas the depletion of Pak1 interferes with the heregulin-mediated dephosphorylation of cofilin, the depletion of Pak2 does not. The depletion of Pak1 also has a stronger inhibitory effect on lamellipodial protrusion than does the depletion of Pak2. Interestingly, Pak1 and Pak2 play opposite roles in regulating the phosphorylation of the myosin light chain (MLC). Whereas the depletion of Pak1 decreases phospho-MLC levels in heregulin-stimulated cells, the depletion of Pak2 enhances MLC phosphorylation. Consistent with their opposite effects on MLC phosphorylation, Pak1 and Pak2 differentially modulate focal adhesions. Pak2-depleted cells display an increase in focal adhesion size, whereas in Pak1-depleted cells, focal adhesions fail to mature. We also found that the depletion of Pak2, but not Pak1, enhances RhoA activity and that the inhibition of RhoA signaling in Pak2-depleted cells decreases MLC phosphorylation and restores cell invasion. In summary, this work presents the first comprehensive analysis of functional differences between the Pak1 and Pak2 isoforms.

p21-associated kinases (Paks) are found in most eukaryotes and play an evolutionarily conserved role in regulating the actin cytoskeleton (6, 17). There are six Paks in mammals, which are divided into group A (Pak1 to Pak3) and group B (Pak4 to Pak6). The group A Paks are better characterized and have been shown to be directly regulated by small GTPases including Rac, Cdc42, and Wrch1. Paks can also be regulated in a GTPase-independent fashion by sphingosine lipid and caspase-3 cleavage (7, 38). In addition to their function as kinases, Pak proteins can also serve as scaffolds. The N-terminal region of the group A Paks contain several polyproline (PXXP) motifs, which serve as binding sites for SH3 domain-containing proteins such as Nck, bPIX, and Grb2.

An increase in the Pak protein level has been observed in several human tumors (21). In breast cancer, a correlation between high grade, protein level, and the kinase activity of Pak1 has been reported (53). Cell invasion is a complex process involving extensive remodeling of the actin cytoskeleton, the disruption of cell-cell junctions, and the production of extracellular proteases, which are able to degrade extracellular matrix proteins (14, 39). The expression of a kinase-dead Pak1 mutant inhibits the invasion of the human breast carcinoma cell line MDA-MB-435 (1). Pak proteins have been demonstrated to regulate the actin cytoskeleton during cell motility and invasion. In fibroblasts, Pak1 is concentrated at the leading edge during cell migration and regulates lamellipodial extension and directionality (45, 46) and the formation and breakdown of focal adhesions (24, 29).

Several targets of Pak that are implicated in regulating cytoskeletal dynamics, including LIM kinase (LIM-K) (13), myosin light chain (MLC) kinase (MLCK) (16, 43), MLC (10, 60), Op18/stathmin (57), p41 Arc (Arp2/3) (55), filamin (54), and cortactin (56), have been identified. The actin binding protein ADF/cofilin binds to F-actin and promotes its severing and depolymerization (5). When phosphorylated on Ser-3, cofilin is unable to bind F-actin. Therefore, one of the steps in activating cofilin consists of the dephosphorylation of this residue by phosphatases such as slingshot and chronophin (18). Active Pak1 via its effector LIM-K can lead to the phosphorylation of cofilin on Ser-3 in vitro, which suggests that Pak plays a role in inhibiting cofilin's activity (13). The role of cofilin in actin dynamics appears to be complex, as it has been shown to mediate the disassembly of F-actin filaments, yet it promotes lamellipodial assembly by creating fresh barbed ends at sites of chemotactic stimuli and controls the polarity of a moving cell (15, 20).

Myosin II is a critical generator of contractile force during cell migration. Myosin II-mediated contractility is involved in several aspects of cell motility including the retraction of the trailing edge, the formation of stress fibers, and "squeezing" through matrix filaments during the so-called "amoeboid" mode of invasion (40). Actomyosin-based contractility has also been shown to be important for the maturation of nascent focal complexes (35). The MLC subunit, when phosphorylated, is thought to promote myosin II assembly and increase the actomyosin-based contractility necessary for the generation of stress fibers and the maturation of focal adhesions. Perhaps the best-characterized pathway that controls myosin II is regulated

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by the RhoA small GTPase, which, through its effector ROCK, phosphorylates and inactivates the myosin phosphatase, resulting in an increase in MLC phosphorylation, and directly phosphorylates the MLC itself (27, 52). Pak proteins have the potential to inhibit and promote myosin II function. Pak phosphorylation of MLCK leads to its inhibition and results in decreased MLC phosphorylation (43). Conversely, Paks can activate myosin II-mediated contractility by the direct phosphorylation of the MLC subunit (10, 48, 60). How Paks regulate these pathways in a precise temporal-spatial fashion during cell motility, however, is poorly understood.

Currently, there is limited information addressing a potential isoform-specific role(s) for group A Paks. The use of kinase-dead or other mutant Pak proteins may not be appropriate to identify these isoform-specific functions. Kinase-dead mutants act in a dominant negative fashion by associating nonproductively with their substrates, but it has been shown that Pak1 and Pak2 have virtually identical substrate specificities in vitro (31). Furthermore, it has been shown that protein overexpression can override isoform specificity (9, 59).

In this study, we used small interfering RNA (siRNA) to investigate the isoform-specific roles of Pak1 and Pak2. We used mainly T47D cells, a relatively well-differentiated human breast carcinoma cell line that responds to the ErbB3 ligand heregulin, which, by activating ErbB2/HER2, elicits potent actin cytoskeletal reorganization and chemoinvasion (26). We examined the roles of Pak1 and Pak2 in a number of signaling events that have been implicated in cell migration and invasion, including the phosphorylation of cofilin and MLC and the formation of lamellipodia and focal adhesions.

MATERIALS AND METHODS

Reagents. siRNA oligonucleotides were purchased from Dharmacon: 5′-AA CGTACGCGGAATACTTCGA for GL2 luciferase; 5′-GAAGAAATATACA CGGTTT (catalog number D-003521-01; siGENOME), P1-A, 5′-CATCAAAT ATCACTAAGTCTT (catalog number D-003521-03; siGENOME), and P1-B for Pak1; 5′-AGAAGGAACTGATCATTAA (catalog number D-003597-05; siGENOME), P2-A, 5′-GAAACTGGCCAAACCGTTATT (catalog number D-003597-07; siGENOME), and P2-B for Pak2; 5′-ATGGAAAGCAGGTAGA GTTTT (catalog number D-003860-01; siGENOME) for RhoA; and 5′-TGCC CTCTATGATGCAACC for cofilin. The following antibodies were used for Western blotting: anti-Pak1 (Zymed), anti-Pak2, anti-phospho-Pak1(Ser144) Pak2(Ser141), anticofilin, anti-phospho-cofilin(Ser3), anti-MLC2, anti-phospho-MLC2(T18/S19) (Cell Signaling Technology), antivinculin (Sigma), anti-dynamin, and anti-RhoA (Santa Cruz). Heregulin was obtained from B&D. The inhibitors Y-27632 and blebbistatin were obtained from Calbiochem. Hepatocyte growth factor (HGF) was obtained from Chemicon.

Cell culture and transfection conditions. T47D breast carcinoma cells were cultured at 37°C in a humidified 5% CO_2 incubator in RPMI medium supplemented with 10% fetal bovine serum (FBS) and 10 μg/ml insulin (Sigma). DAOY medulloblastoma cells were cultured at 37°C in a humidified 5% CO_2 incubator in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. For siRNA transfection, T47D cells were plated at a density of 1×10^5 cells per well in a six-well plate for 4 h and then transfected using 20 nM of siRNA duplex with 2.2 μg/ml Lipofectamine 2000 (Invitrogen). For siRNA transfection, cells were plated at a density of 1×10^5 cells per well in a six-well plate for 4 h and then transfected using 20 nM of siRNA duplex with 1.3 μg/ml Lipofectamine 2000. Cells were transfected overnight, and fresh medium was added 24 h posttransfection.

Invasion assays. Matrigel invasion chambers (B&D) were hydrated in serum-free RPMI medium for 2 h and then placed in RPMI medium with 0.5% bovine serum albumin (BSA) containing 1 nM heregulin (B&D). Three days after transfection with siRNA, T47D cells were plated in the top chamber at a density of 2×10^5 cells in a 500-μl volume of RPMI medium plus 0.5% BSA (in the absence of heregulin). DAOY cells were plated onto Matrigel chambers at a density of 1×10^4 cells in a 500-μl volume of medium with 10% serum or in DMEM–0.1% BSA. Cells were allowed to invade for 24 h in the presence of a 1 nM heregulin or a 25-ng/ml HGF gradient before fixation in 3.7% formaldehyde (Sigma) in phosphate-buffered saline (PBS) for 15 min and stained with 0.2% crystal violet in 2% ethanol for 10 min. The level of invasion was quantified by visual counting of the total cells on the underside of the filter.

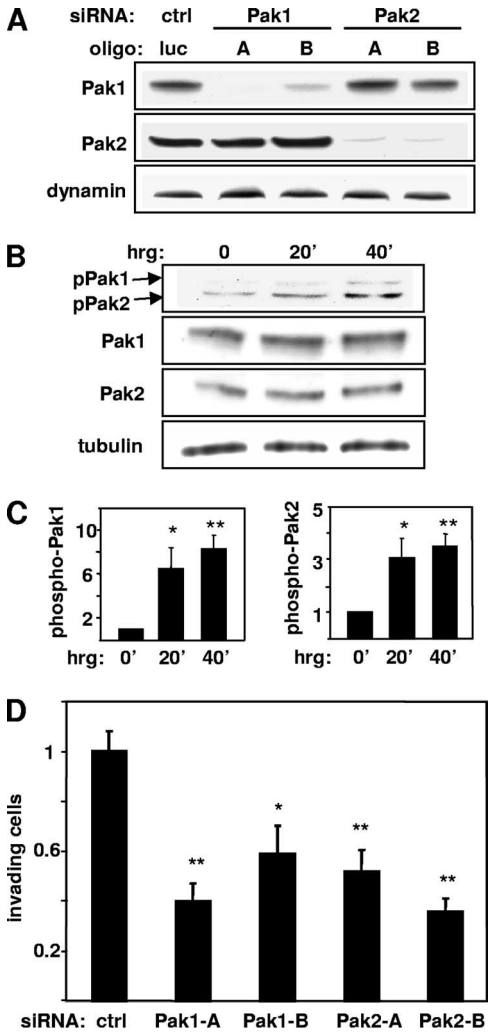

FIG. 1. Both Pak1 and Pak2 are necessary for T47D invasion. (A) Pak1 and Pak2 siRNA-mediated knockdown was determined 3 days after transfection by Western blotting with Pak1- and Pak2-specific antibodies. Results shown are representative of data from at least three experiments. (B) Heregulin (hrg)-stimulated Pak1 and Pak2 activation was determined by Western blotting using a phospho-Pak1 (pPak1)/Pak2 antibody as described in Materials and Methods. The blot shown is representative of three experiments. (C) Intensities of phospho-Pak1 and phospho-Pak2 signals were quantified using NIH ImageJ software and normalized first to tubulin controls and second to phospho-Pak in the absence of heregulin. Data shown represent the means ± standard errors of the means (SEM) of data from at least three independent experiments. **, $P < 0.005$; *, $P < 0.05$ (two-tailed *t* test). (D) T47D cells were transfected with siRNA directed against either luciferase (control [ctrl]) or two independent siRNAs for Pak1 (P1-A and P1-B) and Pak2 (P2-A and P2-B) plated onto Matrigel-coated transwell chambers for 24 h in RPMI with 0.5% BSA against a 1 nM heregulin gradient. Invasion was quantified as described in Materials and Methods. Results shown are normalized to control cell invasion and represent the means ± SEM of data from nine experiments. **, $P < 0.001$; *, $P < 0.01$ (two-tailed *t* test).

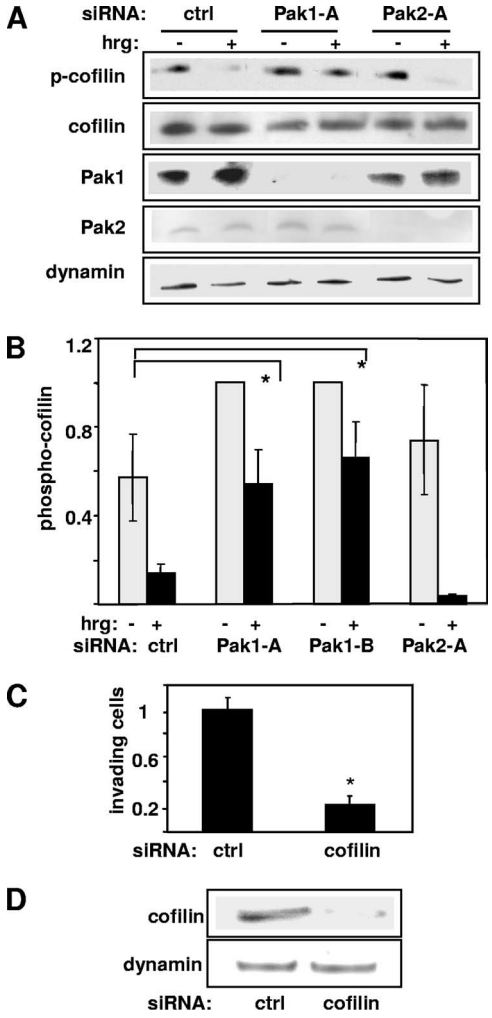

FIG. 2. Pak1, but not Pak2, is required for cofilin dephosphorylation. (A) T47D cells were transfected with siRNA directed against luciferase (control [ctrl]), Pak1, or Pak2 and then starved overnight in RPMI medium plus 0.5% BSA. Cofilin phosphorylation (p-cofilin) was determined by lysing cells 15 min after 1 nM heregulin (hrg) treatment in Laemmli sample buffer, followed by Western blotting analysis with an anti-phosphocofilin(Ser3) antibody. Results shown are representative of data from five experiments. (B). Intensity of the phosphocofilin signal was quantified using NIH ImageJ software and normalized first to total cofilin or dynamin and second to the phosphocofilin signal in unstimulated Pak1-depleted cells. *, $P < 0.05$ (two-tailed t test). Data shown represent the means ± standard errors of the mean (SEM) of data from three experiments for the Pak1-B oligonucleotide and five experiments for the other oligonucleotides. (C) T47D cells were transfected with luciferase (control) or cofilin siRNA and assayed for invasion as described in the legend of Fig. 1. Results shown are normalized to control cell invasion and represent the means ± SEM of data from two experiments. *, $P < 0.001$ (two-tailed t test). (D) Efficacy of cofilin knockdown by siRNA was assessed by Western blot analysis using an anticofilin antibody. Data shown are representative of data from two experiments.

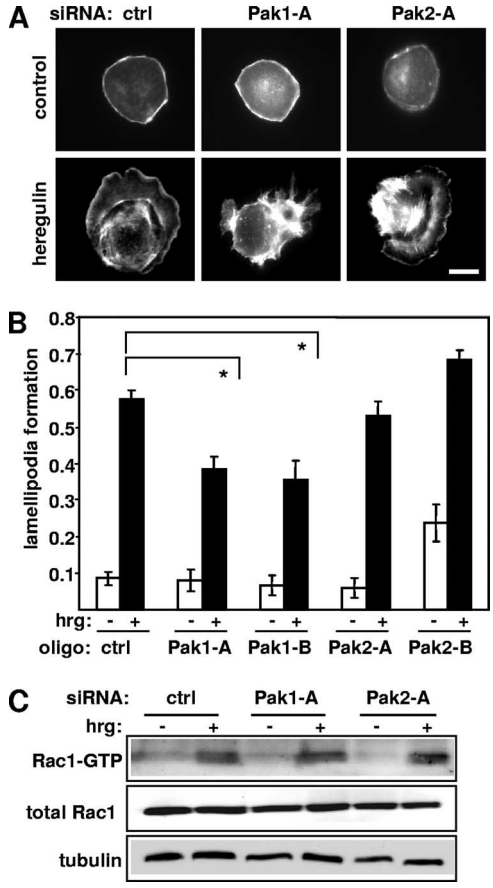

FIG. 3. Pak1 is required for heregulin-induced lamellipodia. (A) T47D cells were transfected with siRNA directed against either luciferase (control [ctrl]) or two independent siRNAs for Pak1 (P1-A and P1-B) and Pak2 (P2-A and P2-B) and then starved overnight in medium with 0.5% BSA. Cells were treated with medium alone or with 1 nM heregulin for 20 min and subsequently fixed in formaldehyde. The actin cytoskeleton was visualized using rhodamine-conjugated phalloidin. Bar, 10 μM. (B) Lamellipodial production was scored as detailed in Materials and Methods. Data shown are the means of data for at least 40 cells ± standard errors of the mean for each treatment condition. *, $P < 0.001$ (two-tailed t test). (C) T47D cells transfected with luciferase (control), Pak1, or Pak2 siRNA were starved overnight in RPMI medium with 0.5% BSA and stimulated with 1 nM heregulin (hrg) for 20 min. Rac1-GTP levels were determined using the EZ Detect Rac activation kit protocol (Pierce). Rac1 was detected using an anti-Rac antibody (UBI). Extracts were prepared from the cell lysates prior to GST-Pak immunoprecipitation to determine total Rac levels. The blot shown is representative of data from two independent experiments.

Western blotting. Three days posttransfection, T47D cells were starved overnight in RPMI medium plus 0.5% BSA and stimulated with 0.25 to 1 nM heregulin for the indicated times. Cell extracts were prepared by washing the cells in cold PBS on ice immediately prior to harvesting and lysed in Laemmli sodium dodecyl sulfate (SDS) sample buffer containing β-mercaptoethanol (Bio-Rad). For the Pak activity assays, cells were lysed in a lysis buffer containing 0.5% Triton X-100, 50 mM Tris-HCl, 0.1 mM EDTA, 150 mM NaCl, 50 mM NaF, 1 mM dithiothreitol, and 0.2 mM sodium orthovanadate. Proteins were resolved on 10 or 12% SDS-polyacrylamide gel electrophoresis (PAGE) gels and transferred onto polyvinylidene difluoride membranes for Western blotting.

Immunostaining. T47D cells were plated onto coverslips precoated with 25 μg/ml collagen (B&D) in sterile PBS for 2 h. Cells on coverslips were starved in RPMI medium plus 0.5% BSA overnight, treated with 1 nM heregulin for 5, 10, or 20 min, and then fixed in 4% formaldehyde for 15 min. Fixed cells were permeabilized in PBS containing 0.1% Triton X-100. F-actin was visualized by staining cells with rhodamine- or fluorescein isothiocyanate (FITC)-phalloidin for 15 min. Focal adhesions were visualized by indirect immunofluorescence using an anti-vinculin antibody. Images were collected using an IX70 Olympus inverted microscope equipped with a 60× (1.4-numerical-aperture) objective, an Orca II cooled charge-coupled-device camera (Hamamatsu Photonic Systems, Bridgewater, NJ), and ESee (Inovision, Raleigh, NC) image analysis software. Lamellipodia were quantified by mea-

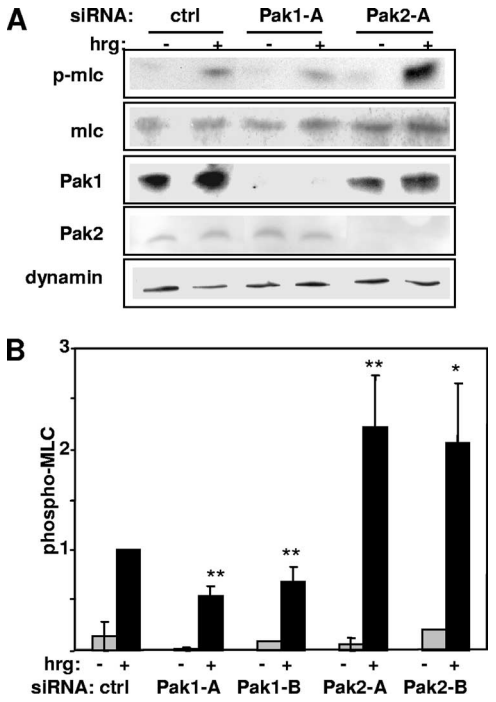

FIG. 4. Pak1 and Pak2 have opposing effects on MLC phosphorylation. (A) T47D cells transfected with luciferase (control [ctrl]), Pak1, or Pak2 siRNA were starved overnight in RPMI medium with 0.5% BSA and were then stimulated with medium alone or 1 nM heregulin (hrg) for 15 min, lysed directly in Laemmli sample buffer, and subjected to Western blotting analysis. MLC phosphorylation (p-mlc) was determined by blotting with an anti-MLC antibody. The blot shown is representative of four experiments. (B) The intensity of the phospho-MLC signal was quantified using NIH ImageJ software and normalized first to total MLC or dynamin and second to heregulin-stimulated controls. Data shown represent the means ± standard errors of the mean standard errors of the mean of data from at least four independent experiments. **, $P < 0.005$; *, $P < 0.01$ (two-tailed t test).

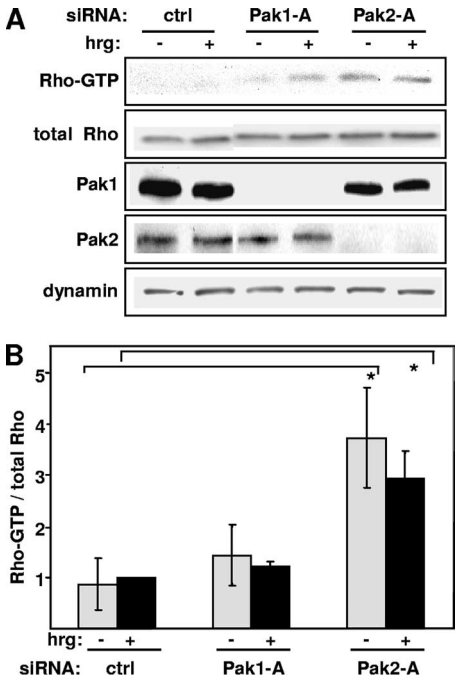

FIG. 5. Depletion of Pak2 activates RhoA. (A) T47D cells transfected with luciferase (control [ctrl]), Pak1, or Pak2 siRNA were starved overnight in RPMI medium with 0.5% BSA and stimulated with 1 nM heregulin (hrg) for 20 min. RhoA-GTP levels were determined using the EZ Detect Rho activation kit protocol (Pierce). RhoA was detected using an anti-Rho antibody (Pierce). Extracts were prepared from the cell lysates prior to GST-rhotekin immunoprecipitation to determine total RhoA levels. The blot shown is representative of three independent experiments. (B) RhoA-GTP levels were quantified using NIH ImageJ software and normalized first to total RhoA and second to heregulin-stimulated controls. Data shown represent the means ± standard errors of the mean of data from three independent experiments. *, $P \leq 0.05$ (two-tailed t test).

suring the fraction of the cell circumference that scored positive for characteristic lamellipodial actin staining. Focal adhesion area and number were determined using NIH ImageJ software. In cases where two focal adhesions were touching or in close proximity to each other, they were scored as distinct if an intensity profile revealed that the intensity at the trough between the two focal adhesions was less than 60% of the average intensity of the two peaks.

GST-Pak immunoprecipitations. Cells were assayed for Rac activity using the EZ Detect Rac activation kit (Pierce). Beads carrying glutathione *S*-transferase (GST) fused to the Rac binding domain of Pak were used to immunoprecipitate GTP-Rac from cell lysates treated or not treated with 1 nM heregulin for 20 min. Lysates were incubated with GST-Pak beads for 1 h at 4°C with continuous rotation. Beads were then washed several times, and the bound material was eluted using sample buffer for 5 min at 90°C. Eluate was resolved on 12% SDS-PAGE gels and analyzed by Western blotting as described in Materials and Methods.

GST-rhotekin immunoprecipitations. Cells were assayed for Rho activity using the EZ Detect Rho activation kit (Pierce). Beads carrying GST fused to the Rho binding domain of rhotekin were used to immunoprecipitate GTP-Rho from cell lysates treated with 1 nM heregulin for 20 min. Lysates were incubated with GST-rhotekin beads for 1 h at 4°C with continuous rotation. Beads were then washed several times, and the bound material was eluted using sample buffer for 5 min at 90°C. The eluate was resolved on 12% SDS-PAGE gels and analyzed by Western blotting as described above.

RESULTS

Both Pak1 and Pak2 are required for heregulin-stimulated T47D breast carcinoma cell invasion. The T47D human breast carcinoma cell line expresses readily detectable levels of both Pak1 and Pak2 proteins (Fig. 1A). Using RNA interference, we were able to achieve at least a 75% knockdown of Pak1 and Pak2 (Fig. 1A). Importantly, Pak1 siRNA does not affect Pak2 protein levels nor vice versa. Heregulin stimulates the activation of both Pak1 and Pak2, as determined by Western blotting using a phospho-Pak1/Pak2 antibody (Fig. 1B and C). The depletion of either Pak1 or Pak2 using two independent siRNA oligonucleotide duplexes for each gene results in approximately 50% inhibition of T47D invasion through Matrigel compared to cells transfected with a control siRNA directed against luciferase (Fig. 1D). The depletion of Pak1 or Pak2 did not significantly affect cell proliferation or survival in the presence of serum (data not shown). These results demonstrate a role for both Pak1 and Pak2 during cell invasion and suggest that Pak1 and Pak2 play nonredundant roles in this process.

Pak1, but not Pak2, is required for heregulin-induced dephosphorylation of cofilin. Since Pak proteins have been implicated in the regulation of cofilin phosphorylation (13), we next investigated the effect of depleting Pak1 and Pak2 on the phosphorylation state of cofilin. The stimulation of serum-starved T47D cells with heregulin for 15 min resulted in a substantial decrease in cofilin phosphorylation (Fig. 2A and B), consistent with the previous finding that heregulin stimulates cofilin ac-

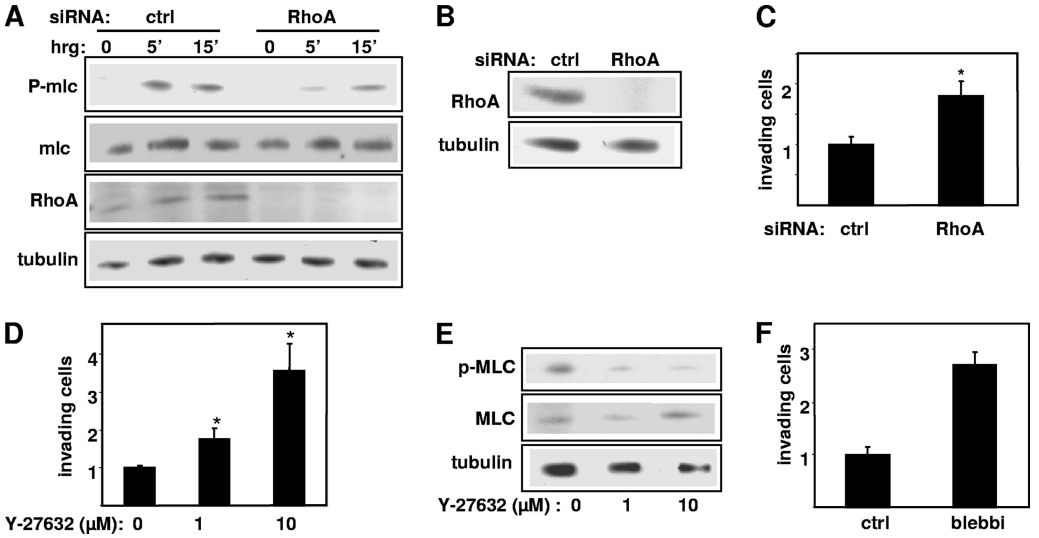

FIG. 6. The Rho/ROCK/myosin II pathway inhibits T47D invasion. (A) RhoA is essential for heregulin-induced MLC phosphorylation. Cells were treated with 1 nM heregulin (hrg) for the indicated times. The effect of RhoA depletion on heregulin-stimulated MLC phosphorylation (P-mlc) was determined by Western blotting as described in the legend to Fig. 4. (B) RhoA siRNA efficiency was determined by Western blotting using an anti-RhoA-specific antibody (Santa Cruz). Results shown are representative of data from three experiments. (C) Depletion of RhoA stimulates T47D cell invasion. T47D cells were transfected with siRNA against luciferase (control [ctrl]) or RhoA and examined for invasion as described in the legend to Fig. 1. Data shown are normalized to the luciferase control and represent the means ± standard errors of the mean (SEM) of data from two experiments. *, $P < 0.05$ (two-tailed t test). (D) Inhibition of ROCK stimulates T47D invasion. T47D cells were plated onto Matrigel-coated Transwell chambers for 24 h in RPMI medium with 0.5% BSA against a 1 nM heregulin gradient with the ROCK-specific inhibitor Y-27632 at the indicated concentrations. Invasion was determined as described in the legend to Fig. 1. Results shown are the means ± SEM of data from six experiments. *, $P < 0.005$ (two-tailed t test). (E) ROCK is essential for heregulin-induced MLC phosphorylation (p-MLC). T47D cells were pretreated with the indicated concentrations of Y-27632 for 45 min and treated with medium alone or with 1 nM heregulin for 15 min. Phospho-MLC levels were determined as described in the legend to Fig. 4. (F) Blebbistatin stimulates T47D invasion. T47D cells were plated onto Matrigel-coated Transwell chambers for 24 h in RPMI with 0.5% BSA against a 1 nM heregulin gradient with medium alone or 10 μM blebbistatin (blebbi). Invasion was determined as described in the legend to Fig. 1. Results shown are the means ± SEM of data from three experiments.

tivity (28). In Pak1-depleted cells, heregulin-induced cofilin dephosphorylation is substantially reduced, whereas in Pak2-depleted cells, heregulin-induced cofilin dephosphorylation is similar to that of controls (Fig. 2A and B), indicating that Pak1, rather than Pak2, mediates heregulin-induced cofilin dephosphorylation. Similar observations were obtained with an additional Pak1 siRNA.

To examine the role of cofilin in T47D invasion, we depleted cofilin using siRNA. Cofilin-depleted cells are dramatically impaired in their ability to invade Matrigel (Fig. 2C). These results are consistent with a model in which Pak1 mediates heregulin-stimulated cell invasion by, at least in part, mediating the dephosphorylation of cofilin.

Heregulin induction of lamellipodia is dependent on Pak1. The formation of lamellipodia is part of the migratory behavior of cells (34), and Pak proteins have been implicated in this process. We therefore investigated the roles of Pak1 and Pak2 in heregulin-induced lamellipodial formation. Heregulin induces extensive actin remodeling in T47D cells, with maximal lamellipodial extension occurring around 20 min after treatment (Fig. 3). Serum-starved T47D cells plated onto collagen are radially symmetric and display a ring of F-actin, which runs around the cell perimeter (Fig. 3A). Control (luciferase siRNA-transfected) cells treated with heregulin for 20 min displayed lamellipodia over more than 50% of their circumference (Fig. 3B). Pak1-depleted cells were significantly inhibited in lamellipodial formation and, at most, formed small, narrow protrusions. In contrast, Pak2-depleted cells were still able to generate a lamellipodial extension. These data demonstrate an isoform-specific role for Pak1 in the generation of lamellipodia.

Pak proteins bind to PIX/Cool proteins that serve as guanine nucleotide exchange factors (GEFs) that in turn can activate Rac1 (25, 51). Since Rac1 is critical for lamellipodium formation (9), we examined the possibility that Rac1 activity could be diminished in Pak1-depleted cells, thereby explaining the inhibition in lamellipodium formation in these cells. The Rac activation status was determined by selectively extracting GTP-bound Rac proteins from cell lysates using a GST-Pak1 fusion protein (3). No significant changes in Rac activity were observed by knocking down either Pak1 or Pak2 (Fig. 3C). These observations indicated that Pak1 does not control lamellipodium formation by modulating Rac activity.

Pak1 and Pak2 play opposing roles in the regulation of MLC phosphorylation. Previous work (using both dominant negative and constitutively active mutants of Pak) has shown that Pak proteins may either promote or inhibit the phosphorylation of the regulatory light chain subunit (MLC) of nonmuscle myosin II (10, 43, 48, 60). Treatment with heregulin for 15 min induces an increase in MLC phosphorylation (Fig. 4). In Pak1-depleted cells, there is a modest but significant inhibition of heregulin-induced MLC phosphorylation. However, in Pak2-depleted cells, heregulin-stimulated MLC phosphorylation is significantly enhanced. These data indicate that Pak1

promotes MLC phosphorylation in heregulin-stimulated T47D cells, whereas Pak2 represses MLC phosphorylation and thus limits myosin II activity. Similar results were obtained with two independent siRNAs for both Pak1 and Pak2.

Although there is very little MLC phosphorylation in unstimulated control cells (Fig. 4), long exposure of the film revealed a significant increase in MLC phosphorylation in Pak2-depleted, but not in Pak1-depleted, cells under serum-starved conditions in comparison to control cells (data not shown).

Pak2 suppresses RhoA activity. We considered several mechanisms that could underlie the Pak2-mediated suppression of MLC phosphorylation. One scenario is that Pak2 decreases myosin II activity by phosphorylating and inactivating MLCK (16, 43). Another possibility is that Pak downregulates Rho activity (2), which in turn modulates MLC phosphorylation (52). The pharmacological inhibition of MLCK using ML7 had no effect on MLC phosphorylation in either control or Pak2-depleted cells (data not shown), implying that MLCK does not play a significant role in the control of MLC in T47D cells. We therefore measured the level of active Rho proteins in heregulin-stimulated cells using the Rho binding fragment of the Rho effector rhotekin to immunoprecipitate GTP-bound Rho from cell lysates (32). Whereas the depletion of Pak1 does not affect RhoA activation, the depletion of Pak2 significantly increases RhoA activity levels over those of controls under both unstimulated and heregulin-stimulated conditions (Fig. 5). RhoC activation levels in T47D cells are insignificant in comparison to those of RhoA under all the conditions that we examined (data not shown). These results suggest that Pak2 suppresses MLC phosphorylation, at least in part, by inhibiting the Rho/ROCK pathway.

Notably, heregulin does not increase Rho activity in control cells or in cells depleted of Pak1 or Pak2, although heregulin significantly stimulates MLC phosphorylation under these conditions. This lack of correlation indicates that heregulin can stimulate MLC in a Rho-independent fashion.

RhoA/ROCK/myosin II signaling negatively regulates heregulin-induced T47D invasion. To confirm the role of Rho proteins in the regulation of MLC phosphorylation in T47D cells, we inhibited Rho function using siRNA. RhoA-depleted cells exhibited a marked reduction in MLC phosphorylation induced by heregulin (Fig. 6A). To investigate the role of the Rho/ROCK/myosin II axis in heregulin-induced invasion, we first examined the effect of the siRNA-mediated depletion of RhoA in T47D cells. The inhibition of RhoA causes a significant increase in invasion (Fig. 6C). The depletion of RhoC slightly inhibits Matrigel invasion (data not shown). Furthermore, the pharmacological inhibition of ROCK causes a dramatic enhancement of cell invasion with a concomitant decrease in MLC phosphorylation (Fig. 6D and E). Finally, we examined the role of myosin II in T47D invasion using blebbistatin, an inhibitor of the myosin II ATPase (50). Blebbistatin at 10 μM resulted in a strong increase in heregulin-mediated invasion (Fig. 6F). We note that at much higher concentrations, blebbistatin has been shown to also have nonspecific targets (47). Together, these results indicate that the RhoA/ROCK pathway is a critical mediator of myosin II activity in T47D cells and that myosin II-based actomyosin contractility plays a potently anti-invasive role in these cells.

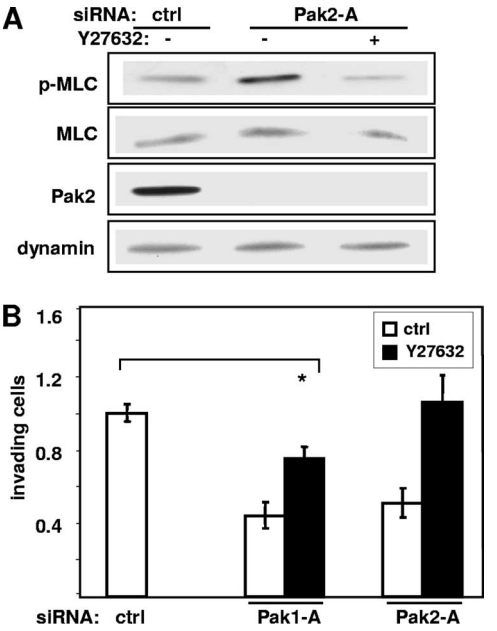

FIG. 7. Inhibition of ROCK restores invasion in Pak2-depleted cells. (A) T47D cells transfected with siRNA against luciferase (control [ctrl]), Pak1, or Pak2 were starved overnight in RPMI medium plus 0.5% BSA, pretreated with 1 μM Y-27632 or medium alone for 45 min, and treated with 1 nM heregulin for 15 min. Determination of MLC phosphorylation (p-MLC) was carried out as described in the legend to Fig. 4. (B) T47D cells were transfected as described above (A) and plated onto Matrigel-coated Transwell chambers for 24 h in RPMI medium with 0.5% BSA against a 1 nM heregulin gradient with medium alone or 1 μM Y-27632. Invasion was determined as described in the legend of Fig. 1. Results shown are the means ± standard errors of the mean of data from seven experiments. *, $P < 0.01$ (two-tailed t test).

Inhibition of the ROCK pathway rescues invasion in Pak2-depleted cells. To further investigate the role of Rho/ROCK signaling in Pak2-mediated invasion, we examined whether inhibiting ROCK signaling in Pak2-depleted cells could restore invasion. We first titrated the concentration of Y-27632 to a level that lowers the phospho-MLC signal in Pak2-depleted cells approximately down to the level observed in control cells (1 μM) (Fig. 7A). This concentration of Y-27632 restored the level of cell invasion to that of control cells (Fig. 7B). Notably, Y-27632 has a smaller stimulatory effect on cell invasion in Pak1-depleted cells than in Pak2-depleted cells, further underlining the significant contribution of RhoA inhibition to the role of Pak2 in the invasive behavior of T47D cells.

Pak1 and Pak2 regulate the size and number of focal adhesions. Since the Rho/ROCK/myosin II pathway is known to be critical for the maturation of focal adhesions (35, 52), we next examined the effect of Pak1 and Pak2 depletion on focal adhesions. Pak proteins have been shown to localize to focal adhesions via the PIX/GIT/PKL/paxillin complex (8, 23, 61), but the precise roles of Pak proteins in focal adhesion generation and maturation are still unclear. Using immunofluorescence with antibodies against the focal adhesion protein vinculin, we analyzed the effect of inhibiting Pak1 and Pak2 on focal adhesion size and number.

Heregulin treatment causes an increase in both the number

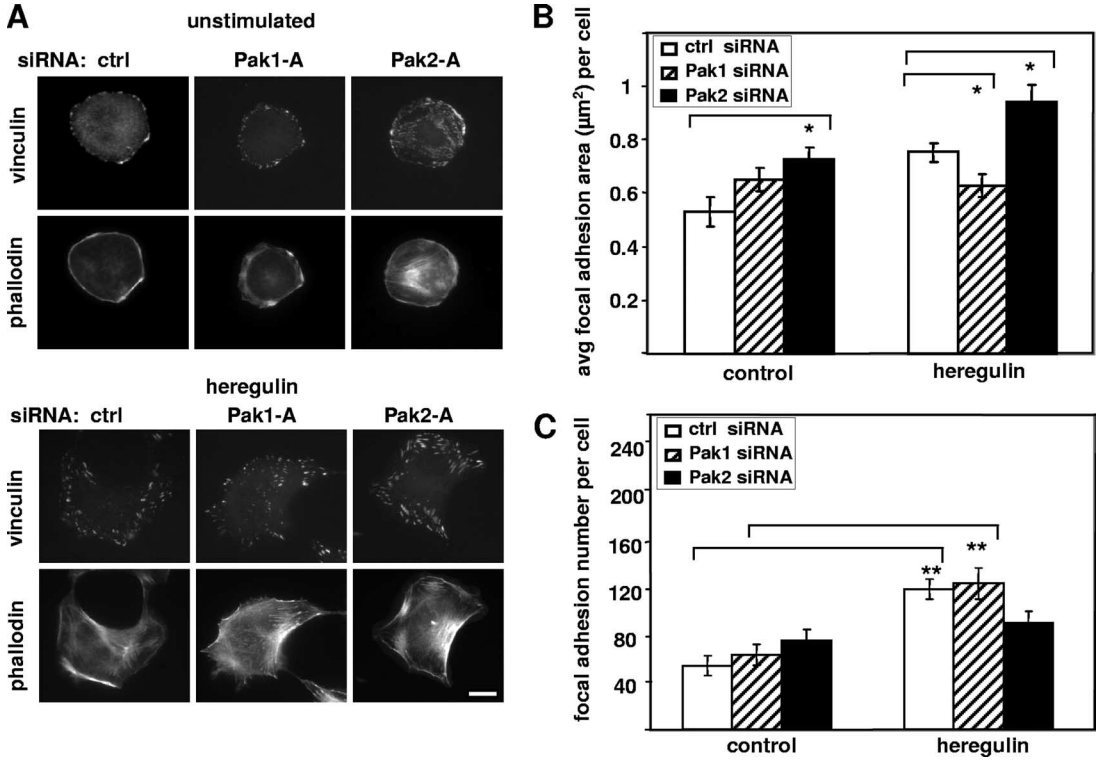

FIG. 8. Pak1 and Pak2 differentially regulate focal adhesions. (A) T47D cells were transfected with siRNA against luciferase (control [ctrl]), Pak1, or Pak2; starved overnight in RPMI plus 0.5% BSA on collagen-coated coverslips; and treated with medium alone or 1 nM heregulin for 20 min. Subsequently, cells were fixed and processed for immunofluorescence using antivinculin antibodies and FITC-phalloidin as described in Materials and Methods. Bar, 10 μM. (B and C) Focal adhesion size and focal adhesion number per cell were quantified as described in Materials and Methods. Results shown are the means ± standard errors of the mean of data for at least 20 cells. **, $P < 0.0005$; *, $P < 0.05$ (two-tailed *t* test).

and size of focal adhesions. Focal adhesion size was measured according to criteria outlined in Materials and Methods. Whereas the depletion of Pak1 appears to slightly increase the sizes of focal adhesions in unstimulated cells (although this difference did not reach significance), it significant inhibits the increase in focal adhesion size caused by heregulin (Fig. 8B). However, the number of focal adhesions per cell was not significantly affected by Pak1 depletion (Fig. 8C). In contrast, in serum-starved Pak2-depleted cells, the focal adhesion size was larger than that seen in control cells (Fig. 8B), but the number of focal adhesions per cell did not significantly increase upon heregulin stimulation (Fig. 8C).

Since Pak proteins have been implicated in cell spreading (6), we also examined the effect of knocking down Pak1 or Pak2 on the spread area of cells under serum-starved and heregulin-stimulated conditions. We observed that the depletion of Pak2 reduces the spread area of heregulin-stimulated cells by 32%, whereas the depletion of Pak1 has a smaller inhibitory effect on the spread area (data not shown). However, after recalculating the number of focal adhesions on a per-spread-area basis, rather than on a per-cell basis, heregulin still induced a marked increase in the density of focal adhesions in Pak1-depleted cells but not in Pak2-depleted cells (data not shown).

Thus, taken together, these results suggest that Pak1 is mainly required for the maturation of focal adhesions, whereas Pak2 is needed to generate new focal adhesions. Pak2 also appears to limit the sizes of focal adhesions.

Consistent with what has been reported previously in the literature (4, 37), the inhibition of ROCK with Y-27632 results in a decrease in the sizes of focal adhesions in heregulin-stimulated cells (Fig. 9). These observations are consistent with the notion that Pak2 limits the size of focal adhesions by inhibiting the activation state of RhoA. Interestingly, however, Y-27632 does not affect the number of focal adhesions per cell (Fig. 9), suggesting that the role of Pak2 in the generation of novel heregulin-induced focal adhesions is independent of the inhibitory effect of Pak2 on RhoA activation.

Differential control of cofilin and MLC by Pak1 and Pak2 in DAOY medulloblastoma cells. To extend our observations on the distinct roles of Pak1 and Pak2 in the regulation of cofilin and MLC to an additional cell line, we used DAOY medulloblastoma cells. Medulloblastoma is a highly invasive pediatric brain tumor (44). We have examined the invasive behavior of DAOY cells under two different conditions, either stimulated by a gradient of HGF or in the presence of serum in the top and bottom transwell chambers. Under both conditions, the depletion of either Pak1 or Pak2 results in a significant inhibition of Matrigel invasion (Fig. 10A and B), showing that the roles of Pak1 and Pak2 in tumor cell invasion are not restricted to the heregulin-stimulated invasion of breast carcinoma cells.

In line with the results obtained using T47D cells, we ob-

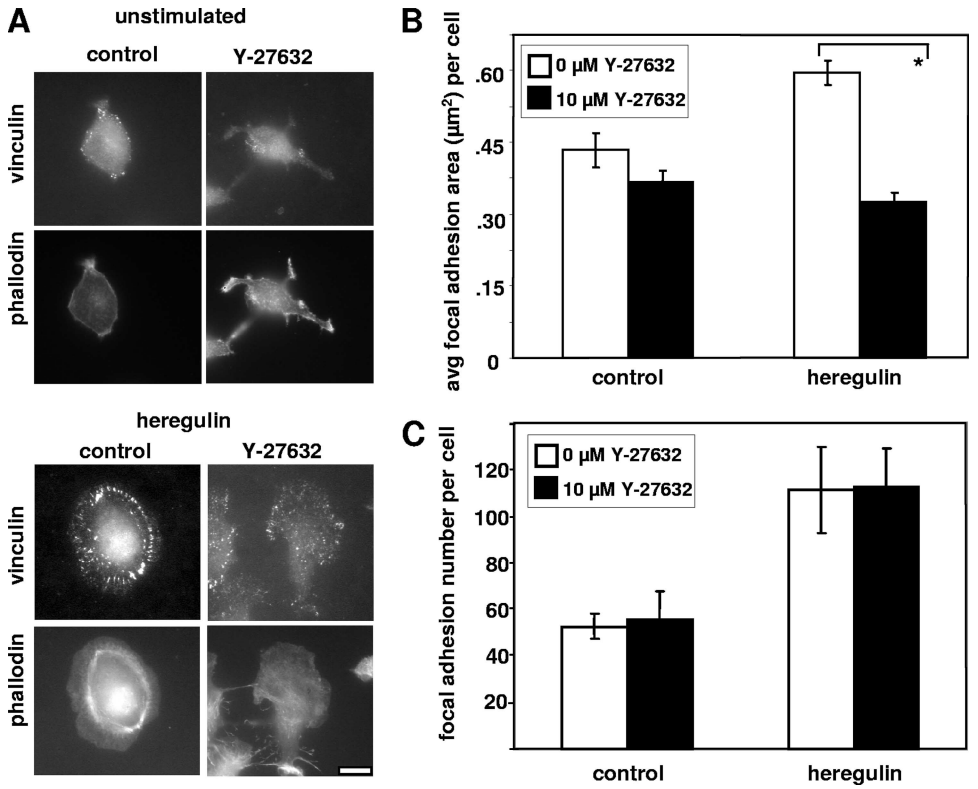

FIG. 9. Inhibition of ROCK results in smaller focal adhesions. (A) T47D cells were either not pretreated or pretreated with 10 μM compound Y-27632 and were either untreated or treated with 1 nM heregulin for 20 min, fixed in formaldehyde, and stained with antivinculin and FITC-phalloidin as described in Materials and Methods. Bar, 10 μM. (B and C) Focal adhesion size and focal adhesion number per cell were quantified as described in Materials and Methods. Data shown represent the means ± standard errors of the mean of data for at least 10 cells. *, $P \leq 0.0005$ (two-tailed t test).

served that serum-starved Pak1-depleted DAOY cells have higher levels of phosphocofilin than control cells, whereas the depletion of Pak2 had no significant effect on cofilin phosphorylation (Fig. 10D and E). In addition, the depletion of Pak2, but not Pak1, resulted in an increase in phospho-MLC levels similar to that seen in T47D cells (Fig. 10F and G). These results suggest that our findings on the differential roles of Pak1 and Pak2 in tumor cell invasion may be relevant for a wide range of cell types.

DISCUSSION

In this study, we have shown that both Pak1 and Pak2 are important for cell invasion and exert these effects through distinct signaling mechanisms. Pak1-depleted T47D breast carcinoma cells are inhibited in the heregulin-mediated stimulation of lamellipodia and cofilin activation, whereas Pak2 plays no significant role in these functions. Pak1 and Pak2 also differentially modulate focal adhesions. In Pak1-depleted cells, focal adhesions do not increase in size upon stimulation by heregulin, whereas Pak2-depleted cells contain significantly larger focal adhesions and are unable to generate new focal adhesions upon heregulin stimulation. The distinct effects of Pak1 and Pak2 on focal adhesion size are mediated in part by their opposing effects on MLC phosphorylation. Pak1- and Pak2-depleted cells that are stimulated by heregulin have lower and higher phospho-MLC levels, respectively, than controls. The inhibitory effect of Pak2 on MLC phosphorylation is mediated, at least in part, by downregulating RhoA, and blocking RhoA-mediated signaling restores invasion in Pak2-depleted cells.

We also have extended our observations to DAOY medulloblastoma cells and showed that their invasive behaviors under two different conditions are strongly dependent on both Pak1 and Pak2, indicating that the roles of Pak1 and Pak2 in cell invasion may be quite general. Furthermore, the depletion of Pak1, but not Pak2, in DAOY cells decreases cofilin activation, and conversely, the depletion of Pak2, but not Pak1, increases phospho-MLC levels, thereby generalizing our observations that Pak1 and Pak2 control distinct signaling pathways.

The Pak kinase family members in *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and *Caenorhabditis elegans* have been found to play at least partially nonoverlapping roles in development and motility (17, 22). Thus far, very little data are available with respect to the isoform-specific functions of group A Paks in mammals. For example, Pak2, but not Pak1, is cleaved and activated by caspase-3 and plays a role in mediating some of the morphological changes associated with apoptosis (38). In addition, in 3T3-L1 fibroblasts, Pak2 is activated by infrared, UV, AraC, and cisplatin, whereas Pak1 is activated only by UV and cisplatin (36).

It has been shown that Pak1 and Pak2 have virtually iden-

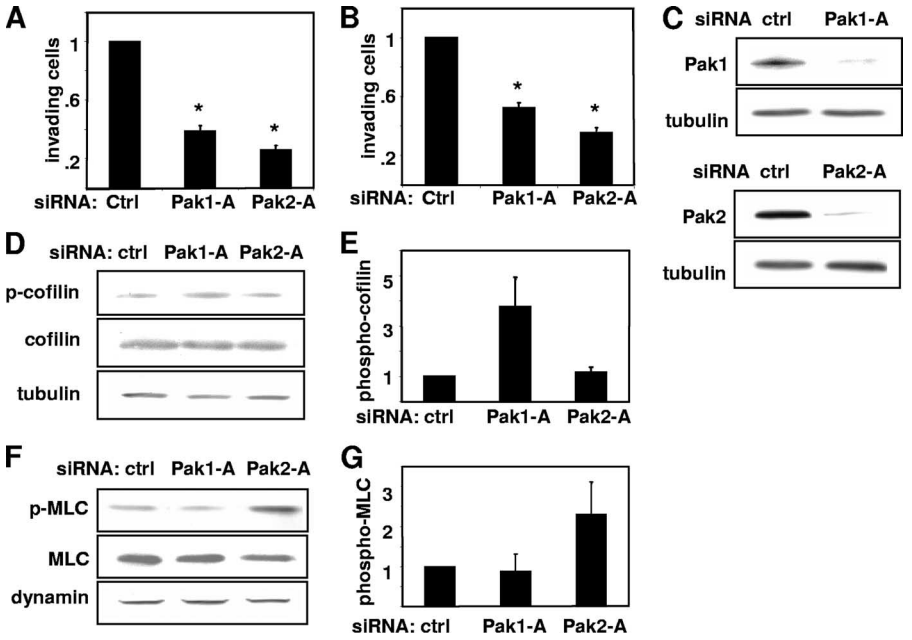

FIG. 10. Both Pak1 and Pak2 mediate invasion in DAOY medulloblastoma cells. (A and B) DAOY cells were transfected with siRNA directed against either luciferase (control [ctrl]), Pak1, or Pak2 and then plated onto Matrigel-coated Transwell chambers for 24 h in DMEM with 10% FBS in the top and bottom chambers (A) or in DMEM plus 0.1% BSA with 25 μg/ml HGF in the bottom chamber (B). Invasion was quantified as described in Materials and Methods. Results shown are normalized to data for control cell invasion and represent the means ± standard errors of the mean (SEM) of data from three experiments (A) or five experiments (B). *, $P < 0.001$ (two-tailed t test). (C) Pak1 and Pak2 siRNA-mediated knockdown was determined 3 days after transfection by Western blotting with Pak1- and Pak2-specific antibodies. Results shown are representative of at least three experiments. (D) DAOY cells were transfected with siRNA directed against luciferase (control), Pak1, or Pak2 and then starved overnight in DMEM with 0.1% BSA. Cofilin phosphorylation (p-cofilin) was determined by lysing cells in Laemmli sample buffer, followed by Western blotting analysis with an anti-phosphocofilin(Ser3) antibody. Results shown are representative of data from five experiments. (E) The intensity of the phosphocofilin signal was quantified using NIH ImageJ software and normalized first to total cofilin or dynamin and second to the control sample. Data shown represent the means ± SEM of data from five experiments. (F) DAOY cells transfected with luciferase (control), Pak1, or Pak2 siRNA were starved overnight in DMEM with 0.1% BSA, lysed directly in Laemmli sample buffer, and subjected to Western blotting analysis. MLC phosphorylation (p-MLC) was determined by blotting with an anti-MLC antibody. The blot shown is representative of four experiments. (G) Intensities of phospho-MLC signals were quantified using NIH ImageJ software and normalized first to total MLC or dynamin and second to the control sample. Data shown represent the means ± SEM of data from four independent experiments.

tical substrate specificities in vitro (33). It is therefore likely that the isoform-specific functions of the group A Paks are mediated by their participation in distinct molecular complexes and their localization to distinct subcellular structures. There is evidence for a differential localization of group A Paks, as Pak1 has been found to be associated with cortical actin structures in platelet-derived growth factor-stimulated fibroblasts, whereas Pak2 localizes to the endoplasmic reticulum in COS-7 and 293T cells (12, 19). However, there is also evidence indicating that both Pak1 and Pak2 localize to focal adhesion structures by virtue of their association with the Rac/Cdc42 guanine-nucleotide exchange factor PIX, which mediates Pak binding to a complex containing PKL (also called GIT) and paxillin (8, 23, 46, 49, 61). The majority of sequence differences among group A Paks are located in the N-terminal regulatory region, which likely governs interactions between Pak proteins and their binding partners. Pak1, for instance, has five canonical SH3-binding PXXP sites, whereas Pak2 has only two. Thus, Pak1 and Pak2 are likely to have different binding partners and to assemble into distinct molecular complexes.

Our finding that the depletion of Pak1 inhibits heregulin-induced lamellipodium formation is consistent with previous observations that Pak1 overexpression stimulates lamellipodium formation (45). Those studies indicated that the Pak1 kinase activity is not required for generating lamellipodial protrusions per se but might play a role in limiting the number of protrusions per cell, possibly by increasing phospho-MLC levels and the consequent stabilization of focal contacts (45).

The observation that the depletion of Pak1 in T47D cells results in an increase in phosphocofilin levels suggests that the Pak substrate LIM-K (13) does not mediate the heregulin-induced dephosphorylation of cofilin (at least during an acute stimulus). It is more likely that Pak1 either directly or indirectly activates a cofilin phosphatase. The stimulation of MCF7 breast carcinoma cells with heregulin was shown previously to activate the cofilin phosphatase slingshot (28). In that same study, Rac was shown to mediate F-actin polymerization in lamellipodia, which may serve to localize and activate slingshot. It is therefore possible that in heregulin-stimulated cells, Pak1 functions downstream of Rac1 to promote actin polymerization and the subsequent activation of slingshot.

Our observations that Pak1 and Pak2 play distinct roles in the regulation of MLC phosphorylation and focal adhesions strongly suggest that these functions of Pak1 and Pak2 are facilitated by the participation of Pak1 and Pak2 in distinct molecular complexes. The result that the depletion of Pak1

inhibits MLC phosphorylation is consistent with observations that Pak proteins can directly phosphorylate MLC in other systems (10, 48, 60). The observation that the depletion of Pak2 enhances the heregulin-induced phosphorylation of MLC by stimulating the activity of the RhoA/ROCK axis presents a novel mechanism for the regulation of actomyosin contractility by a Pak protein. This observation also couples the proinvasive activity of Pak2 to the anti-anti-invasive role of RhoA in T47D cells. Notably, depending on the tumor cell line examined, the RhoA/ROCK pathway is either pro- or anti-invasive (40, 41, 58), suggesting that the precise role of Pak2 in cell invasion may also be cell type dependent.

The fact that Pak2 is able to suppress Rho activity is consistent with several previous reports that demonstrated an antagonism between Rac and Rho signaling (30, 42). One potential mechanism by which Pak proteins could interfere with Rho activation is suggested by the finding that Pak proteins can phosphorylate and inhibit the activity of a Rho-specific GEF (2). phosphorylation by Pak1 inhibits the Rho GEF activity of NET1 in vitro and blocks the NET1-mediated generation of stress fibers in cells (2).

Pak proteins have been implicated in the control of focal adhesion dynamics, although their precise role is still unclear (8, 29, 61). Early studies demonstrated that the overexpression of constitutively active Pak results in the abolishment of focal adhesions and stress fibers (24). The attenuation of heregulin-stimulated MLC phosphorylation and the observation that focal adhesions do not increase in size upon stimulation by heregulin in Pak1-depleted cells suggest that Pak1 may play a role in promoting focal adhesion maturation, at least in part, by stimulating MLC phosphorylation (11).

The depletion of Pak2 has a dual effect on the behavior of focal adhesions in T47D cells. One is that the focal adhesions are abnormally large, and the other is that the generation of new focal adhesions upon heregulin stimulation is inhibited. The increase in focal adhesion size in Pak2-depleted cells is likely due to the increase in myosin II activation, which is observed in both unstimulated and heregulin-stimulated cells. The inhibition of new focal adhesion formation in the absence of Pak2 may reflect a direct requirement of Pak2 for focal adhesion generation or, alternatively, may be an indirect consequence of a limiting component that is necessary for focal adhesion generation that is tied up in the oversized preexisting focal complexes. Thus, taken together, our data suggest that Pak1 and Pak2 cooperate to ensure optimal focal adhesion generation and maturation during migration.

In summary, our functional analysis of Pak1 and Pak2 using RNA interference reveals isoform-specific roles of Pak1 and Pak2 in the organization of the actin cytoskeleton. These findings are also relevant for the molecular dissection of signaling pathways that are governed by these kinases.

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