Akt/PKB is a crucial regulator of diverse cellular processes and contributes to cancer progression. Activation of Akt is essentially dependent on phosphatidylinositol (PI) 3-kinase signaling. Here, we describe a novel mediator of Akt that is independent of PI 3-kinase. This mediator, PIKE-A, is a PIKE isoform and contains GTPase, pleckstrin homology, ArfGAP, and ankyrin repeats domains. PIKE-A directly binds to activated Akt but not PI 3-kinase in a guanine nucleotide-dependent way and stimulates the kinase activity of Akt. Overexpression of PIKE-A enhances Akt activity and promotes cancer cell invasion, whereas dominant-negative PIKE-A and PIKE-A knockdown markedly inhibit these processes. Our results demonstrate that PIKE-A is a physiologic regulator of Akt and an oncogenic effector of cell invasion.

Phosphatidylinositol 3-kinase enhancer (PIKE) is a recently discovered protein, which possesses GTPase activity and stimulates the lipid kinase activity of nuclear PI 3-kinase (1). In PC12 cells treated with nerve growth factor, activation of nuclear PI 3-kinase is mediated by PIKE. PIKE was originally identified in a yeast two-hybrid screening searching for binding partners of the C-terminal domain of protein 4.1N. Nerve growth factor elicits 4.1N to translocate to the nucleus where it binds PIKE and regulates the effects of PIKE on PI 3-kinase. Phospholipase C-γ1 binds PIKE on its third proline-rich domain and acts as a physiological guanine-nucleotide-exchange factor for PIKE. This action is not dependent on the lipase activity of the phospholipase C-γ1 but appears instead to involve its SH3 domain (2–4).

Recently, we identified an alternatively spliced form of PIKE, PIKE-L (bankit475414 (AY128689, human)), which is several hundred amino acids longer than the original form of PIKE, which we now designate PIKE-S. In addition to the GTPase and PH domains shared by PIKE-S and PIKE-L, PIKE-L contains an ArfGAP domain and two ankyrin repeats. PIKE-L binds to Homer, an adaptor protein known to link metabotropic glutamate receptors (mGluR1) to multiple intracellular targets including the inositol 1,4,5-trisphosphate receptor (5–8). We have shown that activation of mGluR1 enhances formation of an mGluR1-Homer-PIKE-L complex leading to activation of PI 3-kinase activity and prevention of neuronal apoptosis (9).

Akt or protein kinase B is a major downstream target of the PI 3-kinase pathway, and plays a key role in a wide variety of cellular functions including metabolism, cell survival, and proliferation (10–12). Akt is fully activated by phosphorylation of threonine 308 and serine 473 upon activation of PI 3-kinase signaling. Akt is frequently constitutively active in many human cancers. Constitutively active Akt occurs because of amplification of Akt genes or as a result of mutations in components of the signaling pathway that activates Akt (13). Akt is a crucial player in regulating diverse tumorigenic activities such as angiogenesis and tissue invasion/metastasis (14).

In the present study we have identified a novel form of PIKE, designated PIKE-A. PIKE-A is co-amplified with CDK4 in a variety of human cancers, and specifically binds to activated Akt but not PI 3-kinase in a GTP-dependent manner, and stimulates Akt kinase activity. The PIKE-A/Akt interaction is mediated through the GTPase domain of PIKE-A and the C-terminal regulatory domain and a portion of the catalytic domain of Akt. Overexpression of wild-type PIKE-A enhances Akt activity, whereas dominant-negative PIKE-A and PIKE-A knockdown inhibit it. Overexpression of wild-type and dominant-negative PIKE-A and PIKE-A knockdown show that PIKE-A regulates human cancer cell invasion, which is dependent upon the Akt.

MATERIALS AND METHODS

Cells and Reagents—HEK293 cells and human glioblastoma LN-Z308, LN487, LN382, SF188, SF767, and U87MG cells were maintained in Dulbecco’s modified Eagle’s medium, whereas neuroblastoma NGL-127 and sarcoma CBL-201 cells were cultured in RPMI1640, supplemented with 10% fetal bovine serum, 2 mg/ml glutamine, and 100 units of penicillin-streptomycin at 37 °C with 5% CO2 atmosphere in a humidified incubator. Mouse monoclonal anti-HA-horseradish peroxidase, anti-Myr-horseradish peroxidase, and anti-GST antibodies were from Sigma. Mouse monoclonal anti-Ser21/9, anti-Thr308, and anti-CDK4 antibodies were from Cell Signaling. Rabbit polyclonal anti-p85 and p110 antibodies were from Santa Cruz Biotechnology, Inc. Anti-PI3K-C and anti-PI3K-N antibodies were raised against GST-PIKE-L (amino acids 1095–1186) and His-PIKE-L (amino acids 268–384) recombinant proteins. Protein A/G-conjugated agarose beads were from Calbiochem. Glutathione-Sepharose 4B was supplied by Amerham Biosciences. GST-GSK3 fusion protein and Crosstide were from Cell Signaling. All the chemicals not included above were from Sigma.

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Fluorescent in Situ Hybridization (FISH) Staining—Experimental procedures for FISH staining on human cancer cell lines are as described (15). One unique sequence 12q13 Bac = 66N19 was employed as a probe for PIKE and one α satellite (repetitive sequence) was used as a probe for chromosome 12 centromere.

Co-immunoprecipitation and in Vitro Binding Assay—Experimental procedures for co-immunoprecipitation and in vitro binding assays are as described (16). For the GTP-dependent association between GST-PIKE-A and Akt, assays were performed as described (17).

In Vitro Immunocomplex Kinase Assay for Akt Kinase—HEK293 cells or glioblastoma cells were infected with adenovirus expressing PIKE-A and Akt, assays were performed as described (17).

Cell Invasion Assay—Cancer cells were plated on 10-cm tissue culture dishes. Approximately 16 h later, cells were infected by Ad-PIKE-A-WT, Ad-PIKE-A-DN, and mock with 25 multiplicity of infection of each adenovirus in 2 ml of media. After 24 h infection, cells were harvested and used for quantification. Cells were counted and resuspended in culture medium at 2 × 10^6 cells/ml. Two ml of cell suspension was added to each chamber (containing Matrigel matrix). After 24 h incubation at 37 °C in 5% CO_2 atmosphere. The assay was performed in duplicate. The membrane was stained with 0.5% crystal violet, and random fields were counted under the light microscope.

Penetratin 1-conjugated Oligonucleotide Preparation—Sense (5'-CTGGGACATCAGATCCGCAATG-3') and antisense (5'-CTGGGACAT-CATGTTGCCGAG-3') oligonucleotides of PIKE-A containing an SH group at the 5' end were conjugated to Penetratin 1 as described previously (19). The oligonucleotides were resuspended in deionized water, an equimolar ratio of Penetratin 1 (Onco) was added, and the mixture was incubated at 37 °C for 1 h. The yield of the reaction, estimated by SDS-PAGE followed by Coomassie Blue staining, was >50%. The conjugated Penetratin 1 was added into serum-starved cells 6 h before treatment.

Statistical Analysis—Results are presented as mean ± S.D. of three or four independent experiments and the statistics were analyzed with Student's t test.

RESULTS

PIKE Is Amplified in Human Malignancy—The chromosome 12q13–15 region is frequently amplified in human sarcomas and brain tumors (20, 21). 12q13–15 amplicon contains two separate core regions, one containing MDM2 and the other containing CDK4 (22–24). Blast search reveals that the PIKE gene is localized at 12q13.3 adjacent to CDK4 (25, 26). In our evaluation of PIKE amplification in the human glioblastoma multiforme, we have identified a new PIKE form (PIKE-A), which was originally identified in the human genome sequencing effort as KIAA0167 (27), and was independently identified by Liu and collaborators (28). PIKE-A, -L, and -S arise from the same PIKE gene. Recently, we have shown that PIKE-L and -S are alternatively spliced variants of PIKE (9). Gene structure analysis reveals that PIKE-A results from different transcription initiation sites from that of PIKE-L and -S. PIKE-A contains the GTPase, PH, ArfGAP, and two ankyrin repeat domains present in PIKE-L but lacks the proline-rich domain containing the N terminus, which binds protein 4.1N, PI 3-kinase, and phospholipase C-γ1 (Fig. 1A).

To investigate PIKE gene amplification, we performed FISH on a variety of human sarcoma and brain tumor cell lines. PIKE is substantially amplified (Fig. 1, arrow, red) in both the neuroblastoma cell line NGP-127 (Fig. 1B) and glioblastoma cell line LN-Z308 (Fig. 1C), with a normal copy of PIKE (arrowhead) on chromosome 12 (green). We have observed similar results on the human glioblastoma cell line CRL-2061 (data not shown), and in primary brain tumors (green, PIKE; red, chromosome 12, Fig. 1D). The percentage of samples with PIKE amplification is summarized in Table I. To further examine amplification of PIKE in these cancer cell lines, we conducted Northern blotting with probes specific for the PIKE-A isoform (nucleotides 1–216; amino acids 1–72). PIKE-A mRNA is highly expressed in NGP-127, CRL-2061, and LN-Z308 (Fig. 1E). Glyceraldehyde-3-phosphate dehydrogenase was employed as a loading control. By contrast, Northern blotting analysis with a probe from the proline-rich domain region of PIKE-L and -S (nucleotides 1–1152, amino acids 1–384) reveals two bands with ~5 and 4 kb in brain sample, corresponding to PIKE-L and -S. Compared with the robust PIKE-L signal in brain, negligible PIKE-L is observed in LN-Z308 cells in the absence of PIKE-S expression. Western blotting analysis with anti-PIKE-C antibody (against C terminus) demonstrates that PIKE-A is overexpressed in these cancer cells in the absence of PIKE-L expression (Fig. 1F, top panel). However, substantial PIKE-L but not PIKE-A is expressed in the brain sample (Fig. 1F, lower right panel). Consistent with Northern blotting analysis, anti-PIKE-N antibody (against residues: 268–384 in PIKE-L and -S) reveals negligible PIKE-L in LN-Z308 cells compared with brain sample (Fig. 1F, lower left panel).

PIKE-A Binds Akt—PIKE-S binds to and activates nuclear PI 3-kinase, which is a portion of the signaling cascade that acts through the mitogenic protein kinase Akt (PKB) (10–12). However, PIKE-A lacks the N-terminal domain that binds to PI 3-kinase. Because PIKE-A, but not PIKE-L or -S, is overexpressed in human tumors, we hypothesized that PIKE-A might interact directly with Akt in the absence of PI 3-kinase inter-
To test this hypothesis, we performed a co-immunoprecipitation assay in human cancer cell lines. Immunoprecipitation with an antibody to Akt leads to the co-precipitation of PIKE-A in two human cancer cell lines, NGP-127 and CRL-2061, which overexpress endogenous PIKE-A. Co-precipitation was not observed in a third cell line, U87MG, which does not overexpress PIKE-A (Fig. 2A, upper left and middle panels). Reciprocal co-immunoprecipitation from LN-Z308 cells using anti-PIKE-C antibody demonstrates that Akt specifically associates with PIKE-A (Fig. 2A, upper right panel). Akt is frequently constitutively active in many types of human cancer. Western blotting analysis with mouse monoclonal anti-phospho-Ser473 reveals that Akt is phosphorylated in all of these cells (Fig. 2A, lower right panel). To determine whether exogenously transfected PIKE-A and Akt colocalize in host cells, we co-transfected Myc-PIKE-A and RFP-Akt into the sarcoma cell line CRL-2061, and conducted immunofluorescent staining. Immunohistochemistry analysis reveals substantial co-localization of the two proteins in punctate particles distributed throughout the cytoplasm and nucleus (Fig. 2B, top panel). N-terminal tagged RFP-Akt translocates to the plasma membrane upon EGF stimulation (Fig. 2B, second panel, middle). Accordingly, both transfected RFP-Akt and endogenous Akt were phosphorylated in cells treated with EGF (Fig. 2B, second panel, lower right), indicating that N-terminal-tagged Akt acts as the endogenous Akt. Staining for endogenous Akt and PIKE-A in the same cells demonstrates sporadic co-localization in both the cytoplasm and nucleus (Fig. 2B, third panel). The colocalization discrepancy between the cotransfected and endogenous proteins in glioblastoma cells could be because of the overexpressed PIKE-A, resulting from PIKE gene amplification, overwhelms the endogenous Akt level, but in cotransfected cells, both exogenous PIKE-A and Akt are overexpressed. In contrast to the exclusively nuclear localization of PIKE-S, PIKE-A occurs in both the cytoplasm and nucleus, consistent with the previous observation that transfected FLAG-tagged PIKE-A distributes in both cytoplasm and nu-
FIG. 2. PIKE-A associates with endogenous Akt in human cancer cells. A, PIKE-A co-immunoprecipitates with Akt in NGP-127, CRL-2061, and LN-Z308 cells but not in U87MG cells. One mg of cell lysate of NGP127, CRL-2061, and U87MG cells was incubated with 1 μg of rabbit polyclonal anti-Akt antibody or rabbit IgG employed as a negative control, and 25 μg of whole cell lysate was employed as input. The co-immunoprecipitated proteins were analyzed with anti-PIKE-C antibody (left and middle panels). Reciprocal co-immunoprecipitation was also performed in LN-Z308 cells with anti-PIKE-C antibody (right panel). The coprecipitated proteins were analyzed with anti-Akt antibody. Endogenous PIKE-A specifically associates with Akt in NGP-127, CRL-2061, and LN-Z308 cells but not in U87MG cells. Western blotting analysis of PIKE-A, CDK4, and phospho-Akt in these three cell lines (lower panels). B, PIKE-A and Akt co-localize in cancer cells. PIKE-A and Akt colocalize in sarcoma CRL-2061 cells. Sarcoma cells were cotransfected with Myc-PIKE-A and RFP-Akt, and stained with mouse monoclonal anti-Myc antibody and fluorescein isothiocyanate-conjugated anti-mouse secondary antibody. Cotransfected Myc-PIKE-A and RFP-Akt were punctately distributed and colocalized in CRL-2061 cells (top panel). Transfected RFP-Akt translocates to plasma membrane and is phosphorylated upon EGF treatment (second panel). Endogenously overexpressed PIKE-A and Akt also punctately distribute and colocalize in 2061 cells (third panel). Endogenously overexpressed PIKE-A localizes in both the cytoplasm and nucleus, whereas coamplified CDK4 displays a different, predominantly cytoplasmic distribution (bottom panel). Bar scale: 10 μm.
cleus (28). By contrast, CDK4, which is co-amplified together with PIKE-A in numerous tumors, displays a different, predominantly cytoplasmic distribution (Fig. 2B, bottom panel).

PIKE-A Specifically Interacts with Activated Akt in a Guanine Nucleotides-dependent Manner—PIKE-S associates with PI 3-kinase in a GTP-dependent way (1). We wondered whether PIKE-A binding to Akt is also regulated by guanine nucleotides. To explore this possibility, we conducted an in vitro binding assay employing purified recombinant GST-PIKE-A protein. We incubated glutathione beads bound to GST-PIKE-A with lysates of HEK293 cells transfected with HA-Akt. The transfected cells were stimulated with or without IGF-1 for 5 min. After 3 h incubation at 4 °C, the associated proteins were analyzed by Western blotting with anti-HA antibody. GST-PIKE-A-WT associates with active HA-Akt in the presence of GTP or GDP but not in the absence of guanine nucleotides. Binding by active HA-Akt appears preferential with GTP than GDP. By contrast, GST-PIKE-A-WT faintly interacts with inactive HA-Akt under both control and guanine nucleotides conditions (top panel). Equal amounts of GST fusion proteins were employed (middle panel). Phosphorylation of Akt correlates its association with GST-PIKE-A-WT glutathione beads (bottom panel). B, GST-PIKE-A-DN associates with both active and inactive HA-Akt in the presence of guanine nucleotides. Purified GST-PIKE-A-DN even binds active HA-Akt but not inactive HA-Akt in the absence of guanine nucleotides (top panel). Equal amounts of GST fusion proteins were employed (middle panel). Phosphorylation of Akt correlates with its association with GST-PIKE-A-DN glutathione beads (bottom panel). C, GST alone does not bind to either the active or inactive Akt. D, Akt phosphorylation status in HEK293 cells stimulated with or without 50 ng/ml IGF-1. E, Akt phosphorylation mediates its association with PIKE-A. HA-Akt wild-type and mutant (T308A,S473A) were, respectively, cotransfected with Myc-PIKE-A into HEK 293 cells, and treated with or without IGF-1. Transfected Akt proteins were immunoprecipitated with anti-HA antibody, the coprecipitated proteins were analyzed with anti-Myc antibody. IGF-1 stimulates wild-type but not mutant Akt that binds to PIKE-A (top panel). Equal amounts of PIKE-A and Akt were expressed (middle and bottom panels).

FIG. 3. PIKE-A binds to activated Akt in a guanine nucleotide-dependent way. A, in vitro binding assay. Purified GST-PIKE-A-WT and GST-PIKE-A-DN were loaded with 1 mM GTPγS or GDPβS, and incubated with the cell lysate of HEK293 cells, which were transfected with HA-Akt. The transfected cells were stimulated with or without IGF-1 for 5 min. After 3 h incubation at 4 °C, the associated proteins were analyzed by Western blotting with anti-HA antibody. GST-PIKE-A-WT associates with active HA-Akt in the presence of GTP or GDP but not in the absence of guanine nucleotides. Binding by active HA-Akt appears preferential with GTP than GDP. By contrast, GST-PIKE-A-WT faintly interacts with inactive HA-Akt under both control and guanine nucleotides conditions (top panel). Equal amounts of GST fusion proteins were employed (middle panel). Phosphorylation of Akt correlates its association with GST-PIKE-A-WT glutathione beads (bottom panel). B, GST-PIKE-A-DN associates with both active and inactive HA-Akt in the presence of guanine nucleotides. Purified GST-PIKE-A-DN even binds active HA-Akt but not inactive HA-Akt in the absence of guanine nucleotides (top panel). Equal amounts of GST fusion proteins were employed (middle panel). Phosphorylation of Akt correlates with its association with GST-PIKE-A-DN glutathione beads (bottom panel). C, GST alone does not bind to either the active or inactive Akt. D, Akt phosphorylation status in HEK293 cells stimulated with or without 50 ng/ml IGF-1. E, Akt phosphorylation mediates its association with PIKE-A. HA-Akt wild-type and mutant (T308A,S473A) were, respectively, cotransfected with Myc-PIKE-A into HEK 293 cells, and treated with or without IGF-1. Transfected Akt proteins were immunoprecipitated with anti-HA antibody, the coprecipitated proteins were analyzed with anti-Myc antibody. IGF-1 stimulates wild-type but not mutant Akt that binds to PIKE-A (top panel). Equal amounts of PIKE-A and Akt were expressed (middle and bottom panels).

PIKE-A Mediates Cell Invasion via Up-regulating Akt

In proteins of the Ras family, guanine nucleotide binding is critically dependent on a specific lysine and serine, with mutation of one or the other or both leading to dominant-negative proteins that bind to but fail to activate their targets (1). We constructed an analogous dominant-negative by mutating the critical lysine (K84A) and serine (S85N) of PIKE-A. This construct, PIKE-A-K84A,S85N, designated as PIKE-A-DN, binds Akt promiscuously even in cells not treated by the growth factor IGF-1 and, in treated cells, binding occurs even in the absence of guanine nucleotides (Fig. 3B). The phosphorylation status of bound Akt correlates with its association with recombinant GST-PIKE-A (Fig. 3, A and B, bottom panel). As a control, we performed the same experiment with GST alone, and observed no binding regardless of guanine nucleotides or IGF-1 treatment (Fig. 3C). We have also verified the phosphorylation of Akt in cells treated with or without IGF-1 (Fig. 3D).

PIKE-A strongly binds to activated Akt, indicating that Akt phosphorylation might play a role in mediating its association...
Akt phosphorylation. PIKE-A fragments and GST-Akt. E by guanine nucleotides or Akt activation status. The interaction between the GTPase domain and Akt is not influenced by growth factor stimulation, suggesting that the extreme N-terminal portion manipulates PIKE-A binding activity in response to upstream stimuli. We have verified the expression of the transfected constructs in lysates of HEK293 cells (Fig. 4D). Phosphorylation of GST-Akt occurs in growth factor-stimulated cells but not in serum-starved cells (Fig. 4E). We have observed the same binding activity with IGF-1 treatment (data not shown).

To further examine whether the GTPase domain itself binds Akt, we conducted an in vitro binding assay with GST-GTPase. We incubated glutathione beads bound to GST-GTPase with lysates of HEK293 cells transfected with HA-Akt, which was stimulated with or without EGF for 5 min. The interaction between the GTPase domain and Akt is not influenced by the presence of GTP·S or GDP·S, regardless of Akt activation. As a control, we employed GST alone to perform the binding assay, and we did not observe any Akt binding (Fig. 4F). This result demonstrates that the GTPase domain alone is sufficient to bind Akt. The full-length wild-type PIKE-A that binds Akt upon growth factor stimulation indicates that their interaction is regulated under physiological condition.

**PIKE-A Mediates Cell Invasion via Up-regulating Akt**

with PIKE-A. To examine this idea, we cotransfected HEK293 cells with Myc-PIKE-A and HA-Akt wild-type or mutant with threonine 308 and serine 473 to alanine, and stimulated the transfected cells with or without IGF-1. Immunoprecipitation reveals that robust binding by PIKE-A to wild-type but not mutant Akt is demonstrable in cells treated with IGF-1 compared with untreated cells (Fig. 3E, top panel). Equal amounts of transfected PIKE-A and Akt are verified (Fig. 3E, middle and bottom panels).

**The GTPase Domain of PIKE-A Directly Binds Akt**—To ascertain the portion of PIKE-A that binds Akt, we co-transfected HEK293 cells with various Myc-tagged fragments of PIKE together with GST-Akt, and stimulated the serum-starved cells with or without EGF for 5 min (Fig. 4, A–C). The transfected GST-Akt protein was pulled down by glutathione beads. Immunoblotting analysis of the co-precipitated proteins reveals that the activated Akt binds robustly to PIKE-A in growth factor-treated cells compared with the serum-starved cells. By contrast, binding is similar for the dominant-negative PIKE-A-DN and PIKE-A fragment lacking the N-terminal 71 amino acids in both cells. Binding is dependent on the GTPase domain of PIKE-A, as it is completely abolished in the form of PIKE-A lacking this domain (Fig. 4B, lane 4). Surprisingly, the C-terminal fragment containing ArfGAP and two ankyrin repeat domains alone associates with Akt whether or not it has been activated. This observation indicates that the PH domain somehow masks the C-terminal region mediating its interaction with Akt. The form of PIKE-A with the N-terminal 71-amino acid deletion strongly associates with Akt regardless of growth factor stimulation, suggesting that the extreme N-terminal portion manipulates PIKE-A binding activity in response to upstream stimuli. We have verified the expression of the transfected constructs in lysates of HEK293 cells (Fig. 4D). Phosphorylation of GST-Akt occurs in growth factor-stimulated cells but not in serum-starved cells (Fig. 4E). We have observed the same binding activity with IGF-1 treatment (data not shown).

To further examine whether the GTPase domain itself binds Akt, we conducted an in vitro binding assay with GST-GTPase. We incubated glutathione beads bound to GST-GTPase with lysates of HEK293 cells transfected with HA-Akt, which was stimulated with or without EGF for 5 min. The interaction between the GTPase domain and Akt is not influenced by the presence of GTP·S or GDP·S, regardless of Akt activation. As a control, we employed GST alone to perform the binding assay, and we did not observe any Akt binding (Fig. 4F). This result demonstrates that the GTPase domain alone is sufficient to bind Akt. The full-length wild-type PIKE-A that binds Akt upon growth factor stimulation indicates that their interaction is regulated under physiological condition.

**PIKE-A Associates with Akt through Its Regulatory Domain and Partial Catalytic Domain**—To assess which portion of Akt is required for its association with PIKE-A, we prepared a variety of GST-tagged Akt fragment constructs (Fig. 5A), and cotransfected them into HEK293 cells together with Myc-PIKE-A. The transfected cells were serum starved overnight and stimulated with EGF for 5 min. The transfected GST-Akt fusion proteins were pulled down by glutathione beads, and immunoblotting analysis of the co-precipitated proteins demonstrates that full-length Akt and Akt containing the regulatory domain but not on fragment 5 (data not shown). The expression of transfected Akt fragments and PIKE-A were confirmed by Western blotting (Fig. 5C).

Phosphorylation of Akt mediates its association with PIKE-A and the regulatory domain (Fragment 5) is required for this interaction. To examine whether this portion is phosphorylated upon growth factor treatment, we transfected HEK293 cells with various GST-tagged Akt fragments and stimulated with EGF. Western blotting analysis of the GST pull-down proteins reveals that serine 473 is phosphorylated on fragments containing the regulatory domain but not on fragment 7, which lacks it (Fig. 5D, top panel). Equal amounts of GST-Akt truncates were verified (Fig. 5D, bottom panel).

**PIKE-A Enhances Akt but Not PI 3-Kinase Activity**—To explore the functional consequence of PIKE-A/Akt binding, we evaluated Akt kinase activity in HEK293 cells infected with adenovirus expressing PIKE-A or dominant-negative PIKE-A-DN (Fig. 6). EGF elicits a 2–3-fold augmentation of Akt activity. Infection of wild-type PIKE-A leads to a further dou-
bling of activity. By contrast, infection with PIKE-A-DN decreases Akt activity by more than 50%. In cells not treated with growth factor, PIKE-A-DN exposure also markedly reduces basal Akt activity (Fig. 6A). Similar results were observed with IGF-1 (data not shown). We have verified equal levels of immunoprecipitated endogenous Akt for in vitro kinase assay and expression of infected PIKE-A constructs (Fig. 6B).

The GTPase domain and C-terminal ArfGAP-ankyrin repeat domains of PIKE-A bind Akt (Fig. 4, B, C, and F). To evaluate which domain is responsible for increasing kinase activity, we conducted the in vitro kinase assay with GST-GSK3 as a substrate on purified Akt (Cell Signaling, Inc.) in the presence of various PIKE-A fragments. GST-GTPase but not GST-PH or GST-ArfGAP/Ank recombinant protein robustly stimulates Akt activity regardless of guanine nucleotides (Fig. 6C, top panel). GST alone has no effect on Akt activity (data not shown). Equal amounts of GST fusion proteins were employed (Fig. 6C, middle panel). To investigate the possibility that full-length PIKE-A binds Akt under both GTP and GDP conditions, but only GTP-bound PIKE-A enhances Akt activity, we performed the in vitro binding assay with active Akt and purified GST-PIKE-A, loaded with 1 mM ATPγS, GTPγS, and GDPβS. Substantial Akt is associated with guanine nucleotide-bound PIKE-A. By contrast, faint amounts of Akt bound to the no nucleotide added control condition. ATPγS caused modest binding of Akt to PIKE-A that was considerably less than caused by GTPγS or GDPβS (Fig. 6C, third panel). Compared with ATPγS or control, demonstrable Akt kinase activity occurs under the GDPβS condition. Quantitative analysis with densitometry reveals that stimulation caused by GDPβS is about 29% that caused by GTPγS with phospho-GSK3 values normalized against bound Akt values (Fig. 6C, bottom panel). Thus, these data indicate that the GTPase domain of PIKE-A stimulates Akt kinase activity in the presence of GTP, consistent with the observation that PIKE-A enhances Akt activity in transfected cells regardless of growth factor stimulation (Fig. 6A).

PIKE-A binds active Akt and enhances its activity probably through maintaining Akt active conformation or initiating its activation. To test this possibility, we employed antennapedia peptide (Penetratin 1)-mediated intracellular delivery, a technique that has been widely and successfully used for gene knockdown (19). U87MG cells were treated with Penetratin 1-conjugated antisense oligonucleotide to knockdown PIKE-A, whereas sense oligonucleotide was utilized as a control. EGF-stimulated Akt phosphorylation is substantially diminished in antisense but not sense-treated cells (Fig. 6D, top panel). Equal amounts of Akt were employed (Fig. 6D, middle panel). Compared with sense oligonucleotide, Penetratin 1-conjugated antisense markedly decreases the expression of PIKE-A. As a control, the expression of poly(ADP-ribose) polymerase is not affected (Fig. 6D, bottom panel). Similar results were observed in LN-Z308 cells as well (data not shown). These data demonstrate that PIKE-A plays an essential role in maintaining and initiating Akt activation.

PIKE-A might stimulate Akt by binding and augmenting...
FIG. 6. PIKE-A stimulates Akt kinase but not PI 3-kinase activity. **A**, *in vitro* Akt kinase assay. HEK293 cells were infected with adenovirus expressing PIKE-A WT or DN, and the infected cells were serum starved overnight. The infected cells were treated with or without EGF or IGF-1 for 5 min, and the endogenous Akt was immunoprecipitated with immobilized anti-Akt antibody. 30 μM Crossstide was employed as substrate in the kinase assay in the presence of 10 μCi of [γ-32P]ATP. After 30 min incubation at 30 °C, 25 μl of solution was spotted on P81 cellulose paper. After extensive washing with 0.75% phosphoric acid and 75% ethanol, the radiolabeled samples were analyzed in a liquid scintillation counter. **B**, equal amounts of Akt was immunoprecipitated and employed in the kinase assay (*top panel*); similar levels of PIKE-A-WT and DN were expressed (*bottom panel*). **C**, GTPase domain of PIKE-A stimulates Akt activity. *In vitro* kinase assay with the purified Akt in the presence of various PIKE-A fragments and 1 mM GTP, GDP, or buffer alone. GST-GTPase but not GST-PH or GST-ArfGAP/Ank recombinant protein robustly stimulates Akt activity regardless of guanine nucleotides (*top panel*). Equal amounts of GST fusion proteins were employed (*middle panel*). Purified GST-PIKE-A (full-length), loaded with 1 mM ATPγS, GTPγS, GDPγS, or buffer alone, was incubated with purified Akt and 1 μg/ml bovine serum albumin. After extensive wash, the glutathione bead-associated Akt was analyzed by immunoblotting analysis (*third panel*). Akt kinase activity was conducted with GST-GSK3 as substrate and analyzed with specific phospho-GSK3 antibody. The relative intensity of Akt and phospho-GSK3 bands was determined with densitometry analysis, and the values for both Akt and phospho-GSK3 were set at 1.0 under control conditions (*bottom panel*). **D**, PIKE-A initiates and maintains Akt activation. Serum-starved U87MG cells were treated with Penetratin 1-conjugated antisense and sense oligonucleotides of PIKE-A. After 6 h, the cells were stimulated with EGF for various time points, and analyzed by Western blotting with anti-phospho-Akt antibody. Antisense but not sense oligonucleotide diminishes Akt activation (*top panel*). Equal amounts of Akt were employed (*middle panel*). The expression of PIKE-A was decreased by antisense but not sense oligonucleotide. By contrast, the expression of poly(ADP-ribose) polymerase (PARP) is not changed (*bottom panel*). **E**, PIKE-A does not bind PI 3-kinase. HEK293 cells were cotransfected with HA-tagged PI 3-kinase and Myc-tagged PIKE-S or PIKE-A, respectively, PIKE-S and PIKE-A were immunoprecipitated with anti-Myc antibody. The co-precipitated proteins were analyzed with anti-HA antibody, both p85 and p110 or Myc-tagged PIKE-S and -A were expressed. **F**, PIKE-A does not affect PI 3-kinase activity. HA-tagged PIKE-A or PIKE-S were cotransfected with PI 3-kinase into HEK293 cells, respectively. PI 3-kinase activity assay was performed with the immunocomplex. Wortmannin was included for verifying the specificity of the PI 3-kinase activity. The relative amount of PI-3-P was quantitated and presented at the bottom of the TLC plate.
activity of nuclear PI 3-kinase, part of the Akt activation cascade. We examined this possibility by co-immunoprecipitation of transfected HA-tagged p85 and p110 subunits of PI 3-kinase with Myc-tagged PIKE-S or PIKE-A. As expected, PIKE-S binds p85 and p110 subunits of PI 3-kinase (Fig. 6E). However, PIKE-A fails to bind to either protein, consistent with its lacking the N-terminal portion of PIKE-S that mediates such binding (1). The expression of transfected PI 3-kinase and PIKE-A constructs has been confirmed (Fig. 6F). Consistent with its failure to bind PI 3-kinase, PIKE-A does not affect PI 3-kinase activity. By contrast, PIKE-S markedly enhances PI 3-kinase activity. As a control, PI 3-kinase activity was measured using Matrigel invasion assay (Fig. 6G). The in vitro Akt kinase assay was performed with the immunoprecipitated endogenous Akt. GST-GSK3 was employed as substrate and its phosphorylation status was analyzed with specific phospho-GSK3 antibody (fourth panel). The cell lysate is also analyzed by Western blotting with anti-phospho-Erk1/2 and phospho-JNK antibodies. Immunoblotting analysis demonstrates that Erk1/2 but not JNK kinase cascade was up-regulated upon PIKE-A overexpression (fifth and bottom panels). Consistent with PIKE-A knockdown, Akt phosphorylation was also diminished in antisense but not sense oligonucleotide-treated cells (bottom panel).

FIG. 7. PIKE-A enhances cancer cell invasion through up-regulating Akt. A, cell invasion assay. Human glioblastoma line LN-Z308 and U87MG were, respectively, infected with adenovirus expressing wild-type PIKE-A and PIKE-DN. After 24 h infection, the infected cells were analyzed with Matrigel invasion assay. In PIKE-A amplified LN-Z308 cells, overexpression of PIKE-A stimulates cell invasion compared with control cells. In U87MG cells, overexpression of PIKE-A substantially stimulates cell invasion. By contrast, infection with dominant-negative PIKE-A markedly diminishes cell invasion in both cells (top panel). The mean ± S.D. for each condition is shown (p < 0.005 versus control, Student’s t test). For the cell proliferation assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay the same amount of both cells were seeded into a 24-well plate. After 12 h incubation, the cell number was determined every 4 h utilizing colorimetric assay (bottom panel). B, PIKE-A stimulates Akt activity in glioblastoma cells. LN-Z308 and U87MG cells were, respectively, infected with adenovirus expressing PIKE-A-WT and PIKE-DN, after 24 h infection, the cell lysate was analyzed with phospho-threonine 308 and phospho-serine 473 antibodies. Robust phosphorylation on Thr308 and Ser473 of Akt was observed in wild-type PIKE-A but not control adenovirus-infected cells. By contrast, the phosphorylation on both residues was completely inhibited by dominant-negative PIKE-A-DN (second and third panels), although equal amounts of Akt were present in all samples (top panel). C, dominant-negative Akt abolishes LN-Z308 and U87MG cell invasion. D, PIKE-A knockdown decreases cell invasion. Serum-starved cells were treated with Penetratin 1-conjugated antisense and sense oligonucleotides of PIKE-A. After 6 h, the treated cells were analyzed with Matrigel invasion assay. E, PIKE-A expression was selectively decreased by antisense but not control sense oligonucleotide in both LN-Z308 and U87MG cells (top panel). By contrast, the poly(ADP-ribose) polymerase (PARP) expression level was not changed (middle panel). Consistent with PIKE-A knockdown, Akt phosphorylation was also diminished in antisense but not sense oligonucleotide-treated cells (bottom panel).
sion effects for LN-Z308 and U87MG cells might be because of the difference in cell growth rates over the 16-h invasion experiment, we monitored the cell proliferation rate and observed a virtually identical growth curve for both LN-Z308 and U87MG cells (Fig. 7A, bottom panel). Furthermore, expression of different PIKE-A constructs does not significantly affect the proliferation rates over the 16-h experiment period (data not shown).

In the LN-Z308 and U87MG cells employed for cell invasion studies, we determined the phosphorylation status of Akt. Infection with wild-type PIKE-A markedly increases levels of phospho-Akt-308 and phospho-Akt-473 in both cell lines (Fig. 7B). Akt kinase activity, monitored by the phosphorylation of GSK3α, a downstream target of Akt, is also augmented by wild-type PIKE-A. By contrast, we detect negligible phospho-Akt or phospho-GSK3α in cells infected with PIKE-A-DN. In both cells the basal levels of phospho-Akt and phospho-GSK3α are depleted by treatment with PIKE-A-DN. To examine the specificity of Akt activation by PIKE-A, we determined the phosphorylation of MAP kinases, the phosphorylation of p44/42 Erk1/2 but not JNKs is increased by wild-type PIKE-A, indicating that mitogenic proteins are selectively up-regulated by PIKE-A.

To examine further whether PIKE-A stimulates cell invasion through Akt, we employed adenovirus expressing dominant-negative Akt (K179A,T308A,S473A). Compared with control samples, dominant-negative Akt substantially diminishes cell invasion for both LN-Z308 and U87MG (Fig. 7C). To determine further the role of PIKE-A in cell invasion, we knocked down PIKE-A utilizing Penetratin 1-conjugated antisense oligonucleotide (19). Cell invasion is markedly decreased in antisense but not sense oligonucleotide-treated cells (Fig. 7D). Antisense but not sense oligonucleotide abolishes PIKE-A expression in both cells. By contrast, poly(ADP-ribose) polymerase expression is not affected. Consistent with this observation, Akt phosphorylation is decreased in antisense but not sense oligonucleotide-treated cells (Fig. 7E).

**DISCUSSION**

Our main finding is that PIKE-A preferentially binds to Akt in a guanine-nucleotide-dependent way, stimulating its kinase activity, and is a physiologic mediator of Akt actions on cellular invasion. We examined the interactions of PIKE-A with Akt, because of the pronounced overexpression of PIKE-A in a variety of human tumors and speculate that its activation of Akt may contribute to the progression of these tumors. Because both PIKE-S and PIKE-L possess the GTPase domain that accounts for the kinase enhancement effect. PIKE-A binds to Akt in both GTP- and GDP-bound stages. It differentially bind to their downstream effectors. However, PIKE-A binds to Akt in both GTP- and GDP-bound stages. It has been shown before that Arafaptin (Por protein) interacts with Rac GTPase with the same affinities under both GTP and GDP conditions (34, 35). The non-bound status transiently exists as an intermediate during the transition. Interestingly, PIKE-A-DN associates with Akt from IGF-stimulated cells, but not from unstimulated cells, in the absence of any added guanine nucleotides (Fig. 3B, top panel). Presumably, phosphorylation of Akt elicits different conformations, leading to the binding motif of Akt accessible for GTPase-mutated PIKE-A. This observation is further supported by the selective interaction between PIKE-A and GST-Akt with and without EGF treatment (Fig. 4, B and C).

Both the N-terminal GTPase domain and C-terminal ankyrin repeat domain of PIKE-A are involved in association with the regulatory and partial catalytic domains of Akt (Figs. 4 and 5). Full-length PIKE-A preferentially binds active Akt in the presence of guanine nucleotides, however, its N-terminal fragment (residue 1–356) containing the GTPase domain binds both active and inactive Akt in a nucleotide-independent manner (Fig. 4P). Presumably, in the full-length PIKE-A protein, the binding domains might be sheltered from Akt in the absence of guanine nucleotides. When bound to GTP or GDP, the N or C terminus of PIKE-A might be accessible for active Akt because of the conformational change. However, the binding domain in the truncate (amino acids 1–356) might be constitutively exposed. This conformational explanation is indirectly supported by the observation that the fragment of PIKE-A (341–836) failed to bind to active Akt, even though it contains the ankyrin repeats domain (Fig. 4B, lane 4). Although both N-terminal GTPase and C-terminal ankyrin repeat domains bind to Akt, only the GTPase domain stimulates both active and inactive Akt kinase activity (Fig. 6C), suggesting it is the GTPase domain that accounts for the kinase enhancement effect.
PIKE-A Mediates Cell Invasion via Up-regulating Akt

Binding to wild-type PIKE-A increases phosphorylation of Akt on both Ser\textsuperscript{473} and Thr\textsuperscript{308}, with a corresponding augmentation in \textit{in vitro} Akt kinase activity. By contrast, the dominant-negative PIKE-A inhibits phosphorylation of Akt and substantially diminishes its kinase activity (Fig. 7B). The molecular mechanism of how PIKE-A regulates Akt remains to be established. The likely scenario is that wild-type PIKE-A preferentially binds phosphorylated Akt on the C terminus (Fig. 5), where both phosphorylated Thr\textsuperscript{308} and Ser\textsuperscript{473} residues are located. PIKE-A-WT may mask the phosphorylated region and shield it away from protein phosphatases, so that the phosphorylated Akt is preferentially accumulated. However, dominant-negative PIKE-A interacts with both active and inactive Akt, insulating bound Akt from phosphorylation.

Numerous proteins bind Akt, but the extent to which they physiologically regulate Akt activity is unclear (11, 36–44). Our discovery that growth factor stimulation triggers the phosphorylated Akt to associate with PIKE-A in a guanine nucleotide-dependent way demonstrates that GTPase PIKE-A provides a new mechanism whereby growth factors and other cellular stimuli regulate Akt. Importantly, our studies with dominant-negative PIKE-A establish that PIKE-A determines the basal, physiologic activity of Akt as well as being responsible for its response to growth factor stimulation.

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