Opposing Roles for Akt1 and Akt2 in Rac/Pak Signaling and Cell Migration*

Received for publication, January 25, 2006, and in revised form, September 12, 2006. Published, JBC Papers in Press, October 1, 2006, DOI 10.1074/jbc.M600788200

Guo-Lei Zhou‡, David F. Tucker§, Sun Sik Bae∥, Kanav Bhatheja‡, Morris J. Birnbaum∥, and Jeffrey Field‡×

From the ‡Department of Pharmacology and the ∥Howard Hughes Medical Institute, Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

The Akt/PKB isoforms have different roles in animals, with Akt2 primarily regulating metabolic signaling and Akt1 regulating growth and survival. Here we show distinct roles for Akt1 and Akt2 in mouse embryo fibroblast cell migration and regulation of the cytoskeleton. Akt1-deficient cells responded poorly to platelet-derived growth factor while Akt2-deficient cells had a dramatically enhanced response, resulting in a substantial increase in dorsal ruffling. Swapping domains between Akt1 and Akt2 demonstrated that the N-terminal region containing the pleckstrin homology domain and a linker region distinguishes the two isoforms, while the catalytic domains are interchangeable. Akt2 knock-out cells also migrated faster than wild-type cells, especially through extracellular matrix (ECM), while Akt1 knock-out cells migrated more slowly than wild-type cells. Consistently, Akt2 knock-out cells had elevated Pak1 and Rac activities, suggesting that Akt2 inhibits Rac and Pak1. Both Akt2 and Akt1 associated in complexes with Pak1, but only Akt2 inhibited Pak1 in kinase assays, suggesting an underlying molecular basis for the different cellular phenotypes. Together these data provide evidence for an unexpected functional link between Akt2 and Pak1 that opposes the actions of Akt1 on cell migration.

Cell movement requires the coordinated rearrangement of the actin cytoskeleton to extend the leading edge of the cell, attach the leading edge to substrates, and then contract the cell body behind the cell. Small hair-like protrusions called filopodia are not well characterized but they are likely to help polarize cells and form a leading edge. Filopodia are followed by much larger extensions called lamellipodia, which often fold back over the peripheral edges to form ruffles. In addition to these peripheral ruffles, ruffles can take the shape of distinct waves over the dorsal surface of cells called dorsal ruffles or circular ruffles. Peripheral ruffles help direct lateral movement while the dorsal ruffles promote movement off of the plane of the cell. All of these structures are rich in filamentous actin (F-actin) and the actin cytoskeleton plays a central role in cell motility through filopodia and lamellipodia at the leading edge as well as promoting contraction at the trailing edge.

Coordinating the actions of the cytoskeleton in response to growth factors and chemo-attractants requires the activation of multiple cell signaling pathways through phosphatidylinositol (PI)3 3-kinase (PI 3-kinase). PI 3-kinase-generated phospholipids serve as second messengers that activate small GTPases from the Rho family to regulate the actin cytoskeleton. Rho, Rac, Cdc42, and their targets are among the best characterized molecules that direct cell motility. Cdc42 regulates filopodia, Rac regulates lamellipodia and ruffles, while Rho regulates stress fibers and cell contraction (1). In addition, signals from PI 3-kinase promote both cell survival and glucose metabolism through a distinct effector, the Akt/protein kinase B (PKB).

The Akt family consists of three isoforms: Akt1 (α), Akt2 (β), and Akt3 (γ). All Akt isoforms contain an N-terminal pleckstrin homology (PH) domain for phospholipid binding followed by a short linker domain, a catalytic domain and a C-terminal regulatory tail domain. The isoforms share many common substrates through their preferential phosphorylation of a motif with the sequence of RXRXX(S/T) (2). The cell survival substrates include Bad and Forkhead (3–5), while metabolic signaling targets regulate the insulin-responsive glucose transporter GLUT4 (6). Although the survival and metabolic signals are relatively well characterized, more recent studies suggest that Akt also regulates cell migration (7–11). Akt1 is also rapidly recruited to the leading edge of migrating cells and is required for cell polarization (8, 12, 13). Conversely, while Rac regulates motility, it can protect cells from apoptosis through its effector, the p21-activated protein kinase (Pak), to stimulate phosphorylation of Bad (14–17). Thus, a growing body of literature has emerged showing that the cytoskeletal and cell survival pathways downstream of PI 3-kinase are interconnected by several overlapping signals.

Isoform-specific knock-out mice have revealed distinct roles for two of the isoforms, Akt1 and Akt2. Akt1 knock-out mice are small throughout their lifespan, and cells derived from them have high rates of apoptosis suggesting that Akt1 regulates growth and survival (18, 19). Akt2 knockouts have metabolic defects resembling diabetes mellitus including elevated blood glucose (20). In addition, adipocytes from Akt2, but not Akt1

---

* This work was supported in part by Grants GM48241 (to J. F.) and DK56886 (to M. J. B.) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by a National Scientist Development grant from the American Heart Association (0630394N).

2 To whom correspondence should be addressed: Dept. of Pharmacology, University of Pennsylvania School of Medicine, 3620 Hamilton Walk, Philadelphia, PA 19104. Tel.: 215-898-1912; Fax: 215-573-2236; E-mail: field@pharm.med.upenn.edu.

3 The abbreviations used are: PI, phosphatidylinositol; Pak, p21-activated protein kinase; DMEM, Dulbecco’s modified Eagle’s medium; GTPyS, guanosine 5’-O-(thiotriphosphate); PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; WT, wild type; GST, glutathione S-transferase; ECM, extracellular matrix; 1KO, Akt knock-out; 2KO, Akt2 knock-out; DKO, double knock-out.

NOVEMBER 24, 2006 • VOLUME 281 • NUMBER 47

THE JOURNAL OF BIOLOGICAL CHEMISTRY © 2006 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

36443
Akt2 Is a Negative Regulator of Rac/Pak Signaling

knock-out mice, display significant defects in uptake of hexose and glucose transporter 4 translocation suggesting that Akt2 is the major isoform used for insulin signaling (6). The isoforms may also have differential effects on cell motility depending on the cell type (10, 11, 21, 22).

Pak kinases are serine/threonine protein kinases identified through screens for direct effectors of the small GTPases Rac and Cdc42. Pakks are divided into two groups, A and B, based on sequence similarities. Group A consists of three closely related family members, Pak1 (Pakα), Pak2 (Pakγ), and Pak3 (Pakβ). Group B contains Pak4, Pak5, and Pak6 (23–25). Activation of Pak1 stimulates some of the changes in the actin cytoskeleton associated with Rac and Cdc42, including stimulation of cell ruffling, especially dorsal ruffles, and inhibition of stress fibers (26–29). The cell ruffling phenotypes are likely to be regulated by a Pak/LIMK/cofilin pathway (30), while the effects of Pak on stress fibers are caused by phosphorylation and inhibition of a Rho GEF (31). However, the precise role of Pak in regulating these structures is complicated by feedback activation of Rac, which occurs when Pak localizes to the membrane, so in some experimental paradigms even kinase-dead Pak can stimulate ruffling by activating Rac (32).

Whereas most of the regulation of Pak is through small GTPases, Pak kinases are also regulated by GTPase-indepen- dent mechanisms. Growth factor receptors recruit Pak to the membrane through the adaptor proteins Nck and Grb2 (33–36). The PIX proteins also bind Pak through SH3 domains (37, 38). PIX proteins are guanine exchangers for Rac and Cdc42 but cannot activate Pak through both GTPase-dependent and GTPase-independent mechanisms (39). PIX is also responsible for the feedback activation of Rac by membrane-targeted Pak (32).

Several protein kinases also regulate Pak. PDK-1 phosphor- ylates Pak at threonine 423, a site that is also autophosphoryl- ated when Pak is activated by Rac or Cdc42 (40). Cdc2 phos- phorylates Pak at threonine 212 (41, 42). Another protein kinase, Akt1, can also stimulate Pak by phosphorylation of ser- ine 21, but other mechanisms contribute to Akt activation of Pak too (9, 14, 43, 44).

We report here that Akt2, unlike Akt1, is a negative regulator of Pak1. Cells lacking Akt2 have enhanced dorsal ruffling in response to the growth factor PDGF as well as elevated levels of activated Rac and Pak1. Akt2 knock-out cells also have increased migration, especially invasion through extracellular matrix (ECM). These studies suggest opposing roles for Akt1 and Akt2 in regulation of Rac/Pak signaling, the actin cytoskel- eton, and cell migration.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, trypsin-EDTA (1 ×), recombinant human platelet-derived growth factor-BB (PDGF-BB) were from Invitrogen (Carlsbad, CA). FuGENE 6 transfection reagent, Complete protease inhibitor mixture tablets, histone 4 and fibronectin were from Roche Applied Science. Recombinant Akt1 and Akt2 and the Rac activation assay kit were from Upstate Biotechnology Inc. (Charlottesville, VA). Rabbit polyclonal Pak1 antibodies (N20 and its agarose-conjugated form, for Western blot and immunoprecipitation, respectively) and protein A/G PLUS agarose were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Rabbit polyclonal Pak1 antibody for immunofluorescence was from Cell Signaling Technology (Beverly, MA). Highly cross-absorbed Alexa-Fluor 488 goat anti-rabbit IgG(H+L), marina blue goat anti-mouse IgG(H+L), and Alexa-Fluor 594 phalloidin were from Molecular Probes (Eugene, OR). Transwell membranes were from Costar (Corning, NY). Matrigel Invasion Chambers (8 micron pore size) were from BD Biosciences (San Diego, CA). [γ-32P]ATP and ECL Western blotting detection reagents were from Amersham Biosciences (Piscataway, NJ). The peptide Bad S112 (ETRSRHHSSYP) was synthesized by Invitrogen.

Construction of Wild Type, Kinase-dead, and Chimeric Akt Retroviral Constructs—Wild-type Akt1 and Akt2 in the retroviral vector, pMIGR1, have been described previously (6). Kinase-dead (KD) Akt2 construct was made based on the pMIGR-mycAkt2 by introduction of a K181M mutation (21). Two primers: CCCCTATTATGCCATGATGATCCCTGCGCAAGAGAAAGACGATGGACTTCCGATC; Akt2Linker (R): GGCCCGTGCCTTGTTGACAGC; Akt2Linker (F): CGGGCCAGGGCCGCCACAC; Akt2Tail (F): TACAACCAGGACCACGAGCGC. The amplified fragments were gel-purified from agarose and served as templates for PCR. All Akt constructs bear an N-terminal c-Myc epitope tag, and lack a native initiation codon. Primer pairs were designed to amplify cDNA sequences corresponding to the following domains of Akt1 and Akt2: PH domain (Akt1 residues 1–113, Akt2 residues 1–112), PH and linker region (Akt1 residues 2–143, Akt2 residues 2–145), PH, linker and catalytic (Akt1 residues 2–349, Akt2 residues 2–350), linker, catalytic, and regulatory tail (Akt1 residues 113–480, Akt2 res- idues 112–481), catalytic (Akt1 residues 144–349, Akt2 res- idues 146–350), catalytic and regulatory tail (Akt1 residues 144–480, Akt2 residues 146–481), and regulatory tail (Akt1 residues 350–480, Akt2 residues 351–481). The primer sequences were as follows: pUC19/EcoRI/c-Myc(F) for the N terminal of both Akt1 and Akt2: CGGCCCAATTCCACCAATGG; pUC19/BamHI/Stop(R) for the C terminal of both Akt1 and Akt2: CTGCACTTGGCTTGGCTGAGGACTCTCTC; Akt1P- H(R):CTGGCCTCTTTAGTCCATCGGCC; Akt1Linker (F): GAAGAACAGAGACGATGGACTTCCATGC; Akt1Linker(R): GTGCTTTGGCTTGGCTGAGGAGG; Akt1Catalytic(C): CGTG- GTGACCATGAACAGTTTTGAG; Akt1Catalytic(R): GAA- GGCCAGGGCCGCGCCACAC; Akt1Tail(F): TACAACCA- GGACCAGGAAGCTGTITTC; Akt2PH(R): CTGGCTTCAG- ACTGTTGGCACCACAT; Akt2Linker(F): CGGCCCACT- TGAGGAG; Akt2Linker(R): GGCCCGTGCTTGGCTGAGGAC; Akt2Tail(R): GAAATGGCAAGCGCGCCACAC; Akt2Tail(F): TACAACCAAGGACACAGGCCG.

The amplified fragments were gel-purified from agarose and subject to blunt-end ligation in the desired combinations. Ligation reactions were used as templates for a second round of PCR to amplify the full-length chimera. PCR products corresponding to full-length chimera were gel-purified from agarose and cloned into pUC19 as EcoRI/BamHI fragments. Chimera integra-
Akt2 Is a Negative Regulator of Rac/Pak Signaling

Enhanced Dorsal Ruffling in Akt2 Knock-out MEFs—Akt1 can stimulate Pak1 through multiple mechanisms (9, 43, 44). However, all of the studies on the interactions between Akt1 and Pak have been based on cell transfection or in vitro studies. To date, there is no genetic evidence for a role of Akt1 in Pak signaling, nor have any studies documented an interaction between Akt2 and Pak. To study the interaction between Akt and Pak we initiated studies with mouse embryo fibroblast (MEF) cell lines derived from Akt knock-out mice as described.
previously (6). The cell lines tested were: wild type (WT), Akt1 knock-out (1KO), Akt2 knock-out (2KO), and Akt1/Akt2 double knock-out (DKO). For reexpression of Akt isoforms, stable cell lines were made by introducing pMIGR-based Akt1 or Akt2 into WT or 2KO backgrounds. These cell lines include: WT cells with empty vector (WTM); 2KO cells with empty vector (2KOM); 2KO cells with Akt2 (2KOM2) and 2KO cells with Akt1 (2KOM1).

We first examined morphological alterations caused by the loss of the Akt isoforms. As shown in Fig. 1A, 1KO cells are larger and more spread than WT and the other two KO lines, 2KO or DKO. Many Akt1 KO cells have a branched structure (Fig. 1A, indicated with an arrowhead). When stained for F-actin with phalloidin, Akt1 KO cells have highly developed stress fibers compared with WT cells (Fig. 1C, arrow). While stress fibers are caused by Rho activation, they are also observed when Pak activity is reduced because Pak phosphorylates and inhibits a Rho guanine nucleotide exchange factor (31). In contrast to the Akt1 KO cells, Akt2 KO cells are smaller, but have more lamellipodia (Fig. 1A, indicated with an arrow), a structure...
associated with high Rac and Pak activity (1). DKO cells are similar to 2KO cells in cell morphology. Most strikingly, when cells were starved and then stimulated with PDGF, ~75% of the 2KO cells formed dorsal ruffles (Figs. 1, B, C, and E), compared with less than 10% of the wild-type cells. When stained with phalloidin, the dorsal ruffles were seen as actin rings (Figs. 1C, arrowheads). Most cells had a single ring, although some cells had 2–3 actin rings (Fig. 1C). In contrast, no dorsal ruffles were observed in the 1KO cells (Fig. 1, C and E). About 50% of the DKO cells formed dorsal ruffles (Fig. 1, C and E). Dorsal ruffles are vertical ruffles that appear directly behind the leading edge on the dorsal surface (46); they are formed when the cell membrane behind the leading edge projects dorsally, and the ruffles move along the dorsal surface until they disappear (47). Dorsal ruffles are also observed when cells express active mutants of Pak (29). In time-lapse microscopy under phase contrast conditions, we observed that most dorsal ruffles initiated ~150–180 s after PDGF stimulation. The dorsal ruffles were first observed as phase dark rings close to the cell periphery. They then moved over the dorsal surface, decreasing in diameter until they disappeared. Most dorsal ruffles were no longer visible 15 min after PDGF stimulation (Fig. 1B, arrowheads), although in rare cases ruffles were visible as long as 30 min after PDGF stimulation (not shown). We also observed an atypical dorsal ruffling pattern in some of the cells. In the atypical cells, the ruffle initially expanded toward the cell periphery, and after reaching the edge, it reversed direction to contract toward the center like a typical ruffle (Fig. 1B, arrow). Thus, loss of either Akt1 or Akt2 caused aberrant cell ruffling, although in different ways.

We performed two types of controls to address the specificity of these observations. First, we reintroduced Akt2 into 2KO cells expressed from a pMIGR vector (2KOM2). Second, we reexpressed Akt1 in the 2KO cells (2KOM1). The expression of Akt1 and Akt2 were verified using isoform-specific Akt antibodies in Western blots (Fig. 1D). As expected, wild-type levels of PDGF-stimulated dorsal ruffling were observed in the cells reexpressing Akt2 (Fig. 1F). However, expressing Akt1 in the Akt2 knock-out cells failed to rescue the dorsal ruffling phenotype (Fig. 1F).

N-terminal Domains Distinguish Akt Isoforms—Because only Akt2 rescued dorsal ruffling, we defined the region essential for rescue using a domain swap strategy. Based on structural studies, Akt1 and Akt2 can be divided into four regions, the N-terminal PH domain, the linker domain, the catalytic domain and the C-terminal regulatory tail domain (Fig. 2A). A series of chimeras between Akt1 and Akt2 were constructed to swap the domains. Three chimeric constructs rescued the dorsal ruffling (Fig. 2B, upper panel). Interestingly all three of these included the N terminus of Akt2 in the fusion, and all constructs with this domain from Akt2 rescued the cells. The construct with the smallest fragment of Akt2 that rescued cell ruffling, Akt2211, contained the N terminus of Akt2, but the catalytic and regulatory domains of Akt1. We next subdivided the N terminus into the PH and linker domains. There was a partial rescue by the Akt2 PH domain by itself (Akt2111), but rescue was best when the linker domain was also included. The PH domain from Akt1 did not prevent Akt2 from rescuing cells (Akt1222). We conclude that the linker domain distinguishes Akt2 from Akt1, while the other domains are largely interchangeable. We also constructed a kinase-dead mutant of Akt2, K181M and tested if the catalytic activity of Akt2 is required for the rescue. The kinase-dead Akt2 failed to rescue 2KO cells demonstrating that the catalytic activity of Akt2 is required to suppress cell ruffling (Fig. 2B, lower panel).

Increased Cell Invasion through ECM by 2KO Cells—Cell ruffles are derived from lamellipodia and are required for cell migration. To determine if cell migration was affected by loss of Akt, we performed cell migration studies. First, we measured cell migration in a transwell assay (9). Cells were loaded into the upper chamber of a two chamber well separated from the lower chamber by a membrane. The lower chamber contained PDGF as a chemo-attractant. After a period of migration, the membrane was fixed and stained and the upper surface of the membrane was wiped clean of cells. The remaining cells, which are now on the lower surface, were counted and scored as migrated cells. We found that 2KO cells harboring an empty vector (2KOM) have higher migration rates than WT or WT cells harboring an empty vector, while 1KO cells had a lower migration rate (Fig. 3A). Reexpression of Akt2 (2KOM2), but not expression of Akt1 (2KOM1) rescued the migration phenotypes (Fig. 3, A and C). 2KO cells overexpressing Akt1 migrated somewhat faster than the 2KO cells consistent with the known role of Akt1 to stimulate migration. Because a recent study found that peripheral ruffles are required for migration within the plane of the cell, while dorsal ruffles are associated with cell invasion through a cell matrix (47), we also performed the invasion studies using chambers coated with the matrix Matrigel. This matrix is rich in collagen fibers and approximates tissue invasion more accurately than the standard membranes. In Matrigel as well, the 2KOM cells migrated significantly faster (more than twice) compared with WT cells, while 1KO cells had a small reduction in migration rates (Fig. 3B). As seen in the ruffling and transwell experiments, reexpressing Akt2 in the 2KO cells, but not Akt1, rescued the migration phenotypes in the Matrigel assays (Fig. 3, B and D). These data suggest that the increased ruffling of 2KO stimulates migration, especially invasion through ECM.

Loss of Akt2 Leads to Activation of Rac and Pak1—Because the predominant phenotypes associated with loss of Akt2 were effects on cell ruffling, we measured Rac and Pak activities in these cells, because both are required for cell ruffling. For Rac activation assays, cells were starved and stimulated with PDGF. Rac activity was then measured by a Pak-PBD pull-down assay. In this assay, the Rac binding domain of Pak (PBD) fused to GST and bound to glutathione-agarose beads was used to capture Rac from cell lysates. Following incubation, the beads were spun down and the Rac captured by the PBD was probed on Western blots. As the Pak domain only binds the GTP-bound form of Rac, only the activated Rac is recovered. Cell lysates preloaded with GTPyS or GDP were used as positive and negative controls, respectively. As shown in Fig. 4A, the 2KO cells had elevated levels of activated Rac compared with WT cells, while no significant differences were observed in either 1KO or DKO cells.
FIGURE 2. The N terminus distinguishes Akt1 from Akt2 in rescue experiments. A, Akt domain structure and chimeras between Akt1 and Akt2. Akt isoforms have 4 domains: PH domain, Linker domain, catalytic domain (Catalytic), and regulatory tail domain (Regulatory tail). The PH domain for Akt1 and Akt2 are residues 1–113 and 1–112, respectively. All constructs have an N-terminal Myc tag and start from residue 2 (see “Experimental Procedures”). B, upper panel, rescue of dorsal ruffle formation in 2 KO cells by Akt chimeras. Akt2KO MEFs were infected twice with retrovirus packaged in BOSC23 cells in the presence of 8 μg/ml polybrene (see “Experimental Procedures” for details). The infection efficiency was estimated to be ~90–95% by observing GFP under a fluorescence microscope. The cells were grown in Nunc chambers overnight, starved ~24 h stimulated with PDGF and then by fixed and stained. The actin cytoskeleton was stained with phalloidin, and the cells were then observed under a fluorescence microscope. Four random fields each with 50–100 GFP-positive cells were counted in each experiment and the experiment was repeated three times with similar results. The data are shown as the mean ± S.E. B, lower panel, rescue of dorsal ruffles by kinase-dead Akt2 and PH domain and linker domain chimeras. The experiment was done as in the upper panel.
Akt2 Is a Negative Regulator of Rac/Pak Signaling

We also measured the activity of Pak in the various cell lines. As we observed maximum levels of dorsal ruffles in 2KO cells 8–10 min after PDGF stimulation (not shown), we treated the cells with PDGF for 10 min before harvesting the cells. Pak was immunoprecipitated from the cell lysates and then used in kinase assays with histone 4 as a substrate. As shown in Fig. 4C, 2KO cells had elevated Pak activity compared with WT cells. Conversely, 1KO cells had slightly lower Pak activity than WT cells. Finally, DKO cells also had elevated Pak activity, but the activation was less than that of the 2KO cells, consistent with a positive role for Akt1 in Pak regulation. These data also demonstrate that, like Rac, Pak is substantially activated in the 2KO cells, while Pak activity is lower in the 1KO cells. The Rac activity (Fig. 4B) and Pak activity (Fig. 4D) both returned to wild-type levels in the 2KO cells reexpressing Akt2 (2KOM2). Taken together, dorsal ruffle formation and Rac/Pak activation are specifically stimulated by loss of Akt2.

Akt2 Associates with Pak1 and Inhibits Its Activity—The elevated levels of activated Rac and Pak in 2KO cells suggested that Akt2 inhibits their activities. To determine the mechanism of inhibition, we first determined if Akt2 phosphorylates Pak or Rac but were unable to detect any phosphorylation with a phosphoserine 71 specific antibody against Rac (48), or ³²P labeling in kinase assays using recombinant proteins (data not shown). Thus, phosphorylation does not appear to be the mechanism for Akt2 regulation of Pak.

As one important mechanism for Pak regulation is through binding of other proteins, we tested if the two Akt isoforms could physically interact with Pak. We first used a transfection system to determine if they co-immunoprecipitate. We transfected 293T cells with HA-Akt1 or HA-Akt2 along with Myc-Pak1 and then performed immunoprecipitations with anti-Myc to immunoprecipitate Pak1 and probed with anti-HA to detect Akt. We found that both Akt1 and Akt2 were found in the immunoprecipitates, suggesting that both isoforms could physically associate with Pak1 (Fig. 5A). Interestingly, WT Pak1 associated more readily with both Akt1 and Akt2 compared with kinase-dead (KD or K299R) Pak. The latter can only be detected with extended exposure. We also tested if Akt2 formed a complex with Pak in MEF cells by immunoprecipitating Pak1 and blot with Akt2 antibody. As shown in Fig. 5B, Akt2 was detected in the precipitates demonstrating that the endogenous proteins associate. We performed pull-down assays using recombinant proteins as well, but were unable to detect a complex between Akt and Pak (data not shown) suggesting that the association may be indirect, or the direct binding is too weak to be detected under these experimental conditions.

To test the effects of Akt1 and Akt2 on Pak1 activity, we performed Pak kinase assays using either recombinant proteins or Pak from Akt co-transfected cells. As a substrate, we used a peptide derived from the region surrounding amino acid 112 of Bad. This peptide is readily phosphorylated by Pak1, but not by Akt2 compared with kinase-dead (KD or K299R) Pak. The latter can only be detected with extended exposure. We also tested if Akt2 formed a complex with Pak in MEF cells by immunoprecipitating Pak1 and blot with Akt2 antibody. As shown in Fig. 5B, Akt2 was detected in the precipitates demonstrating that the endogenous proteins associate. We performed pull-down assays using recombinant proteins as well, but were unable to detect a complex between Akt and Pak (data not shown) suggesting that the association may be indirect, or the direct binding is too weak to be detected under these experimental conditions.

To test the effects of Akt1 and Akt2 on Pak1 activity, we performed Pak kinase assays using either recombinant proteins or Pak from Akt co-transfected cells. As a substrate, we used a peptide derived from the region surrounding amino acid 112 of Bad. This peptide is readily phosphorylated by Pak1, but not by Akt, so it permits measurement of Pak1 activity in the presence of Akt. It also lacks the Akt phosphorylation site (Bad S136) so it is not likely to be affected by Akt. In an in vitro kinase assay, we found that the kinase active form of Akt2 strongly inhibited Pak1 (Fig. 5C, lane 5), while the kinase dead form of Akt2 showed only a modest inhibition (Fig. 5C, lane 4). We speculate that like kinase dead Pak, the kinase dead Akt2 probably does not associate with Pak as well as active Akt2 and thus does not inhibit Pak as well. In a transfection assay, we found that Akt2 cotransfection inhibited the basal activity of Pak (Fig. 5D, lane 3 versus lane 2). We also observed marginal activation of Pak by Akt1 (Fig. 5D, lane 4 versus lane 2), consistent with the results from Akt1 KO cells that support a positive role for Akt1 in Pak

---

**FIGURE 3.** Deficiency of Akt2 results in increased migration, especially invasion through ECM.

A, increased migration of Akt 2KO cells in a transwell assay. Cells were grown overnight, starved for ~24 h and detached (see “Experimental Procedures”). —2.5 × 10⁵ cells were plated in the upper well and DMEM containing 30 ng/ml PDGF was added to the bottom well. After ~18 h of incubation, the cells migrating across the membrane were stained and counted. Four random fields were counted and the number of the migrated cells was used as an index for migration. The experiment was repeated at least three times with similar results. The data are presented as the mean ± S.E. B, increased invasion of Akt 2KO cells through ECM in a Matrigel assay. Cells were starved for ~24 h and detached. —1.25 × 10⁵ cells were loaded into the upper well, and the experiment carried out as in A. The data are presented as the mean ± S.E. C, rescue of Akt2 KO cells by Akt2 (2KOM2) but not Akt1 (2KOM1). The experiment was done as in A. D, rescue of Akt2 KO cells in Matrigel invasion by Akt2 (2KOM2) but not Akt1 (2KOM1). The experiment was done as in B.
Akt2 Is a Negative Regulator of Rac/Pak Signaling

![Image](https://example.com/image)

FIGURE 4. Elevated Rac and Pak activities in Akt2 KO cells. A, elevated Rac activity in 2KO cells. Whole cell lysates (WCL) were prepared after cells were starved for 24 h and stimulated with 10 ng/ml PDGF for 5 min. Active (GTP-bound) Rac was pulled-down from cell lysates by GST-fused Pak PBD domain and detected in a Western blot with a Rac monoclonal antibody. Cell lysates prepared from WT MEF cells and subsequently loaded with GTPγS or GDP were included to serve as positive and negative controls, respectively (See "Experimental Procedures"). Recombinant His-tagged Rac was used as a marker for molecular weight. B, reexpression of Akt2 in 2KO cells rescued the elevated Rac activity, as detected in a Rac activation assay using PBD pull-down. The experiment was done in a similar way as in A. C, elevated Pak activity in 2KO cells as determined in a Pak kinase assay (KA) using histone 4 as a substrate. Cells were grown overnight, starved for 24 h, and stimulated with PDGF for 10 min before being lysed. Pak was immunoprecipitated with Pak1 antibody and the KA mixture was run on a 10% SDS-PAGE, the phosphate incorporated onto the substrate was detected by autoradiography. D, reexpression of Akt2 in 2KO cells rescued elevated Pak kinase activity. The experiment was done in a similar way with C.

FIGURE 5. Akt association with Pak and effects on Pak activity. A, Akt co-immunoprecipitates with Pak1 in a transfection system. 293T cells were transfected with plasmids expressing a HA-tagged Akt1 or Akt2 and Myc-tagged Pak1, the cells were harvested and Pak was immunoprecipitated from the cell lysates with anti-Myc antibody. The co-immunoprecipitated Akt was detected in a Western blot with anti-HA antibody (irrelevant lanes were cropped out). The experiment was repeated three times with similar results. B, Akt co-immunoprecipitates with Pak in MEF cell lysates. MEF cells were harvested and Pak was immunoprecipitated with a conjugated Pak1 antibody, the co-immunoprecipitated Akt2 was detected in a Western blot with an Akt2 antibody. The recombinant His-Akt2 served as a marker for molecular weight. C, Akt2 strongly inhibits Pak activity in an in vitro kinase assay. Kinase assays contained 0.2 μg of Akt (recombinant, both active and inactive), 0.1 μg of baculovirus Pak and 20 μg of Bad S112 peptide as a substrate in a volume of 25 μl. Pak phosphorylation of the peptides was detected by autoradiography. D, Akt2 inhibits Pak activity in a transfection system. 293T cells were co-transfected with the indicated Akt and Pak plasmids. After expression for 24 h, cells were harvested and Myc-Pak was immunoprecipitated from the lysates. The Pak kinase assay was done as in C. A total of 2 μg of plasmid DNA was used to transfect each well of a 6-well plate, with a ratio of Akt:Pak of 2:1, vector plasmids were used instead of Akt.

DISCUSSION

Akt1, Akt2, and Akt3 are closely related protein kinases with a shared preferred phosphorylation consensus motif. They are all also activated through similar mechanisms involving PI 3-kinase and PDK-1. Yet the different isoforms have distinct roles in the widely studied metabolic and survival pathways as first revealed in knockout mice. In addition to isofom specific roles in the metabolic and survival functions, the isoforms now appear to have different roles in the relatively newly identified cytoskeleton and cell motility functions (8–11, 21, 22, 49).
Akt2 Is a Negative Regulator of Rac/Pak Signaling

Interestingly, where roles for Akt1 and Akt2 in cell motility have been reported, distinct and, in some cases, opposing functions for the two isoforms are often observed. In fibroblasts, Akt1 has repeatedly been found to promote invasion (8–11). Akt1, but not Akt2 is also important for endothelial cell migration through regulation of the nitrous oxide signaling pathway (10). However, three studies found that Akt2, but not Akt1 stimulates motility of breast and ovarian cancer cells, while Akt1 actually inhibits motility in these cells (11, 21, 22). Despite apparently contradictory studies on the roles of the two isoforms, two observations are common to the different studies. The first is that Akt1 promotes motility in fibroblasts and endothelial cells, and secondly, Akt2 promotes motility in epithelial cells. Remarkably, in cells where one isoform stimulates motility, the other isoform usually has a limited or even opposing role.

Several models have been proposed to account for the specificities of closely related isoforms of signaling enzymes such as the Akt family including (1) differential expression in target tissues, (2) distinct effectors, despite common preferred substrates motifs (3), differential compartmentalization of isoforms perhaps through association with unique scaffold proteins.

It is not likely that differential expression of the Akt1 and Akt2 isoforms in target tissues accounts for the different phenotypes observed in the two knock-out cells. This is because we observed similar levels of both isoforms in cells, and more importantly, expression of Akt2 rescued the phenotypes of Akt2 KO cells, both biochemical and biological, whereas overexpression of Akt1 failed to rescue either dorsal ruffling or migration. A similar pattern was observed in studies of hexose transport in adipocytes, where reexpression of Akt2 but not Akt1 rescued the phenotypes of Akt2 knock-out adipocytes (6).

Another model to account for the isoform specificity is subtle differences in the targets of the two Akt isoforms. Since the demonstration of Pak as a downstream target of Akt (14), three mechanisms have been proposed to account for Akt1 activation of Pak: 1) phosphorylation of serine 21 (9), 2) activation of Akt1 rescued the phenotypes of Akt2 knock-out adipocytes (6). When phosphorylation of serine 21 of Pak1 (9). In numerous experiments with Akt2, using either peptide substrates in kinase assays or a serine 21 phosphospecific antibody in Western blots, we have been unable to detect any phosphorylation of Pak at serine 21 by Akt2. While this suggests that the ability to phosphorylate Pak may contribute to the different roles in Pak regulation between the two Akt isoforms, we have also found that there are differences in the ability of different preparations of Akt1 to phosphorylate this site. The differences may, in part, be due to differences in the Akt1 activation procedures. Moreover, because chimeras between Akt1 and Akt2 show that the kinase domains are interchangeable, it is likely that substrate specificity does not distinguish the two isoforms.

A third model for isoform specificity is compartmentalization by binding to specific adaptors or scaffolds. Several scaffold proteins bind preferentially to one of the Akt isoforms. One of them POSH, is an adaptor protein that links the Rac target, MLK to JNK signaling. Of relevance to this study, Akt2 is a negative regulator of the signal through POSH (50). Hence, this signal may lead to some of the observed phenotypes. However, POSH activation does not stimulate motility and cell ruffling, but primarily stimulates JNK and NFκB leading to high levels of apoptosis (51, 52), whereas Akt2 knockouts have elevated levels of cell ruffling and no significant growth or survival phenotypes. This argues against POSH as the primary target in our studies. Another adaptor protein called ArgBP2γ was recently shown to bind to all three Akt isoforms and is specifically phosphorylated by Akt1. Additionally, it binds to Pak and promotes Akt activation of Pak (44). While ArgBP2γ remains an attractive candidate for facilitating activation of Pak by Akt1, it probably does not account for the negative regulation of Rac and Pak by Akt2. We also note that neither POSH nor ArgBP2γ bind the PH and linker domains of Akt, which are critical in distinguishing them. Thus, the relevant scaffolds remain to be identified.

Together, these argue for a distinct mechanism of action by the Akt isoforms on the Rac/Pak pathway based on differences between their PH and linker domains. Interestingly, the linker domain, which is more important in distinguishing the isoforms, is the least homologous domain. In addition, although few biochemical differences are found between the isoforms, Akt2 is predominantly found in the membrane and cytoskeleton, while Akt1 is predominantly found in the cytosol, lending support to the compartmentalization model (6, 21). We speculate that the PH and linker domains of Akt2 direct it to membraneous and cytoskeletal compartments, where Akt2 inhibits Pak. Although we do not find that Akt2 phosphorylates Pak1, it may associate better in its kinase active conformation, since the kinase activity of Akt2 is required for Pak inhibition.

Rac and its effector Pak have been extensively studied and are well established regulators of cell ruffling and cell motility so

FIGURE 6. Roles for Pak and Rac in dorsal ruffle formation. A colocalization of Rac and Pak to the actin ring of dorsal ruffles in PDGF treated 2KO cells. Before stimulation with PDGF following cell starvation, Pak was diffusely located in the whole cell (arrow). In contrast, after stimulation with PDGF, most of the Pak protein translocated to the actin ring of dorsal ruffles (arrow). head. Rac also localized to dorsal ruffles. β, retrovirus expression system was used to express kinase-dead Pak, K299R, in the 2KO cells by establishing stable cell lines through puromycin selection (2KO/KDPak). The cells were starved and stimulated with PDGF. Four random fields each with 50–100 cells were counted, and cells with dorsal ruffling were scored. The data are shown as the mean ± S.E.

4 G. L. Zhou and J. Field, unpublished observations.
they are obvious candidates responsible for the phenotypes in the Akt1 and Akt2 knock-out cells. While most studies have identified activators of Pak, there are a growing number of inhibitors of Rac and Pak. In addition to the traditional GTPase-activating proteins (GAPs) and phosphatases, a number of other proteins inhibit Rac and Pak. The integrin binding protein nischarin binds and inhibits Pak (53). Loss of the scaffold protein VASP also activates Pak and Rac causing increases in cell spreading and reduced cell migration and reorientation, although the direct target of VASP has not been established (54, 55). In addition, merlin, the product of the gene responsible for neurofibromatosis type 2, binds and inhibits Pak. Loss of merlin leads to activation of both Rac and Pak, with subsequent increases in cell ruffling (56, 57). In this case, Pak is probably the primary target of merlin and Rac becomes activated through a feedback loop involving PIX. Where tested, each of these inhibitors acts by binding Pak and inhibiting its kinase activity. Thus, while either Rac or Pak could be the primary target of Akt2, our demonstration that Akt2 can associate with Pak and inhibit its kinase activity suggests that direct association of Akt2 and Pak1 may be responsible for the inhibition. In this case then it is likely that the PIX feedback loop causes the Rac activation in the Akt2 knockouts.

The phenotypes of the 2KO cells also suggest that Pak is a primary target of Akt2. Loss of Akt2 increased migration, especially in the Matrigel assays. Note that the type of ruffle that predominates in the Akt2 knock-out cells is a dorsal ruffle. A study comparing the effects of the two ruffles on cell motility concluded that dorsal ruffles promote invasion through extracellular matrix, while peripheral ruffles primarily promote lateral migration (47). Thus, the more dramatic increase in cell migration in the 2KO cells through an ECM compared with the uncoated membranes is likely to be caused by the increase in dorsal ruffling. Rac is required for cell ruffling, but the roles of its effectors are not as well established. Activation of Rac stimulates peripheral cell ruffling (lamellipodia formation), but not dorsal ruffling (1, 58, 59). However, expression of a dominant negative Rac in cells inhibits dorsal ruffle formation (59). Thus, Rac activity is necessary, but not sufficient for inducing dorsal ruffling. It is likely that only a subset of its effectors mediate dorsal ruffling. Of relevance, expression of hyperactive Pak mutants stimulates dorsal ruffling (29). Thus, the high rates of dorsal ruffling and increased Matrigel invasion also point to Pak as the primary target of Akt2.

In conclusion, we find non-overlapping roles for Akt1 and Akt2 in regulation of Rac/Pak signaling and MEF cell migration. Akt1 is a relatively weak activator of Pak1 while Akt2 is a relatively strong inhibitor of Pak1. Furthermore, the two isoforms play opposing roles in regulating cytoskeleton and cell migration. Akt, especially Akt2, is often over expressed in tumors. Our findings suggest that strategies that inhibit Akt2 alone may increase invasion and metastasis by some tumors.

Acknowledgments—We thank Sung-Ro Jo, Kristin Roovers, and Benjamin Fryer for helpful discussions and Shenghao Jin for the KD Pak1 retrovirus construct.

REFERENCES

1. Bar-Sagi, D., and Hall, A. (2000) Cell 103, 227–238
2. Brazil, D. P., and Hemmings, B. A. (2001) Trends Biochem. Sci. 26, 657–664
3. Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999) Cell 96, 857–868
4. Datta, S. R., Dudek, H., Tao, X., Master, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997) Cell 91, 231–241
5. Downward, J. (1999) Nat. Cell Biol. 1, E33–E35
6. Bae, S. S., Cho, H., Mu, J., and Birnbaum, M. J. (2003) J. Biol. Chem. 278, 49530–49536
7. Morales-Ruiz, M., Fulton, D., Sowa, G., Languino, L. R., Fujiy, Y., Walsh, K., and Sessa, W. C. (2000) Circ. Res. 86, 892–896
8. Higuchi, M., Masuyama, N., Fukui, Y., Suzuki, A., and Gotoh, Y. (2001) Curr. Biol. 11, 1958–1962
9. Zhou, G. L., Zhao, Y., King, C. C., Fryer, B. H., Bokoch, G. M., and Field, J. (2003) Mol. Cell. Biol. 23, 8058–8069
10. Ackah, E., Yu, J., Zoellner, S., Iwakiri, Y., Skurk, C., Shibata, R., Ouchi, N., Easton, R. M., Galasso, G., Birnbaum, M. J., Walsh, K., and Sessa, W. C. (2005) J. Clin. Investig. 115, 2119–2127
11. Yoeli-Lerner, M., Yiu, G. K., Rabinovitz, I., Erhardt, P., Jaulac, S., and Toker, A. (2005) Mol. Cell 20, 539–550
12. Servant, G., Weiner, A. D., Herzmark, P., Balla, T., Sedat, J. W., and Bourne, H. R. (2000) Science 287, 1037–1040
13. Chung, C. Y., Potikyan, G., and Firtel, R. A. (2001) Mol. Cell 7, 937–947
14. Tang, Y., Zhou, H., Chen, A., Pittman, R. N., and Field, J. (2000) J. Biol. Chem. 275, 9106–9109
15. Schurmann, A., Mooney, A. F., Sanders, L. C., Sells, M. A., Wang, H.-G., Reed, J.-C., and Bokoch, G. M. (2000) Mol. Cell. Biol. 20, 453–461
16. Jakobi, R., Moertl, E., and Koeppel, M. A. (2001) J. Biol. Chem. 276, 16624–16634
17. Gnesutta, N., Qu, J., and Minden, A. (2001) J. Biol. Chem. 276, 14414–14419
18. Chen, W. S., Xu, P. Z., Gottlob, K., Chen, M. L., Sokol, K., Shiyanova, T., Roninson, I., Weng, W., Suzuki, R., Tobe, K., Kadowaki, T., and Hay, N. (2001) Genes Dev. 15, 2203–2208
19. Cho, H., Thorvaldsen, J. L., Chu, Q., Feng, F., and Birnbaum, M. J. (2001) J. Biol. Chem. 276, 38349–38352
20. Cho, H., Mu, J., Kim, J. K., Thorvaldsen, J. L., Chu, Q., Crenshaw III, E. B., Kaestner, K. H., Bartolomei, M. S., Shulman, G. I., and Birnbaum, M. J. (2001) Science 292, 1728–1731
21. Arboleda, M. J., Lyons, J. F., Kabbinavar, F. F., Bray, M. R., Snow, B. E., You, C. H., and de la Pompa, J. L. (2005) Mol. Cell 19, 196–206
22. Irie, H. Y., Pearlstein, R. V., Gruneberg, D., Hsia, M., Ravichandran, P., Kothari, N., Natesan, S., and Brugge, J. S. (2005) J. Cell Biol. 171, 1023–1034
23. Manser, E., Leung, T., Salihuddin, H., Zhao, Z.-S., and Lim, L. (1994) Nature 367, 40–46
24. Abo, A., Qu, J., Cammarano, J., Dan, C., Fritsch, A., B. V., Belisle, B., and Minden, A. (1998) EMBO J. 17, 6527–6540
25. Bokoch, G. M. (2003) Annu. Rev. Biochem. 72, 743–781
26. Manser, E., Wang, H.-G., Reed, J.-C., and Bokoch, G. M. (1997) Mol. Cell. Biol. 17, 1129–1143
27. Sells, M. A., and Chernoff, I. (1997) Trends Cell Biol. 7, 162–167
28. Sells, M. A., Knaus, U. G., Bagrodia, S., Ambrose, D. M., Bokoch, G. M., and Chernoff, I. (1997) Cell 89, 202–210
29. Dharmawardhane, S., Schurmann, A., Sells, M. A., Chernoff, J., Schmid, S. L., and Bokoch, G. M. (2000) Mol. Biol. Cell 11, 3341–3352
30. Edwards, D. C., Sanders, L. C., Bokoch, G. M., and Gill, G. N. (1999) Nat. Cell Biol. 1, 253–259
31. Alberts, A. S., Qin, H., Carr, H. S., and Frost, J. A. (2005) J. Biol. Chem. 280, 12152–12161
32. Obermeier, A., Ahmed, S., Manser, E., Yen, S. C., Hall, C., and Lim, L. (1998) EMBO J. 17, 4328–4339
33. Bokoch, G. M., Wang, Y., Bohl, B. P., Sells, M., Quilliam, L. A., and Knaus,
Akt2 Is a Negative Regulator of Rac/Pak Signaling