THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 3, pp. 1439–1447, January 18, 2013

Diacylglycerol Kinase δ Modulates Akt Phosphorylation through Pleckstrin Homology Domain Leucine-rich Repeat Protein Phosphatase 2 (PHLPP2)*

Received for publication, August 2, 2012, and in revised form, November 12, 2012 Published, JBC Papers in Press, November 26, 2012, DOI 10.1074/jbc.M112.407379

Tracy M. Crotty[‡], **Tomoyuki Nakano**[‡], **Diana M. Stafforini**[‡§], **and Matthew K. Topham**[‡§¶1]

From the [‡]*Huntsman Cancer Institute,* [¶]*Department of Oncological Sciences,* [§]*Department of Internal Medicine, University of Utah, Salt Lake City, Utah 84112*

Background: Diacylglycerol kinases phosphorylate diacylglycerol to terminate its signaling.
Results: Diacylglycerol kinase δ regulates a diacylglycerol feedback loop that promotes dephosphorylation of Akt.
Conclusion: Diacylglycerol signaling limits Akt activation through PHLPP2.
Significance: Receptor tyrosine kinases, which initiate Akt signaling, also activate a diacylglycerol signaling pathway that serves to modulate the levels of Akt activity.

Discovering proteins that modulate Akt signaling has become a critical task, given the oncogenic role of Akt in a wide variety of cancers. We have discovered a novel diacylglycerol signaling pathway that promotes dephosphorylation of Akt. This pathway is regulated by diacylglycerol kinase δ (DGKδ). In DGKδ-deficient cells, we found reduced Akt phosphorylation downstream of three receptor tyrosine kinases. Phosphorylation upstream of Akt was not affected. Our data indicate that PKCα, which is excessively active in DGKδ-deficient cells, promotes dephosphorylation of Akt through pleckstrin homology domain leucine-rich repeats protein phosphatase (PHLPP) 2. Depletion of either PKCα or PHLPP2 rescued Akt phosphorylation in DGKδ-deficient cells. In contrast, depletion of PHLPP1, another Akt phosphatase, failed to rescue Akt phosphorylation. Other PHLPP substrates were not affected by DGKδ deficiency, suggesting mechanisms allowing specific modulation of Akt dephosphorylation. We found that β-arrestin 1 acted as a scaffold for PHLPP2 and Akt1, providing a mechanism for specificity. Because of its ability to reduce Akt phosphorylation, we tested whether depletion of DGKδ could attenuate tumorigenic properties of cultured cells and found that DGKδ deficiency reduced cell proliferation and migration and enhanced apoptosis. We have, thus, discovered a novel pathway in which diacylglycerol signaling negatively regulates Akt activity. Our collective data indicate that DGKδ is a pertinent cancer target, and our studies could lay the groundwork for development of novel cancer therapeutics.

Upon activation of receptor tyrosine kinases (RTKs)[2], the enzyme activity of PI3K is stimulated and leads to generation of numerous 3′-phosphoinositide lipids. One of these products, phosphatidylinositol-3,4,5-trisphosphate (PIP_3), recruits signaling proteins with pleckstrin homology (PH) domains to cell membranes where they promote growth, survival, and proliferation. At the center of this PIP_3 signaling cascade is Akt/protein kinase B, a kinase with a PH domain that helps manage the balance of cell survival and apoptosis (1). Akt function is carefully regulated by two sequential phosphorylations on Thr-308 and Ser-473 that promote its activity (2). Once dually phosphorylated, Akt is fully active. To limit potent oncogenic effects, enhanced signaling by Akt is usually short-lived as the kinase is inactivated by phosphatases that remove these critical phosphates. Failure to terminate Akt signaling can lead to unregulated proliferation and inhibition of apoptosis (1).

The PH domain leucine-rich-repeats protein phosphatase (PHLPP) enzymes, PHLPP1 and PHLPP2, were recently identified as Akt phosphatases by virtue of their domain structure that contains both a PH domain and a phosphatase domain (3, 4). These enzymes dephosphorylate Akt to limit its activity. Depletion experiments have demonstrated that PHLPP enzymes suppress phosphorylation of Ser-473 in Akt under basal and activated conditions. In some cases, PHLPPs also suppress phosphorylation of Thr-308 in Akt (4). By virtue of their ability to modulate Akt phosphorylation, PHLPP enzymes control the potentially oncogenic activities of Akt. For example, prostate tumors expressing reduced levels of PHLPP1 mRNA tended to be higher-grade tumors compared with those that harbored normal levels of PHLPP1 mRNA (5). And prostate tumors with reduced protein expression of both PHLPP1 and phosphatase and tensin homolog, which helps modulate PIP_3 levels, were more likely to be highly metastatic compared with prostate tumors that expressed normal amounts of these enzymes (6). As such, the activity levels of PHLPP enzymes need to be tightly controlled. This is accomplished, in part, by

* This work was supported, in whole or in part, by National Institutes of Health Grant R01-CA95463 (to M. K. T.). This work was also supported by the Huntsman Cancer Foundation, the R. Harold Burton Foundation, and by Cancer Center Support Grant P30-CA042014 for support of core facilities.
[1] To whom correspondence should be addressed: 2000 East Circle of Hope, Salt Lake City, UT 84112-5550. Tel.: 801-585-0304; Fax: 801-585-0101; E-mail: matt.topham@hci.utah.edu.
[2] The abbreviations used are: RTK, receptor tyrosine kinase; PIP_3, phosphatidylinositol-3,4,5-trisphosphate; PH, pleckstrin homology; PHLPP, PH domain leucine-rich-repeats protein phosphatase; DAG, diacylglycerol; DGK, diacylglycerol kinase; EGFR, EGF receptor; HGF, hepatocyte growth factor; PMA, phorbol 12-myristate 13-acetate; pAkt, phosphorylated Akt; p70S6K, p70S6 kinase; Myr-Akt, myristoylated Akt; PP2A, protein phosphatase 2A; PARP, poly ADP ribose polymerase.

modulating PHLPP expression through mechanisms that regulate their translation (7) and stability (8). In addition, there are also mechanisms to regulate the ability of PHLPP enzymes to dephosphorylate substrates. For example, their localization is modulated in part by their PH domains and through interactions with scaffolding proteins that bring PHLPP enzymes in close physical proximity to their substrates (9–11). There are likely additional means of regulation, such as modifications that modulate their catalytic activity, but these mechanisms have not yet been identified.

Often simultaneous with formation of PIP_3 is the generation of DAG by phospholipase C enzymes. DAG is a lipid second messenger that, like PIP_3, recruits numerous signaling proteins to membranes and can activate them as well. DAG targets include members of the PKC family, chimaerins, protein kinase D, and RasGRP enzymes (12). Because of its numerous targets, DAG levels must be tightly regulated. It is metabolized by a family of proteins called the diacylglycerol kinases (DGKs), which are lipid kinases that phosphorylate DAG to produce phosphatidic acid. Ten mammalian DGK isoforms have been identified, and gene deletion studies suggest that they have unique functions (13). We discovered that DGKδ deficiency led to reduced phosphorylation of Akt following activation of the epidermal growth factor receptor (EGFR) (14). In additional collaborative work, we found that heterozygous deletion of the gene encoding DGKδ in mice reduced Akt phosphorylation downstream of another RTK, the insulin receptor (15). Collectively, these observations suggested that DGKδ might broadly regulate Akt signaling downstream of RTKs. Akt is an important cancer target, and our observations suggested that we had found a novel way to regulate its signaling. Thus, we set out to discover the mechanism by which DGKδ modulates Akt activation.

EXPERIMENTAL PROCEDURES

Cell Lines, Expression Plasmids, Cell Culture, and Transfection—All cell lines were from the ATCC. HepG2 and HeLa cells were grown in DMEM (Invitrogen) with 10% FBS and antibiotics, whereas H1650 cells were grown in RPMI 1640 (Invitrogen) with 10% FBS and antibiotics. Keratinocytes and mice have been described (23). Full-length, human, wild-type, and kinase-dead DGKδ1 and DGKδ2 were cloned into p3XFLAG (Sigma). The FLAG-tagged β-arrestin 1 and β-arrestin 2 plasmids have been described (16). HA-tagged PHLPP2 (catalog no. 22403) was from Addgene. Wild-type Akt1 and Myr-Akt1 were cloned into pcDNA3. Transfection of expression vectors was performed using Lipofectamine (Invitrogen) according to the instructions. Prior to treating cells with recombinant EGF (catalog no. 236-EG, R&D Systems), HGF (catalog no. PHG0254, Invitrogen), or PDGF (catalog no. PHG0043, Invitrogen), all cells were starved for 4–24 h. In some cases, cells received PMA, calyculin A, or okadaic acid (all from Sigma) prior to growth factors. RNAi was performed using Oligofectamine (Invitrogen) and the following siRNA duplexes. PHLPP1, 5′-GGAAUCAACUGGUCACAUUTT-3′ and 5′-AAUGUGACCAGUUGAUUCCTT-3′; PHLPP2, 5′-CCUAAGUGGCAACAAGCUUTT-3′ and 5′-AAGCUUGUUGCCACUUAGGTT-3′; PLCγ1, 5′-GAAGUCGCAGCGACCCGAGTT-3′ and 5′-CUCGGGUCGCUGCGACUUCTT-3′; and PKCα, 5′-GGCUUCCAGUGCCAAGUUUTT-3′ and 5′-AAACUUGGCACUGGAAGCCTT-3′. The following siRNA duplexes were used for DGKδ: 5′-GGCCAUGGUUCACACAUCGTT-3′ (underlined nucleotides in wild-type DGKδ plasmids were changed using site directed mutagenesis to create RNAi-resistant DGKδ constructs) and 5′-CGAUGUGUGAACCAUGGCCTT-3′, 5′-GGAAGCUCAUCUUGUGUGCTT-3′, and 5′-GCACACAAGAUGAGCUUCCTG-3′. PLCβ1 siRNA duplexes were from Qiagen (catalog no. SI02781184). Scrambled siRNA duplexes were used as controls.

Antibodies, Western Blot Analyses, and Immunoprecipitation—Western blotting was performed according to instructions provided by the suppliers. Anti-EGFR (catalog no. 2232), anti-phospho-EGFR (catalog no. 2234 or 2237), anti-pSer-473 Akt (catalog no. 4060), anti-pThr-308 Akt (catalog no. 2965), anti-pMet (catalog no. 3077), anti-Akt (catalog no. 9272), anti-cMet (catalog no. 3127), anti-pSer-241 PDK1 (catalog no. 3061), anti-pSer PKC substrate (catalog no. 2261), anti-pTxR (catalog no. 2351), and anti-pGab1 (catalog no. 3233) antibodies were from Cell Signaling Technology, Inc. Anti-PKCα (catalog no. sc-208), anti-PARP (catalog no. sc-8007), anti-PLCβ1 (catalog no. sc-205), and anti-PLCγ1 (catalog no. sc-81) were from Santa Cruz Biotechnology, Inc. Anti-FLAG M2 was from Sigma. Anti-pThr-229 p70S6K (catalog no. AF896) was from R&D Systems. Anti-Gab1 (catalog no. 06-579) was from Millipore. Anti-DGKδ has been described (17). Anti-PHLPP1 (catalog no. A300-660A) and anti-PHLPP2 (catalog no. A300-661A) antibodies were from Bethyl Laboratories, and the anti-β-arrestin 1 antibody was from Epitomics (catalog no. 1274-1).

To immunoprecipitate β-arrestins, cells transfected with PHLPP2 and Akt along with empty vector, FLAG-β-arrestin 1, or FLAG-β-arrestin 2 were treated as indicated, collected in lysis buffer (Cellular Signaling Technology, Inc., catalog no. 9803), incubated on ice for 10 min, and then centrifuged to remove debris. The lysates (300 μg of protein) were incubated with anti-FLAG overnight (4° C), and then protein A/G-agarose (25 μl, Santa Cruz Biotechnology, Inc.) was added for 2 h. After three washes with lysis buffer, the pellets and lysates (10 μg) were separated by SDS-PAGE and then immunoblotted to detect PHLPP2, Akt, or FLAG. HA-PHLPP2 was immunoprecipitated similarly using anti-HA antibodies.

Generation of Stable DGKδ Knockdown Cell Lines and Transformation Assays—To generate stable DGKδ knockdown H1650 cell lines, Dharmacon SMARTvector 2.0 lentiviral particles (catalog no. SK-006713-00) were used according to the instructions. Non-targeting particles (catalog no. S-005000-01) were used as a control cell line. Polyclonal stable cell lines were isolated under puromycin (2 μg/ml) selection. Cell proliferation was assessed by plating 10,000 cells in a 10 cm-diameter plate, growing them in 1% FBS with antibiotics for 5–7days, and then counting the number of cells. Cell migration was measured by growing HeLa cells to 80% confluency, performing RNAi, starving the cells overnight, scratching the monolayer with a pipette tip, washing, adding growth factor (25 ng/ml) for 24 h, fixing with 10% formalin for 1 h at 4C, and then staining the cells with 0.1% crystal violet. Growth in soft agar was performed as described (18).

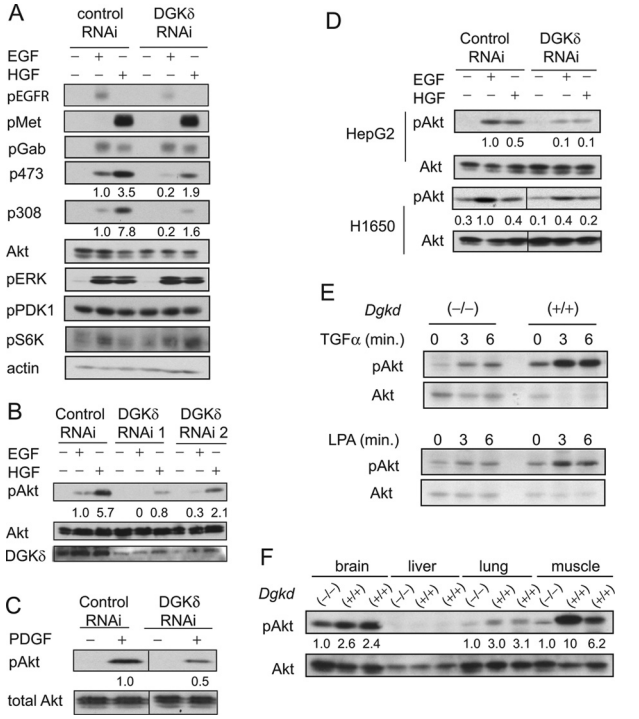

FIGURE 1. **Reduced Akt phosphorylation in DGKδ-deficient cells.** *A,* control or DGKδ knockdown HeLa cells were treated with EGF (10 ng/ml) or HGF (25 ng/ml) for 10 min. The levels of indicated proteins were detected by Western blotting. *p473* and *p308* refer to Akt. *B,* two unique siRNA duplexes were used to knock down DGKδ, and then pAkt (Ser-473) was measured by Western blotting. *C,* PDGF (25 ng/ml) was used to activate HeLa cells, and then pSer-473 Akt was detected by Western blotting. The *vertical lines* indicate that intervening lanes were omitted. *D,* pSer-473 in Akt was measured in H1650 or HepG2 cells. The *vertical lines* indicate that intervening lanes were omitted. *E,* primary keratinocytes isolated from either *Dgkd* (−/−) or *Dgkd* (+/+) newborn mice were treated with either TGFα (5 ng/ml) or lysophosphatidic acid (50 μM) for the indicated times, and then total and pAkt (Ser473) were detected by Western blotting. *F*, tissues harvested from *Dgkd* (−/−) or *Dgkd* (+/+) newborn mice were used for Western blotting to detect total and pAkt (Ser-473). The numbers below the blots throughout this figure indicate relative band intensity normalized to total Akt.

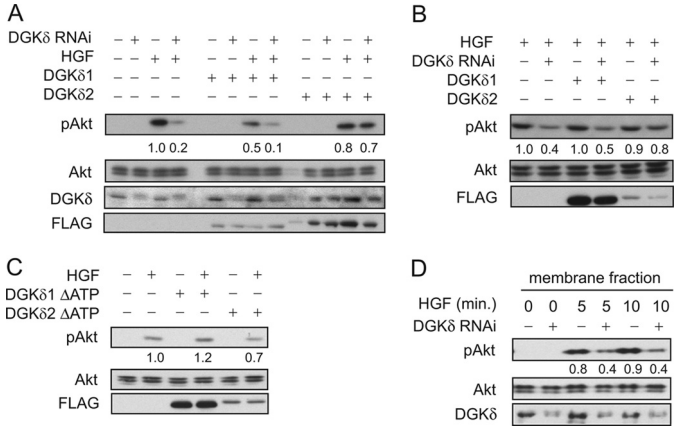

FIGURE 2. **Effects of manipulating DGKδ on Akt phosphorylation.** *A* and *B,* RNAi-resistant constructs of either DGKδ1 or DGKδ2 were used to test for rescue of pSer-473 in Akt. *C,* HeLa cells transfected with control vector or kinase-dead (Δ*ATP*) constructs of DGKδ were treated with 25 ng/ml HGF, and then the indicated proteins were detected by Western blotting. *D,* membrane fractions from control or DGKδ RNAi HeLa cells treated with 25 ng/ml for the indicated times were isolated by 100,000 × *g* centrifugation, and the indicated proteins were detected by Western blotting. Numbers below the blots throughout this figure indicate relative band intensity normalized to total Akt.

RESULTS

DGKδ Deficiency Reduces Phosphorylation of Akt—We first asked whether DGKδ modulated Akt phosphorylation downstream of other RTKs in addition to EGFR and the insulin receptor. We found reduced phosphorylated Akt (pAkt) downstream of c-Met in DGKδ-deficient HeLa cells (Fig. 1*A*). A second siRNA duplex targeting another region of DGKδ similarly reduced pAkt downstream of both EGFR and c-Met (Fig. 1*B*). We also tested PDGF receptor and again found reduced pAkt in DGKδ-deficient cells (Fig. 1*C*). Next, we examined two additional cell lines and found similar reductions in pAkt in DGKδ-deficient cells (Fig. 1*D*). Finally, we assayed primary keratinocytes and tissue harvested from newborn wild-type or DGKδ knockout mice. In the keratinocytes, DGKδ deficiency led to reduced Akt phosphorylation following treatment with either TGFα or lysophosphatidic acid (Fig. 1*E*). In tissue harvested from DGKδ knockout mice, we found reduced pAkt in several organs (Fig. 1*F*). Together, these data indicate that DGKδ deficiency broadly attenuates Akt phosphorylation *in vitro* and *in vivo*.

There are two alternatively spliced products of the DGKδ gene, DGKδ1 and DGKδ2, which differ at their amino termini. The alternative splicing does not change their DGK activity but appears to alter their subcellular localization (19). HeLa cells express both DGKδ splice variants (data not shown). To test which DGKδ splice variant could modulate Akt phosphorylation, we used RNAi to reduce the levels of endogenous DGKδ in HeLa cells and then tested whether we could rescue Akt phosphorylation by expressing RNAi-resistant DGKδ1 or DGKδ2 cDNA constructs. We consistently observed that DGKδ2, but not DGKδ1, rescued Akt phosphorylation (Fig. 2, *A* and *B*), indicating that DGKδ2 was the relevant splice variant. Affirming this conclusion, we found that transient overexpression of kinase-dead DGKδ2 reduced phosphorylation of Akt (Fig. 2*C*). Collectively, these data indicate that DGKδ2 modulates Akt phosphorylation.

The Defect in DGKδ-deficient Cells Occurs at the Level of Akt—Although most RTKs activate PI3K/Akt signaling, they do so using different mechanisms. c-Met uses the adaptor protein Gab1 as a scaffold to recruit PI3K (20), whereas EGFR does not necessarily require Gab1 (21). The diverse mechanisms by which these receptors activate PI3K/Akt signaling contrasted with the similarity of responses to DGKδ depletion suggested that the defect caused by DGKδ deficiency occurred at, or near, the level of Akt. To determine where in the RTK signaling pathway the phosphorylation defect occurred, we focused on signaling from c-Met because, in contrast to its effects on EGFR, DGKδ deficiency does not affect c-Met levels. We first examined the amount of tyrosine-phosphorylated c-Met and found no differences between control and DGKδ siRNA-treated cells (Fig. 1*A*). Next, we assayed the levels of phosphorylated Gab1 and again found no differences in its phosphorylation (Fig. 1*A*), indicating that the defect likely occurred after PI3K was recruited to Gab1. The PIP_3 generated by PI3K serves to recruit numerous signaling proteins to the plasma membrane, including Akt. To determine whether the reduced Akt phosphorylation in DGKδ-deficient cells was due to reduced membrane association of Akt, we examined the abundance of membrane-

bound Akt before and after activation of c-Met and found no differences in membrane-associated Akt between control and DGKδ-deficient cells (Fig. 2*D*). These results, combined with the observation that Akt phosphorylation was significantly reduced in DGKδ-deficient cells, indicated that the defect in Akt phosphorylation occurred after its recruitment to the membrane.

Once Akt associates with the membrane, it is phosphorylated first on Thr-308 by 3-phosphoinositide-dependent protein kinase-1 (PDK1). A second phosphorylation, regulated by mTORC2 at Ser-473 in Akt, yields fully activated Akt (2). We examined phosphorylation of both Thr-308 and Ser-473 in Akt and found that phosphorylation at both of sites was reduced to similar extents in DGKδ-deficient cells (Fig. 1*A*). Next, we examined the levels of phosphorylated Ser-241 in PDK1, which is required for PDK1 activity, and found no differences in phosphorylated Ser-241 between control and DGKδ-deficient cells (Fig. 1*A*), indicating that the defect in Akt phosphorylation occurred at a later point in the signaling cascade. To confirm this possibility, we examined another PDK1 substrate, p70 S6kinase (p70S6K), and found that its phosphorylation on Thr-229 was not reduced in DGKδ-deficient cells (Fig. 1*A*). Additionally, phosphorylation at Thr-389 in p70S6K, a prerequisite for phosphorylation of Thr-229 (22), was also not affected by DGKδ deficiency (not shown). The normal phosphorylation patterns of PDK1 and its downstream target p70S6K were in contrast to the reduced phosphorylation of Akt. This led us to hypothesize that DGKδ might modulate Akt dephosphorylation.

DAG Signaling Promotes Dephosphorylation of Akt—To test whether DGKδ modulated Akt dephosphorylation, we reasoned that DGKδ deficiency would reduce phosphorylation of myristoylated Akt (Myr-Akt), which is associated with the plasma membrane and is constitutively phosphorylated. In DGKδ-deficient cells, we found reduced basal phosphorylation of Myr-Akt and observed that upon activation of c-Met, phosphorylation of Myr-Akt was reduced further (Fig. 3*A*). In contrast, the levels of phosphorylated Myr-Akt in control cells increased upon activation. The reduction in Akt phosphorylation following activation of c-Met in DGKδ-deficient cells suggested that DGKδ deficiency promoted dephosphorylation of Akt.

The rapid dephosphorylation of Myr-Akt in DGKδ-deficient cells following activation of c-Met indicated that receptor activation initiated a phosphatase feedback mechanism to limit Akt phosphorylation. To better understand the dynamics of Akt phosphorylation, we performed a time course experiment and observed, after c-Met activation, that phosphorylation of Akt peaked at 5 min and then returned to near base line by 20 min, whereas phosphorylation of c-Met peaked at 10 min and remained quite high, even after 20 min (Fig. 3*B* and data not shown). The more rapid reduction in phosphorylated Akt compared with phosphorylated c-Met suggested that activation of c-Met initiated a feedback mechanism that led to dephosphorylation of Akt. Although the phosphatases involved, including protein phosphatase 2A (PP2A) and PHLPP enzymes, have been studied thoroughly, the mechanisms that initiate this

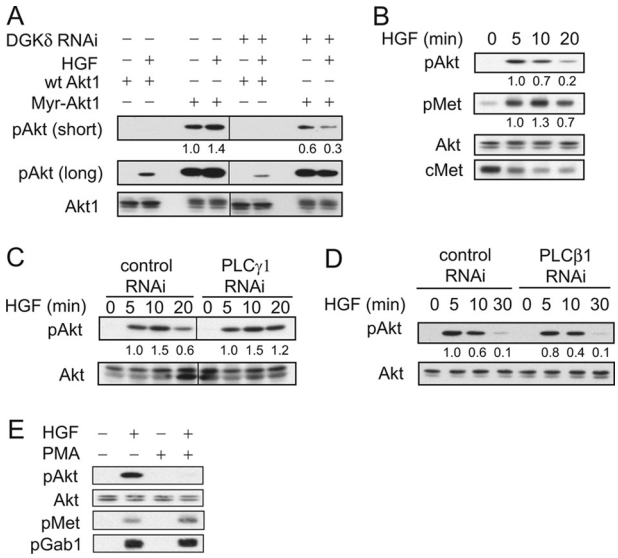

FIGURE 3. **DAG signaling modulates Akt phosphorylation.** *A,* HeLa cells were transfected with wild-type Akt or Myr-Akt followed by control or DGKδ RNAi. The cells then were treated with 25 ng/ml HGF for 10 min, and levels of the indicated proteins were detected by Western blotting. *Short* and *long* refer to the times that the blot was exposed to film. The *vertical lines* indicate that intervening lanes were omitted. *B,* HeLa cells were treated with 25 ng/ml HGF for the indicated times, and then the proteins were measured by Western blotting. *C* and *D,* same as *B,* except that PLCγ1 or PLCβ1 were knocked down using RNAi. The *vertical lines* indicate that intervening lanes were omitted. *E,* HeLa cells were pretreated with 10 nM PMA for 5 min, exposed to 25 ng/ml HGF for 10 min, and then the proteins were detected by Western blotting. The numbers below the blots throughout this figure indicate relative band intensity normalized to total Akt.

phosphatase feedback loop are not well understood. Our data indicate that DGKδ might be involved.

We have shown previously that DGKδ deficiency increases cellular DAG levels (23), which led us to hypothesize that DAG signaling might have a prominent role in modulating Akt dephosphorylation. To test the importance of DAG signaling, we determined the effects of increasing DAG levels by depleting PLCγ1, which is activated downstream of c-Met, or PLCβ1, which is not. We found that PLCγ1 depletion, but not PLCβ1 depletion, prolonged Akt phosphorylation (Fig. 3, *C* and *D*), implicating DAG signaling as a negative regulator of Akt phosphorylation. As a second approach to test the effects of DAG signaling, we treated cells with PMA, a long-lived DAG analog, and found that it reduced the phosphorylation of Akt following activation of c-Met (Fig. 3*E*). Collectively, our data indicate that phosphorylation of Akt is modulated by a DAG negative feedback loop initiated by PLCγ1 and negatively regulated by DGKδ.

DGKδ Modulates Akt Dephosphorylation through PHLPP2—Two different types of phosphatases regulate Akt. Those in the PP2A family predominantly dephosphorylate Thr-308 (3), whereas those in the PHLPP family dephosphorylate Ser-473 under both basal and activated conditions and can also mediate dephosphorylation of Thr-308 under activated conditions (4). We first tested whether we could rescue Akt phosphorylation in DGKδ-deficient cells using calyculin A to inhibit PP2A phosphatases and found that this inhibitor failed to rescue HGF-induced phosphorylation of either Thr-308 or Ser-473 (Fig. 4*A*). Okadaic acid, another PP2A inhibitor, also did not rescue

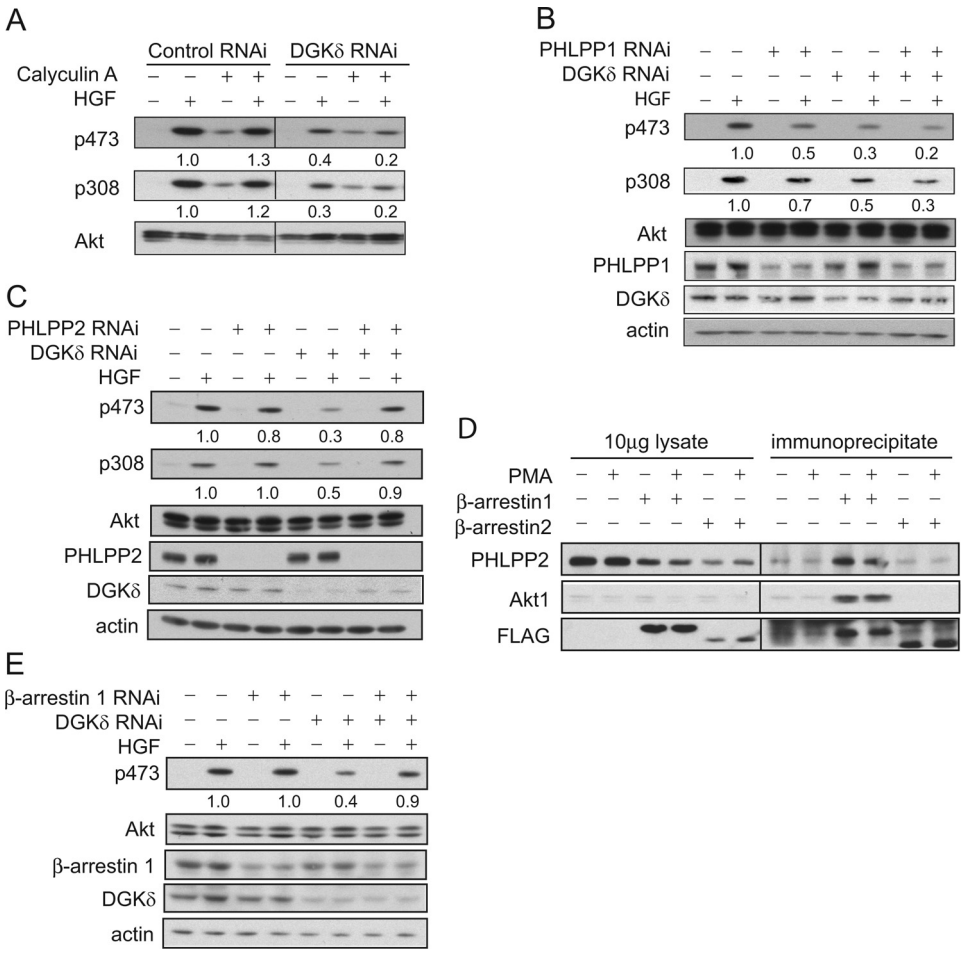

FIGURE 4. **PHLPP2 RNAi rescues Akt phosphorylation.** *A,* HeLa cells pretreated for 10 min with 30 nM calyculin A were exposed to 25 ng/ml HGF for 10 min, and then pSer-473 in Akt was measured by Western blotting. The *vertical lines* indicate that intervening lanes were omitted. *B* and *C,* DGKδ along with PHLPP1 or PHLPP2 were knocked down in HeLa cells by RNAi. The cells were treated for 10 min with 25 ng/ml HGF, and then the indicated proteins were detected by Western blotting. *D,* HeLa cells were transfected with cDNA constructs encoding wild-type Akt and PHLPP2 along with empty vector, FLAG-β-arrestin 1, or FLAG-β-arrestin 2. After treating starved cells with 10 nM PMA for 10 min, β-arrestins were immunoprecipitated with anti-FLAG, and the indicated proteins were detected by Western blotting. The *vertical lines* indicate that intervening lanes were omitted. *E,* DGKδ along with β-arrestin 1 were knocked down in HeLa cells by RNAi. The cells were treated for 10 min with 25 ng/ml HGF, and then the indicated proteins were detected by Western blotting. The numbers below the blots throughout this figure indicate relative band intensity normalized to total Akt.

Akt phosphorylation (not shown). PHLPP enzymes are insensitive to PP2A inhibitors, so we next tested the effects of RNAi knockdown of PHLPP enzymes. We found that RNAi knockdown of PHLPP2, but not that of PHLPP1, rescued Akt phosphorylation in DGKδ-deficient cells (Fig. 4, *B* and *C*). Collectively, these data indicate that the reduced Akt phosphorylation in DGKδ-deficient cells is mediated by PHLPP2.

Specificity in signal transduction is achieved by gathering together signaling proteins in common pathways along with their regulators (24). PHLPP2 has been shown to dephosphorylate Akt 1 but not Akt 2 (4). We hypothesized that a scaffolding protein regulates this specificity by juxtaposing these proteins. β-arrestins act to scaffold Akt to the insulin receptor (25), and disrupting this interaction using molecular approaches or by deleting the gene encoding β-arrestin 2 in mice reduced Akt activation and caused insulin resistance (25). Both reduced Akt activation and insulin resistance were also evident in DGKδ-deficient mice (15), and DGKδ is known to bind β-arrestins 1 and 2 (16). Collectively, these observations indicated that β-arrestins and DGKδ might impact the same Akt signaling pathway. This led us to test the possibility that β-arrestins scaffold PHLPP2 and Akt1. We found that β-arrestin 1, but not β-arrestin 2, coimmunoprecipitated with both PHLPP2 and Akt1 (Fig. 4*D*). This scaffolding interaction was not affected by treatment with the DAG analog PMA, suggesting that DAG signaling likely affects the specific activity of PHLPP2 rather than its ability to access Akt1. Further supporting its role as a scaffold, we found that RNAi mediated depletion of β-arrestin 1-rescued Akt phosphorylation in DGKδ-deficient cells (Fig. 4*E*).

PKCα Is a Target of Excess DAG That Modulates Akt Phosphorylation—Protein kinase C isoforms are major targets of DAG (12). In DGKδ knockout mice we found evidence of increased PKC activation detected by increased phosphorylation of PKC substrates and enhanced phosphorylation of PKC isoforms (14, 23). Additionally, we observed that DGKδ coimmunoprecipitated with several PKC isoforms (23). Together, these data led us to examine whether PKCs were the DAG targets that modulated phosphorylation of Akt downstream of c-Met. Prior data have implicated PKCα as a regulator of Akt signaling (26, 27), and we found that an inhibitor of conven-

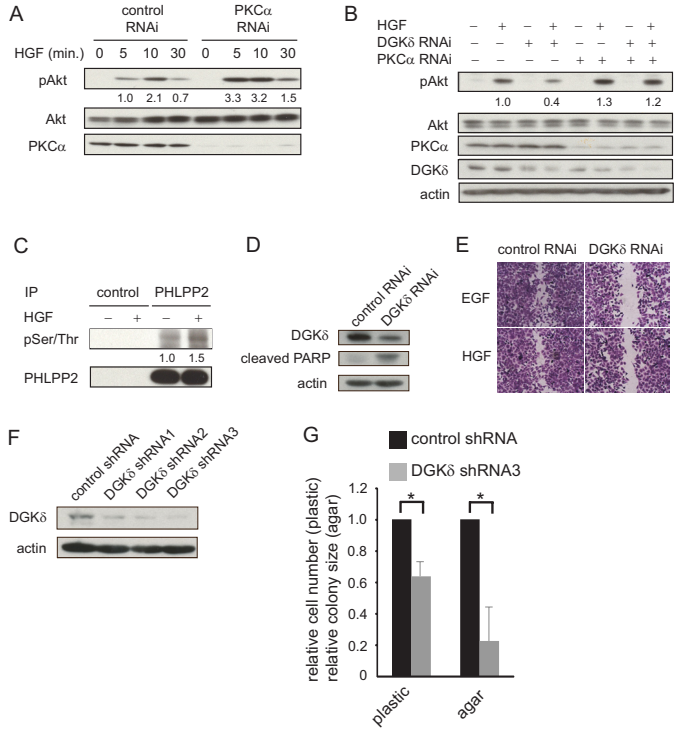

FIGURE 5. **DGKδ deficiency attenuates tumorigenic properties.** *A,* PKCα was knocked down in HeLa cells using RNAi. The cells were treated with 25 ng/ml HGF for the indicated times, and then the proteins were measured by Western blotting. *B,* DGKδ along with PKCα were knocked down in HeLa cells by RNAi. The cells were treated for 10 min with 25 ng/ml HGF, and then the indicated proteins were detected by Western blotting. *C*, PHLPP2 was immunoprecipitated from lysates of HeLa cells transfected with PHLPP2, and then phosphorylation was detected by immunoblotting with a mixture of anti-PKC substrate and anti-pTxR antibodies. The blot was stripped and reprobed to detect PHLPP2. *D,* control or DGKδ knockdown HeLa cells were starved for 48 h, and the indicated proteins were detected by Western blotting. *E,* migration of HeLa cells into a wounded area in the presence of 10 ng/ml EGF or 25 ng/ml HGF (24 h). *F,* Western blot analysis of polyclonal, stable H1650 cell lysates. Three shRNA constructs targeting unique regions of DGKδ were used. *G,* growth of the control and DGKδ shRNA3 polyclonal stable H1650 cell lines was determined in 1% serum ($n = 5$) or in soft agar. $>$ 50 soft agar colonies were assessed in three different experiments. Data are mean $\pm$ S.D. *, $p <$ 0.03. The numbers below the blots throughout this figure indicate relative band intensity normalized to total Akt.

tional PKCs α and β rescued Akt phosphorylation in DGKδ-deficient cells (14). Thus, we focused our efforts on PKCα. To test whether this PKC isoform modulates Akt phosphorylation, we knocked down PKCα in HeLa cells and evaluated the extent of stimulated Akt phosphorylation over 30 min. We found that PKCα knockdown augmented and prolonged Akt phosphorylation in response to HGF stimulation (Fig. 5*A*). This effect on Akt was similar to the effects of knocking down PLCγ1 (see Fig. 3*C*), suggesting that PKCα is an important component of the mechanism by which DAG and DGKδ modulate Akt dephosphorylation downstream of c-Met.

To test this possibility, we reasoned that knocking down PKCα could rescue phosphorylation of Akt in DGKδ-deficient cells. After simultaneously reducing expression of both DGKδ and PKCα, we found that this manipulation normalized Akt phosphorylation (Fig. 5*B*). Collectively, these data suggest that the DAG generated after activation of c-Met modulates Akt phosphorylation in part through PKCα and that DGKδ helps modulates the levels of this DAG. In the context of DGKδ deficiency, DAG levels rise, which activates PKCα and promotes dephosphorylation of Akt. Finally, to test for a link between PKC activity and PHLPP2, we treated cells with HGF and IPed PHLPP2 and then detected phosphorylated PHLPP2 by immunoblotting with PKC substrate antibodies. We found that HGF increased phosphorylation of PHLPP2 (Fig. 5*C*). This observation, together with our coimmunoprecipitation data in Fig. 4*D*, suggests that phosphorylation of PHLPP2 might affect its activity. We are in the process of testing this possibility more rigorously.

DGKδ Depletion Limits Tumorigenic Properties—Because of the reduced Akt phosphorylation in DGKδ-deficient cells, we hypothesized that depleting the levels of DGKδ would attenuate tumorigenic properties of cultured cells. Akt1 modulates cell survival (28), so we first determined whether DGKδ deficiency promoted apoptosis by starving control or DGKδ RNAi knockdown HeLa cells and then assessing the level of apoptosis by measuring cleaved poly ADP ribose polymerase (PARP). We found that knockdown of DGKδ significantly enhanced apoptosis under these conditions (Fig. 5*D*). Cell migration is another important tumorigenic property, so we also tested the ability of control or DGKδ-deficient HeLa cells to migrate into a wounded area and found that the DGKδ-deficient cells migrated more slowly compared with control cells in serum-free conditions (not shown) and during exposure to either EGF or HGF (Fig. 5*E*). Together, these data indicated that DGKδ modulates tumorigenic properties and that inhibiting its function could be used to limit tumor growth or metastasis.

Targeted strategies to limit tumor growth are more likely to succeed if the target is a primary driver of tumor growth. Recently, activating mutations in EGFR have been discovered in lung adenocarcinomas (29). These mutations in EGFR increase its kinase activity, which promotes proliferation and survival, leading to a state of oncogene addiction. Because DGKδ modulates both EGFR expression levels and signaling downstream of EGFR (14, 23), we reasoned that inhibiting the function of DGKδ in cells harboring an activating EGFR mutation would significantly reduce cell proliferation. To test this possibility, we used H1650 lung cancer cells, which harbor an activating mutation in EGFR, to generate cell lines with stable knockdown of DGKδ (Fig. 5*F*). We found that depletion of DGKδ reduced growth of the cells on plastic and in soft agar (Fig. 5*G*). Unfortunately, we could not maintain knockdown of DGKδ during growth of the cell lines as xenografts in immunodeficient mice, so we were unable to examine the effects of DGKδ deficiency on tumorigenesis *in vivo*. However, our collective *in vitro* data strongly suggest that DGKδ deficiency attenuates oncogenic properties, justifying further preclinical studies to assess the therapeutic potential of DGKδ inhibitors.

DISCUSSION

We have discovered a novel mechanism where DAG signaling modulates PHLPP2 to limit the activation of Akt. RTKs initiate DAG signaling by activating PLCγ1, but the effects of this DAG have not been extensively evaluated. Early studies using phorbol esters, which are long-lived DAG analogues, suggested that excessive DAG signaling might universally promote tumorigenesis (30, 31). More recent evidence has indicated that

the effects of excessive DAG signaling depend on the specific DAG targets that are abnormally activated (12). PKCs are major DAG targets, and they offer an enlightening example of the diverse effects that excessive DAG signaling can have. PKCε, for example, generally promotes cell transformation (12, 32, 33). We determined whether DGKδ affects the activity of PKCε by measuring its kinase activity *in vitro* with or without overexpression of DGKδ and found no changes in its activity (not shown). Nor was phosphorylation of the PKCε hydrophobic motif affected by DGKδ deficiency (not shown). Thus, DGKδ does not appear to modulate PKCε.

In contrast to PKCε, PKCα generally attenuates transforming properties (12). The inhibitory effects of PKCα on transformation were evident in a recent study where deletion of the gene encoding PKCα doubled the number of polyps in the $Apc^{Min/+}$ mouse model of familial adenomatous polyposis (34). The mechanisms by which PKCα deficiency promoted tumorigenesis in $Apc^{Min/+}$ mice were not clear, but excessive EGFR signaling was implicated (34).

On the basis of the excessive tumorigenesis in PKCα null mice, one would predict that increasing the activity of PKCα could be a valid therapeutic strategy to limit EGFR and Akt signaling. Specifically increasing PKCα activity is difficult to achieve, given the broad and often tumor-promoting effects of phorbol esters and related compounds that activate PKCs (12). Our data indicate that an alternative approach to capitalize on the antitumorigenic effects of PKCα would be to disrupt DGKδ activity. Indeed, we have found that DGKδ deficiency led to activation of PKCα, which then reduced EGFR expression, limited the specific activity of EGFR, and reduced Akt phosphorylation (Refs. 14, 23 and Figs. 1–5). Collectively, these changes caused *Dgkd* knockout mice to have a phenotype very similar to that of *Egfr* knockout mice (23). Moreover, the effects of disrupting DGKδ were not limited to EGFR signaling. Akt activation was also diminished downstream of the insulin receptor (15), c-Met, and PDGFR (Fig. 1). Given the importance of these RTKs and Akt in cell proliferation, migration, apoptosis, and transformation, our collective data suggest that disrupting the activity of DGKδ could be a viable therapeutic strategy to treat cancer. Consistent with this possibility, we found that DGKδ deficiency reduced proliferation and migration and enhanced apoptosis of cancer cells.

Central to the ability DGKδ to modulate Akt is PHLPP2. We found that depleting PHLPP2, but not PHLPP1, rescued Akt phosphorylation in DGKδ-deficient cells. This specificity of PHLPP function suggested that scaffolding interactions, which are known to regulate PHLPPs (9–11), might be important. Indeed, we discovered that β-arrestin 1 could scaffold PHLPP2 and Akt1. Again, there was specificity in this interaction. β-arrestin 2 did not bind PHLPP1 or Akt1. We also found evidence that PHLPP2 was phosphorylated by PKCs but that PMA did not affect the PHLPP2/β-arrestin/Akt1 interaction. Together, these data indicate that DAG signaling likely modulates the specific activity of PHLPP2 rather than affecting the ability of PHLPP2 to access Akt. We are currently developing an assay that will allow us to measure the specific activity of PHLPP2 in cultured cells.

PHLPP enzymes can also be regulated by means that modulate their abundance (7, 8). We found no changes in the abundance of PHLPP2 in DGKδ or PKCα depletion experiments, suggesting that changes in the abundance of PHLPP2 are not the cause of reduced Akt activation in DGKδ-deficient cells. The rapid dephosphorylation of Myr-Akt that we observed in DGKδ-deficient cells also argues against this possibility and suggests that direct modifications, binding partners, or localization of PHLPP2 are more likely causes. All of these changes can be achieved through appropriate scaffolding interactions, such as the β-arrestin 1 scaffold that we show here.

PHLPP enzymes have been shown to dephosphorylate numerous proteins, including PKCs, Akt, and p70S6K (3, 35, 36). Given the diverse targets of PHLPP enzymes, it is not surprising that their specificity might be modulated by scaffolding interactions. Such interactions could provide distinct targets sets for PHLPP1 and PHLPP2 as well as unique targets for a single PHLPP enzyme that depend on the cell context. Although we found reduced Akt phosphorylation in DGKδ-deficient cells, we found no differences in p70S6K phosphorylation and excessive phosphorylation of several PKC isoforms (23), even though PHLPP enzymes have been shown to dephosphorylate all of these kinases (35, 36). Collectively, these data demonstrating that DGKδ specifically affects Akt lend credence to the possibility that β-arrestin 1 and other scaffolding proteins provide specificity for PHLPP enzymes.

Like PHLPP and PKC enzymes, DGK enzymes appear to have distinct functions. In published work, we demonstrated that similar to DGKδ, DGKζ also inhibited the function of PKCα (37). However, the phenotype of DGKζ-deficient mice was not similar to that of DGKδ null mice, and DGKζ deficiency did not affect EGFR or Akt signaling (Ref. 38 and data not shown). DGKη, a close relative of DGKδ, was recently found to affect ERK phosphorylation downstream of EGFR, but it did not alter Akt signaling (39). DGKα has also been shown to affect RTK signaling (40–42), but like DGKζ knock-out mice, DGKα null mice displayed no evidence of altered EGFR or Akt signaling (43). We and others have additionally generated mouse knockouts of DGKs β, ε, ι, and θ, all of which show no evidence of EGFR or Akt signaling defects [(44–46 and data not shown). Thus, it appears that DGKs have unique functions. We have now discovered that DGKδ specifically regulates Akt signaling downstream of potentially oncogenic RTKs. Its novel mechanism of action offers unique opportunities for therapeutic applications and we believe that further studies are warranted to determine the suitability of DGKδ as a cancer target.

Acknowledgment—We thank Fumio Sakane for providing the DGKδ antibody.

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