A novel acylglycerol kinase that produces lysophosphatidic acid modulates cross talk with EGFR in prostate cancer cells

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The bioactive phospholipids, lysophosphatidic acid (LPA) and phosphatidic acid (PA), regulate pivotal processes related to the pathogenesis of cancer. Here, we report characterization of a novel lipid kinase, designated acylglycerol kinase (AGK), that phosphorylates monoacylglycerol and diacylglycerol to form LPA and PA, respectively. Confocal microscopy and subcellular fractionation suggest that AGK is localized to the mitochondria. AGK expression was up-regulated in prostate cancers compared with normal prostate tissues from the same patient. Expression of AGK in PC-3 prostate cancer cells markedly increased formation and secretion of LPA. This increase resulted in concomitant transactivation of the EGF receptor and sustained activation of extracellular signal related kinase (ERK) 1/2, culminating in enhanced cell proliferation. AGK expression also increased migratory responses. Conversely, down-regulating expression of endogenous AGK inhibited EGF- but not LPA-induced ERK1/2 activation and progression through the S phase of the cell cycle. Hence, AGK can amplify EGF signaling pathways and may play an important role in the pathophysiology of prostate cancer.

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Abbreviations used in this paper: AGK, acylglycerol kinase; DAG, 1,2-dioleoyl-sn-glycerol; DAGK, DAG kinase; ERK, extracellular signal related kinase; GPCR, G protein–coupled receptor; LPA, lysophosphatidic acid; MAGK, monoacylglycerol kinase; MOG, 1-oleoyl-2-sn-glycerol; PA, phosphatidic acid; PPARγ, peroxisome proliferator-activated receptor-γ; PTX, pertussis toxin; S1P, sphingosine-1-phosphate; siRNA, small interfering RNA; SphK, sphingosine kinase.

The online version of this article includes supplemental material.
Figure 1. Lipid kinase activity of recombinant AGK. (A) NIH 3T3 cells were transiently transfected with vector, hSphK1, hSphK2, or hAGK. After 24 h, cells were lysed and sphingosine-phosphorylating activity in cell lysates was measured with 50 μM D-erythro-sphingosine (Sph) substrate added as a BSA complex as described previously (Jiu et al., 2000). (B) Lipid-phosphorylating activity was determined in cell lysates from NIH 3T3 cells transiently transfected with vector (open bars) or hAGK (closed bars). The following lipids were tested: Sph, C6-cer; 1-oleoyl-2-arachidonoyl-glycerol (MOG), 2-arachidonoyl-glycerol (20:4), 2-AG; 1-palmitoyl-2-sn-glycerol (16:0), MPG; 1-stearoyl-2-sn-glycerol (18:0), MSG; or diacylglycerol (1,2-dioleoyl-sn-glycerol), DAG. Where indicated, octyl-glucopyranoside detergent was added to a final concentration of 1.5%. The data are expressed as picomoles of phosphorylated product formed per minute per milligram ± SD. Similar results were obtained in four additional experiments. *, P < 0.05 by t test. (C) TLC separation of products formed with MOG as substrate visualized with a phosphoimager.

Results

A new lipid kinase catalyzes the phosphorylation of acylglycerols to generate LPA and PA

While searching for additional isoforms of sphingosine kinase (SphK), the enzyme that catalyzes the formation of sphingosine-1-phosphate (SIP), another serum-borne lysophospholipid structurally similar to LPA, we cloned a related gene that encodes a 422-amino acid protein (Sugiura et al., 2000), was also a reasonably good substrate (Fig. 1 B). Like the crude bovine brain MAGK activity (Shim et al., 1989), AGK required magnesium for maximal activity, whereas other divalent cations, including Ca²⁺ and Zn²⁺, inhibited phosphorylation of MOG. Similar to brain MAGK, AGK also had higher activity in the presence of 0.03% deoxycholate, although enzymatic activity was completely abolished by most other detergents, including Triton X-100, Triton X-114, CHAPS, and β-octylglucopyranoside (Fig. 1 B and not depicted).

While this manuscript was in revision, Waggoner et al. (2004) showed that AGK expressed in bacteria phosphorylates DAG as well as MOG and ceramide, but not sphingosine, whereas in lysates of AGK-overexpressing cells, ceramide was not phosphorylated (Fig. 1 B), nor did we detect any phosphorylation of ceramide or sphingosine in vivo.

Subcellular localization of AGK

Confocal immunofluorescence microscopy revealed that AGK was distributed in a punctate, reticular pattern in NIH 3T3 cells (Fig. 2 A), which is reminiscent of a mitochondrial localization. There was no significant colocalization with the ER marker calnexin (Fig. 2 A). On the other hand, AGK expression clearly colocalized with mitochondria stained with MitoTracker red (Fig. 2 A). Similar mitochondrial localization of AGK was also observed in HEK 293 and PC-3 cells (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200407123/DC1), indicating that the subcellular distribution was not cell type specific. In agreement, although AGK does not contain a canonical mitochondrial localization signal, the MitoProt II website predicts an 80% probability of mitochondrial localization,
AGK activity was also determined in each subcellular fraction with MOG as substrate. Results are means ± SD of triplicate determinations. Similar results were obtained in two additional experiments. *, P < 0.05 by t test. (D) 400-μg aliquots of lysates from HEK 293 cells transiently transfected with vector (open bars) or V5-AGK (closed bars) were immunoprecipitated with anti-V5 antibody as described in Materials and methods, and AGK activity was determined in the immunoprecipitates. Data are expressed as picomoles of LPA formed in 30 min and are means ± SD of duplicate determinations.

AGK regulates LPA and PA in vivo
To definitively demonstrate that AGK has intrinsic kinase activity rather than affecting the activity of some endogenous lipid kinase, phosphorylation of acylglycerol substrates after specific pull-down of epitope-tagged AGK expressed in HEK 293 cells with V5 antibody was determined. Although the V5 antibody inhibits AGK, phosphorylation of MOG was sixfold greater in immunoprecipitates from V5-AGK transfectants than vector transfectants (Fig. 2 D) and there was no significant phosphorylating activity with other lipid substrates (not depicted).

To identify the phosphorylated lipids produced by AGK in vivo, vector and AGK PC-3 transfectants were incubated with 32P-labeled orthophosphate and labeled phospholipids in isolated mitochondria examined (Fig. 3 A). Expression of AGK resulted in 80% increase of 32P-labeled PA without significantly affecting labeling of the other mitochondrial phospholipids. Because it is known that LPA synthesized in mitochondria can readily exit this organelle (Chakraborty et al., 1999) or be rapidly metabolized to PA, changes in total cellular phospholipids were also examined (Fig. 3, B–D). There were no obvious differences in labeling of the major known cellular phospholipids in AGK-expressing cells compared with the vector cells. However, two-dimensional HPTLC analysis revealed that a labeled phospholipid that comigrated with authentic LPA (Fig. 3, B and C), although barely detectable in vector cells, was increased threefold in AGK-expressing cells. Moreover, this phospholipid was eliminated by treatment with phospholipase B, which hydrolyzes the ester bonds of lysophospholipids, confirming its identity as LPA. Labeled PA was also increased in these transfectants (Fig. 3 D), albeit much less than LPA. Of note, in these cells, AGK mRNA levels relative to 18S RNA were increased by almost twofold over endogenous expression from 1.2 ± 0.1 to 2.3 ± 0.2, as determined by quantitative PCR.

It has previously been shown that cancer cells secrete LPA (Mills and Moolenaar, 2003). Small amounts of labeled lysophospholipids, including LPA, were secreted by vector transfected PC-3 cells. However, secretion of 32P-labeled LPA was significantly increased threefold by overexpression of AGK (Fig. 3, F and G), indicating that AGK increases both intracellular and extracellular levels of LPA. It should be noted that AGK was not detectable in the medium by immunoblotting nor did its expression cause apoptosis of cells, suggesting that appearance of LPA in the media is not a result of cell death.

All members of the DAGK and SphK superfamily have a conserved GDG sequence in the glycine-rich loop of the putative ATP binding region and a single point mutation of the second conserved glycine residue to aspartate has been used to prepare catalytically inactive DAGK (Topham and Prescott, 1999) and SphK (Pitson et al., 2002). Similarly, site-directed mutagenesis of the equivalent residue in AGK (G126E) resulted in a complete loss of phosphorylating activity (Fig. S2 B), and its expression had no discernible effects on 32P-labeled LPA, PA, or other phospholipids (Fig. S2 C). However, like wild-type AGK, this catalytically inactive mutant was localized to the mitochondria (Fig. S2 A)

and the program TMpred predicted one transmembrane region from amino acid 11 to 30.

To further substantiate the localization of AGK, protein expression and enzymatic activity were examined in subcellular fractions prepared by differential centrifugation. V5-Episode--tagged AGK with the predicted MW of 46.4 kD was highly enriched in the P2 mitochondria fraction (Fig. 2 B). Much less AGK was present in the P3 fraction containing intracellular membranes of the ER and Golgi or in the P4 plasma membrane fraction. In concordance with the protein expression pattern, the highest AGK-specific activity was in P2 (Fig. 2 C).
AGK is highly expressed in prostate cancer

As LPA has been most prominently associated with growth-promoting effects and probably contributes to cancer (Mills and Moolenaar, 2003), it was of interest to examine the expression of AGK in normal tissues and cancers. By Northern analysis, a 2.6-kb AGK mRNA was widely expressed (Fig. 4A), most abundantly in heart, kidney, muscle, and brain. Importantly, using a matched human tumor/normal tissue expression array, we found that AGK expression was significantly up-regulated in prostate cancers compared with the normal prostate tissues from the same patient (Fig. 4B). However, not all tumor tissues show an increase in AGK expression. In uterine, cervical, and stomach cancers, there also appeared to be higher expression of AGK compared with the normal tissues, whereas AGK expression seems to be reduced in colon cancer. AGK was also expressed in many types of human cancer cell lines (Fig. 4B), including prostate cancer cells, such as androgen-responsive LNCaP cells, which are more similar to early stage carcinoma, and androgen-insensitive TSU-Pr1 and PC-3 cells, a model for more advanced prostate carcinoma.
AGK expression enhances cell growth through LPA receptors

Growth promotion is one of the most prominent effects mediated by LPA (Mills and Moolenaar, 2003). Consistent with its ability to increase LPA synthesis, transient or stable expression of AGK enhanced proliferation of diverse cell types, including PC-3 cells (Fig. 4, C–E) and NIH 3T3 fibroblasts (Fig. S3 A, available at http://www.jcb.org/cgi/content/full/jcb.200407123/DC1). The growth-promoting effect of AGK was observed even in the presence of suboptimal serum concentrations (Fig. 4 C and Fig. S3 A). Although AGK stimulates growth, it had no cytoprotective effects on apoptosis induced by serum deprivation or the anti-cancer drug doxorubicin (Fig. S3 D). As expected, exogenous LPA increased proliferation of both AGK and vector transfectants to the same extent (Fig. 4 D). Addition of MOG to cells cultured in serum-free medium had a minimal effect on vector transfectants and significantly stimulated proliferation of AGK-expressing PC-3 cells (Fig. 4 E). This result is probably due to rapid metabolism and degradation of MOG, and thus only in AGK-expressing cells is sufficient LPA produced and secreted (Fig. 3, F and G) to stimulate proliferation.

FACS analysis revealed that 83 ± 0.4% of the vector transfectants were in Go/G1 phase and 7.0 ± 0.04 and 10 ± 0.4% were in S and G2/M phases, respectively. Overexpression of AGK reduced the fraction of cells in Go/G1 to 73 ± 0.04% and increased the proportion in the S phase by greater than twofold (15 ± 0.1%), without significantly affecting the proportion in the G2/M phase (12 ± 0.1%).

Although it is well established that the mitogenic effects of LPA in many cell types are mediated by binding to its specific GPCRs (Mills and Moolenaar, 2003), intracellular actions have also been suggested (Hoeks et al., 2001), possibly as an agonist of the nuclear transcription factor peroxisome proliferator-activated receptor-γ (PPARγ; Zhang et al., 2004). To examine the potential involvement of intracellular actions of LPA generated by expression of AGK, its mitogenic effects were examined in rat hepatoma RH7777 cells that do not express LPA34 and do not respond to LPA (Fukushima et al., 1998). However, in contrast to PC-3 (Fig. 4, C–E) and NIH 3T3 (Fig. S3 A) cells, RH7777 cells did not show an increase in DNA synthesis in response to expression of AGK as measured by incorporation of BrdU into nascent DNA (Fig. 4 F). Although RH7777 cells express AGK, it is possible that they do not produce sufficient amounts of LPA if MOG is limiting. However, even addition of MOG did not enhance BrdU incorporation in RH7777 cells expressing AGK (Fig. 4 F).

Similar to a previous paper (Lea et al., 2004), GW9662, a selective antagonist of PPARγ, inhibited proliferation of PC-3 cells (Fig. 5 A). However, importantly, it did not abrogate the mitogenic effect of AGK (Fig. 5 A). Consistent with previous studies (Kue et al., 2002), we found that LPA1, LPA2, and LPA3 are expressed in PC-3 cells (unpublished data). It is known that LPA1 couples to pertussis toxin (PTX)-sensitive Gi, whereas LPA2 and LPA3 couple also to Gq. In PC-3 cells, LPA-regulated mitogenic signaling is mediated by Gβγ subunits derived from PTX-sensitive Gi proteins (Bookout et al., 2003). In agreement, PTX pretreatment not only inhibited growth of vector transfectants but it also markedly decreased the growth-promoting effects of AGK (Fig. 5 A). However, AGK expression enhanced cell proliferation even in the presence of PTX.

and the immunoprecipitates were analyzed by Western blotting using anti-phosphotyrosine or anti-EGFR antibody. Blots were stripped and reprobed with anti-EGFR antibody. Blocking of EGFR signaling suppresses ERK activation and cell growth advantage mediated by AGK. (D) Serum-starved PC-3 cells stably transfected with vector or AGK were preincubated for 60 min in the absence or presence of 200 nM AG1478, and then treated with EGF for 10 min. Cell lysates were analyzed by immunoblotting with phospho-specific ERK1/2 antibody. Blots were stripped and reprobed with ERK2 antibody to demonstrate equal loading. (E) PC-3 cells stably transfected with vector or AGK were cultured in medium supplemented with 1% serum without or with GW9662 (1 μM or 5 μM) or with PTX (100 ng/ml), and cell proliferation was determined after 6 d with WST-1. Asterisks denote significant differences compared with untreated controls (P < 0.05, t test). (B) Enforced expression of AGK enhances EGFR tyrosine phosphorylation and stimulates ERK1/2. Serum-starved PC-3 cells stably transfected with vector or AGK were stimulated without or with 10% serum for 10 min, lysed and immunoblotted with anti-phosphotyrosine, anti-EGFR antibody, or phospho-specific anti-ERK1/2 antibodies. Blots were stripped and reprobed with ERK2 antibody to demonstrate equal loading. (C) AGK expression induces EGFR transactivation. Lysates from cells treated as in B were immunoprecipitated with anti-EGFR antibody and analyzed by Western blotting using anti-phosphotyrosine or anti-EGFR antibody. Blots were stripped and reprobed with EGFR antibody. Blocking of EGFR signaling suppresses ERK activation and cell growth advantage mediated by AGK.
AGK promotes transactivation of EGFR

Many studies have led to the notion that LPA is important in the pathophysiology of prostate carcinoma functioning in an emerging paradigm of cross talk between LPA receptors and the tyrosine kinase EGFR (Prenzel et al., 1999; Mills and Moolenaar, 2003). Therefore, it was of importance to determine whether overexpression of AGK and increased LPA levels resulted in such receptor transactivation leading to enhanced growth.

In serum-starved cells, AGK expression increased tyrosine phosphorylation of several proteins, notably a 170-kD band, which was similarly increased by serum in vector transfectants (Fig. 5 B). Kinetic analysis revealed that the 170-kD tyrosine phosphorylation induced by serum was a rapid event in AGK-expressing cells, clearly evident within 5 min and remaining elevated for at least 60 min (Fig. S4 A, available at http://www.jcb.org/cgi/content/full/jcb.200407123/DC1). The enhanced tyrosine phosphorylation of the 170-kD protein represented activation of the EGFR, as immunoblotting of anti-EGFR immunoprecipitates with anti-phosphotyrosine revealed increased EGFR tyrosine phosphorylation in cells overexpressing AGK, even in the absence of serum (Fig. 5 C).

AGK-induced extracellular signal related kinase (ERK) 1/2 activation requires EGFR

Previously, it has been suggested that EGFR activation is required for signal relay from LPA receptors to ERK1/2 activation in prostate cancer cells (Prenzel et al., 1999; Kue et al., 2002; Raj et al., 2002). AGK expression markedly increased activation of ERK1/2, as determined with a phosho-specific antibody, which was further enhanced by serum (Fig. 5 B) and EGF (Fig. 5 D and Fig. S3 C). To further confirm that activation of the EGFR was necessary for AGK-stimulated ERK activation, we used the specific EGFR tyrosine kinase inhibitor, tyrphostin AG1478. As expected, AG1478 abolished EGFR-induced tyrosine phosphorylation (Fig. S4 B). AG1478 blocked AGK-mediated ERK1/2 phosphorylation (Fig. 5 D) and decreased its mitogenic effect (Fig. 5 E) and also inhibited MOG-stimulated proliferation by 35 ± 4%. Nonetheless, prolonged treatment with AG1478 did not affect AGK protein levels (Fig. 5 E, inset).

Involvement of AGK in motility

Transactivation of the EGFR has also been implicated in motility of cancer cells (Gschwind et al., 2001). In agreement, AGK overexpression enhanced migration of PC-3 cells toward EGF, which was blocked by the EGFR inhibitor AG1478 (Fig. 6 A). AGK also enhanced migration of NIH 3T3 fibroblasts toward serum (Fig. S3 B).

In the Boyden chamber cell migration assay, differences in cell shape and size may affect passage through the pores in the membrane but do not affect the in vitro wound closure assay. AGK expression also enhanced closure of the wounded area, especially in the presence of EGF and the AGK substrate MOG (Fig. 6, B and C). In contrast, wound closure induced by LPA was not affected by AGK expression. AGK-induced wound closure was also blocked by AG1478, supporting a role for EGFR transactivation in AGK-induced migratory responses.

AGK up-regulates IL-8

Expression of the multifunctional cytokine IL-8 correlates with angiogenesis, tumorigenicity, and metastasis of human prostate cancer cells implanted in nude mice (Kim et al., 2001). Similarly, LPA markedly enhanced IL-8 secretion from PC-3 cells. Expression of AGK slightly increased IL-8 release, which was further significantly increased by addition of MOG, the precursor of LPA (Fig. 6 D). The EGFR inhibitor AG1478 only slightly decreased LPA-induced IL-8 secretion, suggesting that this response is independent of EGFR transactivation.

Involvement of endogenous AGK in ERK1/2 activation and cell cycle progression

Serum and EGF induced significant increases in AGK expression as determined by quantitative real-time PCR (Fig. 7 A). It has previously been shown that LPA itself is sufficient to increase its own production in PC-3 cells, indicating the pres-
ence of an autocrine function for LPA, we found that LPA also increased expression of AGK by threefold in naïve PC-3 cells (Fig. 7 A). To examine the physiological function of AGK, its expression was down-regulated with small interfering RNA (siRNA). siAGK, but not control siRNA, markedly reduced AGK mRNA in PC-3 cells, as determined by QPCR, without influencing expression of SphK1 (Fig. 7 B). Consistent with its role in synthesis of LPA and PA, the most striking effect of down-regulating AGK was reduction of mitochondrial PA and LPA by ~30% (Fig. 7 C). Remarkably, siAGK completely blocked stimulation of ERK1/2 induced by EGF (Fig. 7 D). To rule out off-target effects, we used two additional unrelated siRNAs targeted to different sequences of AGK. siAGK2 and siAGK3 markedly and specifically reduced expression of AGK determined by QPCR (0.2 and 0.16 relative to siControl) without reducing expression of SphK1 (1.1 and 1.0 relative to siControl) or SphK2 (1.1 and 1.0 relative to siControl). Importantly, both of these siRNAs also markedly reduced EGF-induced ERK1/2 activation but did not decrease LPA-induced ERK activation (Fig. 7 E), suggesting that LPA can bypass the effects of down-regulation of AGK. In addition, down-regulation of AGK reduced EGF-stimulated tyrosine phosphorylation of the EGFR (Fig. 5 S C).

Down-regulation of AGK reduced EGF-induced wound closure but had no effect on wound closure induced by LPA (Fig. 7 F). siAGK also reduced migration toward EGF but not toward serum (Fig. 7 G). siAGK but not siControl inhibited basal secretion of IL-8 in untreated PC-3 cells and also blocked the small effect of MOG (1.28- and 1-fold stimulation in siControl and siAGK, respectively; Fig. 7 H). However, its effects on EGF or LPA-induced IL-8 secretion were smaller (fold stimulation with EGF is 2.16 and 2.06 and with LPA is 5 and 7.5 in siControl and siAGK, respectively). Similarly, siAGK2 also reduced basal IL-8 secretion without affecting LPA-induced secretion (Fig. 7 H).

Next, we examined the role of endogenous AGK in cell growth regulation. The levels of LPA in serum range from 1 to 6 μM (Baker et al., 2001), and in 10% serum, the level is well below the concentration needed for its mitogenic effects. In agreement with others (Qi et al., 1998), we have found that serum is a more potent mitogen for PC-3 cells than 10 μM LPA (unpublished data). Remarkably, siAGK markedly decreased DNA synthesis as measured by incorporation of BrdU into nascent DNA, whereas nonspecific siRNA had no increased DNA synthesis as measured by incorporation of LPA (unpublished data). Remarkably, siAGK markedly decreased expression of AGK mRNA was determined by quantitative real-time PCR. Data were normalized to expression of 18S RNA and are means ± SD of triplicate determinations. *, P < 0.05 by t test. (A) Expression of endogenous AGK. Naïve PC-3 cells were serum starved for 24 h and treated in DME with or without 10% serum, LPA (10 μM), or EGF (100 ng/ml) for 16 h, and AGK mRNA was determined by quantitative real-time PCR. Data were normalized to expression of 18S RNA and are means ± SD of duplicate determinations. *, P < 0.05 by t test. (B) PC-3 cells were transfected with control siRNA (open bars) or siRNA specific for AGK (hatched bars) and mRNA levels of AGK and SphK1, and 18S RNA was determined by QPCR. (C) Duplicate cultures were labeled with 32P for 12 h. Phospholipids were extracted from mitochondria isolated by differential centrifugation. Equal amounts of 32P-phospholipids were separated by TLC, and the indicated phospholipids were quantified with a phosphoimager. The data are expressed as a percentage of total 32P-labeled phospholipids and are means ± SD of duplicate determinations. *, P < 0.05 by t test. (D) Down-regulation of AGK with siRNA blocks EGF-induced ERK1/2. PC-3 cells were transfected with control siRNA (open bars) or siRNA specific for AGK and treated without [None] or with 10 ng/ml EGF for the indicated times. Equal amounts of lysate proteins were separated by SDS-PAGE, and ERK1/2 activation was determined by immunoblotting with anti-pERK1/2. Blots were stripped and reprobed with anti-ERK2 as a loading control. (E) PC-3 cells were transfected with siControl, siAGK, or siAGK2 (Dharmacon), as described in Materials and methods, and treated with EGF (10 ng/ml) or LPA (10 μM) for the indicated times. Equal amounts of lysate proteins were separated by SDS-PAGE and ERK1/2 activation determined by immunoblotting with anti-pERK1/2. Blots were stripped and reprobed with anti-tubulin as a loading control. Down-regulation of AGK decreases EGF-induced wound closure and migration. (F) Monolayers of PC-3 cells transfected with control siRNA (open bars) or siRNA specific for AGK (hatched bars) were wounded and treated with vehicle [None], EGF (10 ng/ml), or LPA (10 μM), and migration of cells into the wound was determined after 24 h. (G) Cells from duplicate cultures were allowed to migrate for 3 h in Boyden chambers toward vehicle [None], EGF (10 ng/ml), or serum [10%]. The data are means ± SD of two determinations. (H) Down-regulation of AGK with siRNA decreases IL-8 secretion. PC-3 cells were transfected with the indicated control siRNAs (open bars and gray bars) or siRNAs specific for AGK (hatched bars and black bars), incubated in serum-free DME without [None] or with MOG (10 μM), LPA (1 μM), or EGF (10 ng/ml) for 16 h, and IL-8 secretion was measured. *, P < 0.05 by t test.
Figure 8. Involvement of endogenous AGK in cell proliferation. (A) PC-3 cells were transfected with control siRNA (open bars) or siAGK (hatched bars) and serum starved for 8 h. After culturing for an additional 16 h in serum-free medium or in medium supplemented with 10% serum, BrdU was added for 3 h and the fraction of cells incorporating BrdU was determined. Data are means ± SD of duplicate cultures from a representative experiment. At least three different fields were scored with a minimum of 100 cells per field. Similar results were obtained in two independent experiments. (B) Representative fluorescent and phase images of si-Control and siAGK-transfected cells. (C) Cell cycle analysis. PC-3 cells transfected with control siRNA or siAGK were cultured in serum-free medium or in medium supplemented with 10% serum. After 24 h, cellular DNA was stained with propidium iodide and cell cycle analysis was performed with an Epics XL-MCL flow cytometer (Beckman Coulter). Asterisks indicate significant differences from vector-transfected values as determined by t test (P < 0.05).

Discussion

Several lines of evidence suggest that AGK is a bona fide acylglycerol kinase. First, recombinant AGK catalyzes the phosphorylation of both MOG and DAG in vitro, producing LPA and PA, respectively. In agreement, bacterially expressed, purified recombinant AGK phosphorylated DAG as well as MOG (Waggoner et al., 2004). Second, overexpression of AGK increases cellular levels of LPA and PA. Third, and conversely, down-regulation of AGK reduces their intracellular levels. Fourth, overexpression of kinase dead AGK, although localized similarly to the mitochondria as wild-type AGK, does not increase acylglycerol kinase activity nor produce LPA.

Prostate carcinomas often possess an autocrine stimulatory loop in which the transformed cells express high levels of EGFR and also produce activating ligands. One such ligand might be the bioactive phospholipid LPA, which stimulates prostate cancer cell proliferation, migration, and survival, not only by acting on its cognate GPCRs but also by stimulating metalloproteinase activity and proteolytic EGF precursor processing leading to EGFR transactivation (Gschwind et al., 2001). Our identification of AGK as a lipid kinase that produces LPA and PA and is highly expressed in prostate tumors may have important clinical implications with regard to advanced prostate cancer. AGK not only regulates mitogenic EGFR signaling that plays important roles in androgen-refractory metastatic prostate cancer but also stimulates cell motility and EGFR-independent secretion of the pluripotent cytokine IL-8. Indeed, blockade of the EGFR in PC-3 cells inhibited tumor growth and invasion and also down-regulated expression of IL-8 within the tumors (Karashima et al., 2002).

Interestingly, the endocannabinoids anandamide and 2-AG potently inhibit proliferation and cause apoptosis of PC-3 and DU145 prostate cancer cells (Melck et al., 2000). Because AGK can phosphorylate 2-AG converting it to LPA, it may regulate the dynamic levels of these counterregulatory lipids that have been shown to play opposing roles in growth and survival of prostate cancers. Of note, LPA1 was originally cloned from prostate cancer cells (Im et al., 2000), which is concordant with the ability of LPA to induce autocrine proliferation of these cells (Xie et al., 2002). Moreover, PC-3 cells express LPA1,3 receptors, thus providing AGK with the potential to activate numerous downstream growth signaling pathways.

The mitogenic responses of some mammalian cells to LPA may be LPA1,4 independent (Hooks et al., 2001). However, in prostate cancer cells, LPA transduces mitogenic signals via activation of Galpha and GBeta subunits (Kue and Daaka, 2000; Bookout et al., 2003). Importantly, the expression of GRK2ct inhibited PC-3 tumor formation in animals (Bookout et al., 2003). Several lines of evidence indicate that the growth-promoting effects of AGK are mediated via LPA receptors. In RH7777 cells, which are devoid of LPA1,4, expression of AGK had no effect on DNA synthesis, even in the presence of exogenous MOG. Moreover, PTX pretreatment decreased the growth-promoting effects of AGK. Our results are similar to others showing that PTX not only inhibits proliferation of PC-3 cells induced by LPA but also inhibits the effect of serum more drastically (Kue and Daaka, 2000). Furthermore, consistent with a previous study (Lea et al., 2004), GW9662, a selective antagonist of PPARgamma, inhibited proliferation of vector-transfected PC-3 cells, and yet it did not abrogate the mitogenic effect of AGK. Our results are consistent with the notion that production and secretion of LPA by AGK induces Gi-dependent proliferation, likely through LPA receptors. Remarkably, down-regulation of AGK drastically reduced ERK1/2 activation induced by EGF, which, as expected, was bypassed by addition of the AGK product, LPA. Our data suggest that AGK plays an important role in EGF-induced mitogenic ERK signaling. AGK also phosphorylates DAG to produce the bioactive mediator PA, which regulates numerous biological processes including Raf translocation to the plasma membrane (Rizzo et al., 1999) and activation of mTOR (Fang et al., 2001). Our results also imply that specific pools of PA may play important roles in growth signaling.
Although the physiological function of LPA and PA generation in the mitochondria is not clear, a LPA phosphatase with 28.5% amino acid identity to human prostatic acid phosphatase is also localized to the mitochondria (Hiroyama and Takenawa, 1999). This LPA phosphatase has been suggested to regulate lipid metabolism in mitochondria by hydrolysis of LPA to monoacylglycerol (Hiroyama and Takenawa, 1999). Previous studies suggest that mitochondria-produced LPA can leave this organelle and be transported to the ER in the presence of liver fatty acid binding protein, can be secreted, and/or can be converted to PA (Haldar and Lipfert, 1990; Chakraborty et al., 1999; Hiroyama and Takenawa, 1999). In addition, LPA generated by prostate cancer cells in response to mitogenic stimuli can be secreted (Qi et al., 1998; Gibbs et al., 2000; Kue and Daaka, 2000; Kue et al., 2002; Xie et al., 2002).

It has been demonstrated that in both PC-3 and DU145 prostate cancer cells, agonists induce 18:1 LPA formation that is then released into the medium (Xie et al., 2002). As Du145 and PC-3 cells express LPA1, it was suggested that 18:1 LPA can act as an autocrine mediator (Xie et al., 2002), yet the critical enzymes involved have not been identified. Our data suggest that AGK could be a missing link. Production of LPA by AGK, which in turn transactivates the EGFR, can amplify mitogenic and survival signals. Moreover, expression of AGK is stimulated by EGF, serum, and even by LPA itself, thereby providing a positive feed-forward stimulus that could further enhance EGFR-dependent and -independent processes important for cancer progression. Therefore, targeting AGK, which is upstream of the EGFR, could offer additional therapeutic benefits in treatment of androgen-independent prostate cancer.

Materials and methods

Cloning of an AGK

An EST (AW321722) was identified that contained an open reading frame with 25% identity and 50% similarity to hShPK2 from aa 133 to 256. 5’ and 3’ RACE were performed using the GeneRacer kit (Life Technologies) to obtain the sequence of the full-length open reading frame. A cDNA with a complete open reading frame was cloned from a human kidney cDNA library encoding a 422-amino acid polypeptide with a calculated molecular mass of 46,400 D (Fig. S1). A nearly identical mouse homologue (CACO6108) was also identified.

The predicted sequence of this human protein and its mouse homologue identified from the mouse database (CACO6108) are 95% identical and both show sequence similarity to ShPKs, especially in the five conserved ShPK domains (Liu et al., 2000, 2002). These domains of SphK1/SphK2, new lipid kinase/ShPK1, new lipid kinase/ShPK2, new lipid kinase/CERK, and new lipid kinase/DAGK indicated sequence identities of 53, 29, 23, 26, and 24%, respectively. These comparisons suggest that this new lipid kinase may be unique. A search of the human genome database revealed that the gene encoding this lipid kinase is located on chromosome 7q54, whereas ShPK1 and ShPK2 have been localized to chromosomes 17q25.2 and 19q13.2, respectively.

Lipid kinase activity

Lipids (100 nmol) were dried under N2 and resuspended in 180 µl of buffer containing 100 mM MOPS, pH 7.2, 2 mM EGTA, 15 mM NaF, 2 mM orthovanadate, 50 mM NaCl, 250 mM sucrose, 0.03% deoxycholate, and 1,500 diluted protease inhibitor cocktail (Sigma-Aldrich). After brief sonication, 10 µl lysates (10 µg) and 10 µl [32P]ATP (10 µCi, 1 nmol) containing MgCl2 (10 mM) were added and reactions were performed for 30 min at 37°C. 32P-Labeled lipids produced were extracted into 0.8 ml chloroform/methanol/concentrated HCl (100:200:1 vol/vol), and the extraction was confirmed by adding 0.25 µl 2 M NaCl and 0.25 µl CHCl3. Aliquots of the organic phases were analyzed by TLC on silica gel G60 and CHCl3/acetone/methanol/acetic acid/water (10:4:3:2:1, vol/vol). After brief sonication, 10 µl lysates (10 µg) and 10 µl [32P]ATP (10 µCi, 1 nmol) containing MgCl2 (10 mM) were added and reactions were performed for 30 min at 37°C. In preparation for SDS-PAGE, the membranes were cut into small pieces and washed and incubated for a further 2 h in 4 ml of 2% sodium dodecyl sulfate (SDS) gel and electrophoresed on a 10% SDS-PAGE gel. The gels were dried, and the proteins were visualized by autoradiography after 6 h at 4°C. The relative intensity of the bands was measured by densitometric scanning of the autoradiographs.

Cell culture and transfection

Human PC-3 prostate cancer cells (CRL-1435; American Type Culture Collection), NIH 3T3 fibroblasts (CRL-1658; American Type Culture Collection), rat hepatoma RH7777 cells (provided by X. Fang, Virginia Commonwealth University, Richmond, VA), and human embryonic kidney cells (HEK 293; CRL-1573; American Type Culture Collection) were seeded at 4–5 × 105 cells per well in 6-well plates and transfected with Lipofectamine PLUS for NIH 3T3, HEK 293, and RH7777 cells and Lipofectamine 2000 for PC-3 cells, according to the manufacturer’s instructions (Life Technologies).

Catalytically inactive AGK

The QuikChange site-directed mutagenesis kit (Stratagene) was used to prepare catalytically inactive AGK (G126E) by mutating the conserved glycine in the glycine-rich loop of the ATP binding site (forward primer, 5’-TGGAGGAGGAGATGAGACACTGCAGGAGGTT-3’; reverse primer, 5’-AACCTTCGGAGGATGCATCCTCTCCTCCGCAA-3’). The mutation was confirmed by sequencing.

siRNA transfection

AGK expression was down-regulated with sequence-specific siRNA. siRNA target sequence for AGK (siAGK: 5’-AAGACGTAGAGGTCTTCCATCTCAGAGGAGGTT-3’) and control siRNA (5’-TCTTCCGACAGGCTTCCAGGTT-3’) were obtained from QIAGEN. In some experiments, cells were transfected with two additional AGK siRNAs (siAGK1: 5’-GAGCTACCTCTCAGAAGA-3’; siAGK2: 5’-GGAGAAGCAGTATGGTAGA-3’) and siControl (non-targeting siRNA with at least four mismatches to all human and mouse genes from Dharmacon). Cells (3 × 105) were transfected in 6-well dishes for 3–4 h with the RNA duplexes (200 nm) using Oligofectamine (Life Technologies) according to the manufacturer’s protocol. 90 ± 2% of the cells were transfected as determined with siGLO RISC-Free siRNA (Dharmacon).

Real-time PCR

Quantitative real-time PCR was performed on a real-time PCR machine (model Taqman ABI 7900; Applied Biosystems) with the primers/probes: AGK forward primer, 5’-CAGAGGCTGCGCTTCTTTCG-3’; reverse primer, 5’-TTGGCCAGCCTCCACACTC-3’; probe, 5’-CAACA- CAGAGGATGTCGCTCC-3’ (Integrated DNA Technologies); pre-mixed primer-probe set for hShPK1 was purchased from Applied Biosystems. Ribosomal RNA (18S rRNA) measured using TaqMan assay reagents served as endogenous control.

Lipid kinase activity

Lipids (100 nmol) were dried under N2 and resuspended in 180 µl of buffer containing 100 mM MOPS, pH 7.2, 2 mM EGTA, 15 mM NaF, 2 mM orthovanadate, 50 mM NaCl, 250 mM sucrose, 0.03% deoxycholate, and 1,500 diluted protease inhibitor cocktail (Sigma-Aldrich). After brief sonication, 10 µl lysates (10 µg) and 10 µl [32P]ATP (10 µCi, 1 nmol) containing MgCl2 (10 mM) were added and reactions were performed for 30 min at 37°C. 32P-Labeled lipids produced were extracted into 0.8 ml chloroform/methanol/concentrated HCl (100:200:1 vol/vol). The extraction was confirmed by adding 0.25 µl 2 M KCl and 0.25 µl CHCl3. Aliquots of the organic phases were analyzed by TLC on silica gel G60 with CHCl3/acetone/methanol/acidic water (10:4.3:2:1, vol/vol) as solvent and the radioactive spots corresponding to migration of standards were quantified with an FX Molecular Imager (Bio-Rad Laboratories). In some experiments, ShPK1 (Ji et al., 2000), ShPK2 (Ji et al., 2000), and ShPK3 (Sugiera et al., 2002) were measured exactly as described.

AGK activity in immunoprecipitates

HEK 293 cells were seeded in 10-cm dishes and transiently transfected with vector or V5-tagged AGK. 24 h later, cells were lysed by sonication in buffer containing 100 mM MOPS, pH 7.2, 2 mM EGTA, 2 mM orthovanadate, 2 mM β-glycerophosphate, 150 mM NaCl, 250 mM sucrose, and 1,500 diluted protease inhibitor cocktail. Lysates were cleared by centrifugation and 400 µg of protein in 250 µl was incubated with 1 µg anti-V5 (Invitrogen) for 4 h at 4°C. Protein A/G PLUS–Agarose beads (10 µl; Santa Cruz Biotechnology, Inc.) were added and incubated for an additional 1 h. The beads were washed four times with the same buffer, resuspended in 10 µl, and AKG activity was determined.

32P labeling of cellular phospholipids

Vector and kinase PC-3 transfectants were grown to 80–90% confluency in 100-mm dishes, incubated for 2 h with 40 µCi/ml [32P] in phosphate-free DMEM at 37°C, and washed and incubated for a further 2 h in 4 µl

ACYLGLYCEROL KINASE, LPA, AND EGFR SIGNALING • BEKTAS ET AL. 809
phosphate-free DME. Medium was removed and brief centrifugation of the cells was performed. Following centrifugation, 2 ml of a 1:1 vol/vol mix of chloroform/methanol/0.1 M HCl was added, followed by 3 ml of chloroform. This mixture was vortexed, and the resulting debris was centrifuged at 5,000 rpm for 10 min. A total of 0.6 ml of the supernatant was transferred to a clean tube containing 0.5 ml of water and 0.1 M NaCl. The mixture was vortexed, and the resulting debris was centrifuged at 100,000 rpm for 1 h to obtain plasma membranes. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies as described in the figure legends.

**Immunoprecipitation**

PC-3 cells were lysed in buffer containing 25 mM Hepes, pH 7.5, 0.3 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 0.5 mM dihydrostreptomycin, 1 mM PMSF, and 10 μg/ml leupeptin. Lysates were cleared by centrifugation at 10,000 g for 10 min and incubated with 2 μg anti-EGFR antibody for 2 h at 4°C. 20 μl of protein G-Sepharose beads (Santa Cruz Biotechnology, Inc.) were added and incubated for an additional 1 h at 4°C. Sepharose beads were washed and boiled in SDS sample buffer, and bound proteins were analyzed by Western blotting.

**Online supplemental material**

Fig. S1 shows a Clustal W alignment of AGK with other lipid kinases. Fig. S2 demonstrates the mitochondrial localization of AGK and catalytically inactive AGK and confirms that the mutant has no kinase activity. Fig. S3 shows the effect of AGK on growth and survival of NIH 3T3 cells. Fig. S4 shows regulation of EGFR phosphorylation by AGK. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200407123/DC1.

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