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

From the Huntsman Cancer Institute, Department of Oncological Sciences, Department of Internal Medicine, University of Utah, Salt Lake City, Utah 84112

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

Background: Diacylglycerol kinases phosphorylate diacylglycerol to terminate its signaling. Diacylglycerol kinase δ (DGKδ) regulates Akt through pleckstrin homology domain leucine-rich repeats protein phosphatase 2 (PHLPP2). Depletion of DGKδ-deficient cells, 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 2 (PHLPP2). 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.

Results: Diacylglycerol kinase δ regulates a diacylglycerol feedback loop that promotes dephosphorylation of Akt. Diacylglycerol signaling limits Akt activation through PHLPP2.

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 2 (PHLPP2). 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.
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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 PIP3 is the generation of DAG by phospholipase C enzymes. DAG is a lipid second messenger that, like PIP3, recruits numerous signaling proteins of DAG by phospholipase C enzymes. DAG is a lipid second activation.

We discovered that DGK, which are lipid kinases that phosphorylate DAG to produce phosphatidic acid. Ten mammalian DGK isoforms have been identified, these observations suggested that they have unique functions (13). We discovered that DGK 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 DGK in mice reduced Akt phosphorylation downstream of another RTK, the insulin receptor (15). Collectively, these observations suggested that DGK 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 DGK 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 DGKα1 and DGKβ2 were cloned into p3XFLAG (Sigma). The FLAG-tagged β-arrestin 1 and β-arrestin 2 plasmids have been described (16). HA-tagged PHLP2 (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 the instructions. Treatment of cells with PMA, PMA, or okadaic acid (all from Sigma) prior to the instructions. Treatment of cells with PMA, PMA, or okadaic acid (all from Sigma) prior to the instructions. Treatment of cells with PMA, PMA, or okadaic acid (all from Sigma) prior to the instructions. Treatment of cells with PMA, PMA, or okadaic acid (all from Sigma) prior to the instructions. Treatment of cells with PMA, PMA, or okadaic acid (all from Sigma) prior to the instructions. Treatment of cells with PMA, PMA, or okadaic acid (all from Sigma) prior to the instructions. Treatment of cells with PMA, PMA, or okadaic acid (all from Sigma) prior to the instructions. Treatment of cells with PMA, PMA, or okadaic acid (all from Sigma) prior to the instructions. Treatment of cells with PMA, PMA, or okadaic acid (all from Sigma) prior to the instructions. Treatment of cells with PMA, PMA, or okadaic acid (all from Sigma) prior to the instructions.

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-PLC (catalog no. 3127), anti-pSer-241 PKD1 (catalog no. 3061), anti-pSer PKC substrate (catalog no. 2261), anti-pTyr (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-DGKα 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 PHLP2 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 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 PHLP2, Akt, or FLAG. HA-PHLPP2 was immunoprecipitated similarly using anti-HA antibodies.

Generation of Stable DGK Knockdown Cell Lines and Transformation Assays—To generate stable DGK 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 5–7 days, 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 4°C, and then staining the cells with 0.1% crystal violet. Growth in soft agar was performed as described (18).
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. 1A). A second siRNA duplex targeting another region of DGKδ similarly reduced pAkt downstream of both EGFR and c-Met (Fig. 1B). We also tested PDGF receptor and again found reduced pAkt in DGKδ-deficient cells (Fig. 1C). Next, we examined two additional cell lines and found similar reductions in pAkt in DGKδ-deficient cells (Fig. 1D). Finally, we assayed primary keratinocytes and tissue harvested from newborn wild-type or DGKδ-deficient mice. In the keratinocytes, DGKδ deficiency led to reduced Akt phosphorylation following treatment with either TGFα or lysophosphatidic acid (Fig. 1E). In tissue harvested from DGKδ knockout mice, we found reduced pAkt in several organs (Fig. 1F). Together, these data indicate that DGKδ deficiency broadly attenuates Akt phosphorylation in vitro and in vivo.

There are two alternatively spliced proteins 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. 2C). Collectively, these data indicate that DGKδ2 modulates Akt phosphorylation.

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. 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 pAkt (Ser-473) 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 Dgkd1/H9251 or Dgkd2/H9254 (5 ng/ml) or lysophosphatidic acid (50 μM) for the indicated times, and then total and pAkt (Ser-473) were detected by Western blotting. F, tissues harvested from Dgkd1/−/− or Dgkd2/−/− 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.

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 after PI3K was recruited to Gab1. The PIP3 generated by PI3K serves to recruit 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 after PI3K was recruited to Gab1. The PIP3 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. 2D). 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. 1A). 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. 1A), 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 S6 kinase (p70S6K), and found that its phosphorylation on Thr-229 was not reduced in DGKδ-deficient cells (Fig. 22), which is not. We found that 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. 3E). Collectively, our data indicate that phosphorylation of Akt is modulated by a DAG negative feedback loop initiated by PLCγ1 and negatively regulated by DGKδ.

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**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 were then 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 the numbers below the blots throughout this figure indicate relative band intensity normalized to total Akt.

**A**

| Condition | Akt | Myr-Akt |
|-----------|-----|---------|
| Control   | +   | +       |
| DGKδ RNAi | -   | -       |

**B**

| Condition | Akt | Myr-Akt |
|-----------|-----|---------|
| Control   | +   | +       |
| HGF 5 ng/ml | +   | +       |
| HGF 10 ng/ml | +   | +       |
| HGF 20 ng/ml | +   | +       |

**C**

| Condition | Akt | Myr-Akt |
|-----------|-----|---------|
| Control   | +   | +       |
| PLCγ1 RNAi | +   | +       |

**D**

| Condition | Akt | Myr-Akt |
|-----------|-----|---------|
| Control   | +   | +       |
| PLCβ1 RNAi | +   | +       |

**E**

| Condition | Akt | Myr-Akt |
|-----------|-----|---------|
| Control   | +   | +       |
| PMA 10 ng/ml | +   | +       |

**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. 4A). Okadaic acid, another PP2A inhibitor, also did not rescue...
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/H9254-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, communoprecipitated with both PHLPP2 and Akt1 (Fig. 4D). 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. 4E).

**PKCa 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 communoprecipitated 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 PKCa as a regulator of Akt signaling (26, 27), and we found that an inhibitor of conven-
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FIGURE 5. DGKδ deficiency attenuates tumorigenic properties. A, PKCcα 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 PKCcα 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-PKCs substrate and anti-pTyr 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 ± S.D. *p < 0.03. The numbers below the blots throughout this figure indicate relative band intensity normalized to total Akt.

To test this possibility, we reasoned that knocking down PKCcα could rescue phosphorylation of Akt in DGKδ-deficient cells. After simultaneously reducing expression of both DGKδ and PKCcα, we found that this manipulation normalized Akt phosphorylation (Fig. 5B). Collectively, these data suggest that the DAG generated after activation of c-Met modulates Akt phosphorylation in part through PKCcα and that DGKδ helps modulate the levels of this DAG. In the context of DGKδ deficiency, DAG levels rise, which activates PKCcα and promotes dephosphorylation of Akt. Finally, to test for a link between PKCc activity and PHLPP2, we treated cells with HGF and IPed PHLPP2 and then detected phosphorylated PHLPP2 by immunoblotting with PKCc substrate antibodies. We found that HGF increased phosphorylation of PHLPP2 (Fig. 5C). This observation, together with our communoprecipitation data in Fig. 4D, 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. 5D). 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. 5E). 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. 5F). We found that depletion of DGKδ reduced growth of the cells on plastic and in soft agar (Fig. 5G). 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. PKCe, for example, generally promotes cell transformation (12, 32, 33). We determined whether DGKδ affects the activity of PKCe 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 PKCe hydrophobic motif affected by DGKδ deficiency (not shown). Thus, DGKδ does not appear to modulate PKCe.

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

On the basis of the excessive tumorigenesis in PKCa null mice, one would predict that increasing the activity of PKCa could be a valid therapeutic strategy to limit EGFR and Akt signaling. Specifically increasing PKCa 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 PKCa would be to disrupt DGKδ activity. Indeed, we have found that DGKδ deficiency led to activation of PKCa, 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 Egrf 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 deleting 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 Dgkd or PKCa 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, DGKγ also inhibited the function of PKCa (37). However, the phenotype of DGKδ-deficient mice was not similar to that of DGKδ null mice, and DGKδ deficiency did not affect EGFR or Akt signaling (Ref. 38 and data not shown). Dgkd, 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 DGKδ, 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 DGKδ, DGKε, and DGKθ, 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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