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13. ABSTRACT (Maximum 200 Words)

Lysophosphatidic acid (LPA) is a major mitogen in serum that regulates an array of cellular processes related to pathogenesis of cancer, especially ovarian, prostate and breast cancers. Interest in LPA has accelerated recently with the discovery that it is a ligand of a family of three G protein coupled cell surface receptors. Prostate cancer cells express these LPA receptors and it has been suggested that their expression correlates with more advanced prostate cancer. We found that androgen markedly upregulates expression of LPA(3) in LNCaP cells which are androgen-responsive prostate cancer cells, making them more similar to early stage carcinoma. In this grant period, we cloned a novel type of lipid kinase (MDGK) which phosphorylates monoacylglycerols and diacylglycerols to form LPA and PA, respectively. Both have been implicated in growth and survival of prostate cancer cells. Using a matched human tumor/normal tissue expression array, we found that MDGK expression was strikingly upregulated in prostate cancers compared to the normal prostate tissues from the same patient. In contrast, MDGK was similarly expressed in other types of cancers compared to their normal tissue counterparts, including kidney, breast, colon, and stomach cancers. MDGK is highly expressed in human prostate cancer cells, including androgen-responsive LNCaP cells, which are more similar to early stage carcinoma, and androgen-insensitive PTsu-Pr1 and PC-3 cells. Overexpression of MDGK in PC-3 cells results in secretion of LPA from these cells, transactivation of the epidermal growth factor (EGF) receptor, and subsequent activation of ERK1/2. Because of the well known role of the EGF receptor family in androgen-refractory metastatic prostate cancer, the pathophysiological significance of MDGK is to produce LPA which in turn can stimulate the release of mature EGF and thus activate the EGF receptor, amplifying mitogenic and survival signals. Therefore, targeting this kinase, which is upstream of the EGF receptor, offers additional therapeutic benefits in treatment of androgen-independent prostate cancer.

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INTRODUCTION

Originally known for its pedestrian role as an intermediate in intracellular lipid metabolism, LPA is now recognized as a potent lipid mediator that evokes growth factor-like responses and regulates an array of cellular processes related to pathogenesis of cancer (1). These include stimulating proliferation by increasing cell cycle progression, enhancing cell survival, stimulating motility and inducing tumor cell invasion, and regulating neovascularization (1). There are three established LPA receptors, LPA_1, LPA_2, and LPA_3 (1-3), which are differentially expressed, coupled to a variety of G proteins, and thus regulate diverse cellular responses (1,3). Intriguingly, expression of LPA receptors correlates with more advanced prostate cancer cell lines (4) and LPA_2 and LPA_3 are aberrantly expressed in ovarian cancer cells (5,6), indicating a potential role in the pathophysiology of cancer. Moreover, LPA has a novel intracellular function as a high-affinity ligand for peroxisome proliferators-activated receptor-gamma (PPAR-γ), a transcription factor that regulates genes controlling energy metabolism (7) and can exacerbate mammary gland tumor development (8).

In addition to actions through conventional GPCR signaling pathways, LPA receptors can indirectly regulate cell functions by transactivating the EGF tyrosine kinase receptor (9-11). This cross-communication between different signaling systems is not only important for the growth promoting activity of LPA (9,11), it also may be a clue to its pathophysiological role in prostate cancer (10), head and neck squamous cell carcinoma (12), and kidney and bladder cancer (13).

LPA is not only an active component of serum, it also accumulates to high concentrations in malignant effusions (14) and has been proposed to be a marker and mediator of ovarian cancer progression (5,6). Although LPA, formed by acylation of glycerol 3-phosphate, is considered to be a key intermediate in *de novo* glycerolipid synthesis, abundant evidence now indicates that bioactive LPA can also be generated by other pathways. LPA is produced from phosphatidic acid

(PA) in activated platelets and ovarian and prostate cancer cells by phospholipase D and subsequent deacylation by phospholipase A_1 or A_2 (15,16). The recent discovery that LPA is generated in the extracellular milieu from lysophosphatidylcholine by the ecto-enzyme autotaxin, known to be involved in tumor invasion, neovascularization and metastasis (17), further supports the notion that LPA is an important regulator of tumor progression (1,18).

Yet another potential pathway for synthesis of LPA is the phosphorylation of monoacylglycerol by a specific lipid kinase (19), an enzyme that has remained an enigma for more than 40 years. We have now cloned and characterized a novel acylglycerol kinase (AGK) that phosphorylates both diacylglycerol to produce PA and monoacylglycerol to form LPA, which in turn activates the EGF receptor, amplifying mitogenic and survival signals and regulating EGF-directed motility (Appendix 1).

BODY

Cloning and characterization of a novel human lipid kinase

In the previous annual report, we described in detail the cloning of a lipid kinase that phosphorylates monoacylglycerols to form LPA and demonstrated that it is highly expressed in prostate cancers. We also describe characterization of its enzymatic activity, localization, and signaling pathways that it modulates.

AGK expression enhances cell growth through LPA receptors

Growth promotion is one of the most prominent effects mediated by LPA (1). 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. 4C-E, Appendix 1) and NIH 3T3 fibroblasts (Fig. S1A Appendix 1). The growth promoting effect of AGK was observed even in the presence of sub-optimal serum concentrations (Fig. 4C and Fig. S1A Appendix 1). Although

AGK stimulates growth, it had no cytoprotective effects on apoptosis induced by serum deprivation or the anti-cancer drug doxorubicin (Fig. S1D Appendix 1). As expected, exogenous LPA increased proliferation of both AGK and vector transfectants to the same extent (Fig. 4D Appendix 1). 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. 4E Appendix 1). This is probably due to rapid metabolism and degradation of MOG and thus, only in AGK expressing cells is sufficient LPA produced and secreted (Fig. 3F Appendix 1) to stimulate proliferation.

The growth promoting effects of AGK were also examined by DNA flow cytometry. FACS analysis revealed that 83 ± 0.4 % of the vector transfectants were in Go/G1 phase and 7 ± 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 ± 0.1% and increased the proportion in the S phase by more than 2-fold (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 (1), intracellular actions have also been suggested (20), possibly as an agonist of the nuclear transcription factor PPARγ (21). To examine the potential involvement of intracellular actions of LPA generated by expression of AGK, its mitogenic effects were determined in rat hepatoma RH7777 cells that do not express LPA_{1-4} and do not respond to LPA (22). However, in contrast to PC-3 (Fig. 4C-E Appendix 1) and NIH 3T3 (Fig. S1A Appendix 1) 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. 4F Appendix 1). Moreover, although GW9662, a selective antagonist of PPARγ, inhibited proliferation of vector transfected PC-3 cells, it did not abrogate the mitogenic effect of AGK

(Fig. 5A Appendix 1). Consistent with previous results (23,24), we found that LPA_1, LPA_2, and LPA_3 are expressed in PC-3 cells. It is known that LPA_1 couples to pertussis toxin (PTX)-sensitive Gi, whereas LPA_2 and LPA_3 couple also to Gq. In PC-3 cells, LPA-regulated mitogenic signaling is mediated by $G\beta\gamma$ subunits derived from PTX-sensitive Gi proteins (23,25). In agreement, PTX pretreatment not only inhibited growth of vector transfectants, it also markedly decreased the growth promoting effects of AGK (Fig. 5A Appendix 1).

AGK-induced ERK1/2 activation requires EGFR

Previously, it has been suggested that EGFR activation is required for signal relay from LPA receptors to ERK1/2 activaton in prostate cancer cells (10,24,26,27). AGK expression markedly increased activation of ERK1/2, as determined with a phospho-specific antibody, which was further enhanced by serum (Fig. 5B) and EGF (Fig. 5D and Fig. S1C Appendix 1). To further confirm that activation of the EGFR was necessary for AGK-stimulated ERK activation, we utilized the specific EGFR tyrosine kinase inhibitor, tyrphostin AG1478. As expected, AG1478 abolished EGFR-induced tyrosine phosphorylation (Fig. S2B Appendix 1). AG1478 blocked AGK-mediated ERK1/2 phosphorylation (Fig. 5D Appendix 1) and decreased its mitogenic effect (Fig. 5E Appendix 1) and also inhibited MOG-stimulated proliferation by 35 ± 4%. Nonetheless, prolonged treatment with AG1478 did not affect AGK protein levels (Fig. 5E, insert Appendix 1).

Taken together, these results suggest that the tyrosine kinase activity of EGFR contributes to AGK-induced activation of the ERK cascade and that stimulation of DNA synthesis is mediated not only by transactivation of EGFR, but also by direct activation of Gi-coupled LPA receptors.

Involvement of AGK in motility

Transactivation of the EGFR has also been implicated in motility of cancer cells (12). In agreement, AGK overexpression enhanced migration of PC-3 cells towards EGF, which was blocked by the EGFR inhibitor AG1478 (Fig. 6A Appendix 1). AGK also enhanced migration of NIH 3T3 fibroblasts towards serum (Fig. S1B Appendix 1).

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. 6B,C Appendix 1). 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.

Involvement of endogenous AGK in cell cycle progression

Surprisingly, we noticed that serum and EGF induced significant increases in AGK expression as determined by quantitative real-time PCR (Fig. 7A Appendix 1). It has previously been shown that stimulated PC-3 cells produce high levels of LPA and also respond to LPA (16). Moreover, in some cancer cells, LPA itself is sufficient to increase its own production, indicating the presence of an autocrine network (28). Consistent with an autocrine function for LPA, we found that LPA also increased expression of AGK by 3 fold in naïve PC-3 cells (Fig. 7A Appendix 1). Since AGK expression was stimulated by these potent growth factors, it was of interest to examine the physiological function of AGK by knocking down its expression with small interfering RNA (siRNA). siAGK, but not control siRNA, markedly reduced AGK mRNA in PC-3 cells, as determined by quantitative real-time PCR, without influencing expression of SphK1 (Fig. 7B Appendix 1). Consistent with its role in synthesis of LPA and PA, the most striking effect of downregulating AGK was reduction of mitochondrial PA and LPA by

approximately 30% (Fig. 7C Appendix 1). siAGK completely blocked stimulation of ERK induced by EGF (Fig. 7D Appendix 1) and also markedly reduced migration towards EGF (Fig. 7E Appendix 1). siAGK not only inhibited basal secretion of IL-8, it also reduced secretion of IL-8 induced by EGF and LPA (Fig. 7F Appendix 1). This was due to specific downregulation of AGK as treatment with control siRNA did not influence IL-8 secretion.

We next examined the role of endogenous AGK in cell growth regulation. Remarkably, treatment with siRNA targeted to AGK markedly decreased DNA synthesis as measured by incorporation of BrdU into nascent DNA (Fig. 8A,B Appendix 1). In contrast, treatment with non-specific siRNA did not alter BrdU incorporation. In agreement, cell cycle analysis revealed that after one day in serum free medium, more than 75% of PC-3 cells transfected with non-specific RNA were in G_o/G_1 phase and only a small fraction were in S and G_2/M phases, which was similar to untreated cells (Fig. 8C Appendix 1). Transfection with siAGK increased the fraction of cells in G_o/G_1 and decreased the proportion in the S phase and, to a lesser extent, in the G_2/M phase. Even in the presence of 10% serum, which markedly increased the proportion of cells in the S phase and G_2/M phase, siAGK, but not control siRNA, further reduced cells in S phase (Fig. 8C Appendix 1).

KEY RESEARCH ACCOMPLISHMENTS

- Cloning an acyglycerol kinase AGK (previous report)
- Establishing the substrates for this kinase and cellular localization (previous report)
- AGK expression enhances cell growth through LPA receptors
- AGK expression enhances EGF-directed motility
- AGK is involved in transactivation of EGFR
- Downregulation of AGK substantiated its physiological function
- Endogenous AGK regulates cell cycle progression

- Endogenous AGK is critical for EGF-induced ERK1/2 activation

REPORTABLE OUTCOMES (not reported previously)

1. Bektas, M., S.G. Payne, H. Liu, S. Milstien, and S. Spiegel. 2004. A novel acylglycerol kinase that produces lysophosphatidic acid modulates crosstalk with EGFR in prostate cancer cells. J. Cell Biol., in revision (Appendix 1)

2. Payne, S.G., S. Milstien, S.E. Barbour, and S. Spiegel. 2004. Modulation of adaptive immune responses by sphingosine-1-phosphate. Semin. Cell Devel. Biol. 15:521-527. (Appendix 2)

Presentations

1. A novel acylglycerol kinase that produces lysophosphatidic acid modulates crosstalk of growth signals in prostate cancer cells. Bektas, M., SMBL, VCU, July, 2004.

CONCLUSIONS

In this work, we have cloned the long searched for monoacylglycerol kinase (MAGK), a novel type of diacylglycerol kinase which phosphorylates monoacylglycerols, to form LPA. LPA has long been implicated as an autocrine and paracrine growth stimulatory factor for prostate cancer cells. The identification of this novel lipid kinase that regulates its production could provide new and useful targets for preventive or therapeutic measures. Expression of AGK in PC-3 prostate cancer cells markedly increased formation and secretion of LPA. This resulted in concomitant transactivation of the EGF receptor and sustained activation of ERK1/2, culminating in enhanced cell proliferation. AGK expression also increased migratory responses and stimulated secretion of IL-8. Conversely, downregulating expression of endogenous AGK inhibited progression through the S phase of the cell cycle and reduced IL-8 secretion. Hence, AGK can amplify EGF signaling pathways and may play an important role in the pathophysiology of prostate cancer. Because of the well-known role of the EGF receptor in androgen-refractory metastatic prostate cancer, the pathophysiological significance of our novel lipid kinase may be to produce LPA, which in turn can stimulate the release of mature EGF, and thus activate the EGF receptor, amplifying mitogenic and survival signals. Therefore, targeting

this kinase that is upstream of the EGF receptor offers additional therapeutic benefits in treatment of androgen-independent prostate cancer.

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A Novel Acylglycerol Kinase that Produces Lysophosphatidic Acid Modulates Crosstalk with EGFR in Prostate Cancer Cells

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Key words: acylglycerol kinase, LPA, PA, phospholipid metabolism, EGF, signaling, prostate cancer

The online version of this article contains supplemental material.

ABSTRACT

The bioactive phospholipids, lysophosphatidic acid (LPA) and phosphatidic acid (PA), regulate pivotal processes related to the pathogenesis of cancer. Here we report the cloning of a long-searched for lipid kinase, designated acylglycerol kinase (AGK), that phosphorylates monoacylglycerol and diacylglycerol to form LPA and PA, respectively. Confocal microscopy and subcellular fractionation suggests that AGK is localized to the mitochondria. Using a matched human tumor/normal tissue expression array, we found that AGK expression was upregulated in prostate cancers compared to normal prostate tissues from the same patient. Expression of AGK in PC-3 prostate cancer cells markedly increased formation and secretion of LPA. This resulted in concomitant transactivation of the EGF receptor and sustained activation of ERK1/2, culminating in enhanced cell proliferation. AGK expression also increased migratory responses and stimulated secretion of IL-8. Conversely, downregulating expression of endogenous AGK inhibited progression through the S phase of the cell cycle and reduced IL-8 secretion. Hence, AGK can amplify EGF signaling pathways and may play an important role in the pathophysiology of prostate cancer.

Introduction

Originally known for its pedestrian role as an intermediate in intracellular lipid metabolism, LPA is now recognized as a potent lipid mediator that evokes growth factor-like responses and regulates an array of cellular processes related to pathogenesis of cancer (Mills and Moolenaar, 2003). These include stimulating proliferation by increasing cell cycle progression, enhancing cell survival, stimulating motility and inducing tumor cell invasion, and regulating neovascularization (Mills and Moolenaar, 2003). Progress in understanding LPA actions has accelerated with the discovery that it is a ligand of several G protein-coupled cell surface receptors (GPCRs). To date, there are three established LPA receptors, LPA_1, LPA_2, and LPA_3 (Anliker and Chun, 2004; Hecht et al., 1996; Mills and Moolenaar, 2003), which are differentially expressed, coupled to a variety of G proteins, and thus regulate diverse cellular responses (Anliker and Chun, 2004; Mills and Moolenaar, 2003). Intriguingly, expression of LPA receptors correlates with more advanced prostate cancer cell lines (Gibbs et al., 2000) and LPA_2 and LPA_3 are aberrantly expressed in ovarian cancer cells (Fang et al., 2002; Goetzl et al., 1999), indicating a potential role in the pathophysiology of cancer. Recently, a fourth LPA receptor was described (LPA_4/GPR23/P2Y9) which is distinct from the other LPA receptors (Noguchi et al., 2003). Moreover, LPA has a novel intracellular function as a high-affinity ligand for peroxisome proliferators-activated receptor-gamma (PPAR-γ), a transcription factor that regulates genes controlling energy metabolism (McIntyre et al., 2003) and can exacerbate mammary gland tumor development (Saez et al., 2004).

In addition to actions through conventional GPCR signaling pathways, LPA receptors can indirectly regulate cell functions by transactivating the EGF tyrosine kinase receptor (Daub et al., 1996; Luttrell et al., 1999; Prenzel et al., 1999). This cross-communication between different

signaling systems is not only important for the growth promoting activity of LPA (Daub et al., 1996; Luttrell et al., 1999), it also may be a clue to its pathophysiological role in prostate cancer (Prenzel et al., 1999), head and neck squamous cell carcinoma (Gschwind et al., 2002), and kidney and bladder cancer (Schafer et al., 2004).

LPA is not only an active component of serum, it also accumulates to high concentrations in malignant effusions (Xiao et al., 2001) and has been proposed to be a marker and mediator of ovarian cancer progression (Fang et al., 2002; Goetzl et al., 1999). Although LPA, formed by acylation of glycerol 3-phosphate, is considered to be a key intermediate in *de novo* glycerolipid synthesis, abundant evidence now indicates that bioactive LPA can also be generated by other pathways. LPA is produced from phosphatidic acid (PA) in activated platelets and ovarian and prostate cancer cells by phospholipase D and subsequent deacylation by phospholipase A_1 or A_2 (Fourcade et al., 1995; Xie et al., 2002). The recent discovery that LPA is generated in the extracellular milieu from lysophosphatidylcholine by the ecto-enzyme autotaxin, known to be involved in tumor invasion, neovascularization and metastasis (Umezu-Goto et al., 2002), further supports the notion that LPA is an important regulator of tumor progression (Koh et al., 2003; Mills and Moolenaar, 2003).

Yet another potential pathway for synthesis of LPA is the phosphorylation of monoacylglycerol by a specific lipid kinase (Pieringer and Hokin, 1962), an enzyme that has remained an enigma for more than 40 years. We have now cloned and characterized a novel acylglycerol kinase (AGK) that phosphorylates both diacylglycerol to produce PA and monoacylglycerol to form LPA, which in turn activates the EGF receptor, amplifying mitogenic and survival signals and regulating EGF-directed motility. Our results suggest that AGK, which is highly expressed in prostate cancers, might be important for the initiation and progression of

prostate cancer, processes in which LPA plays prominent roles (Guo et al., 2000; Kue and Daaka, 2000; Kue et al., 2002; Mills and Moolenaar, 2003; Prenzel et al., 1999; Xie et al., 2002).

RESULTS

Cloning of an acylglycerol kinase

While searching for additional isoforms of sphingosine kinase (SphK), the enzyme that catalyzes the formation of sphingosine-1-phosphate (S1P), another serum-borne lysophospholipid structurally similar to LPA, we cloned a related gene that encodes a 422 amino acid protein (Fig. 1A). The predicted sequence of this human protein and its mouse homologue identified from the mouse data base (CAC06108) are 95% identical and both show sequence similarity to SphKs, especially in the five conserved SphK domains (Liu et al., 2002; Liu et al., 2000). We previously noted that conserved regions 1-3 of SphKs have high sequence homology with the catalytic domain of diacylglycerol kinases (DAGKc) (Liu et al., 2002). This region (amino acid 65 to 191 of the new kinase) contains the GDGXXXEXXXGXXXRX_nK (n = 7, 8) motif, present in the catalytic domain of SphKs (SphKc) (Liu et al., 2002), which is reminiscent but distinct from the sequence GGDGXXG previously suggested to be part of the ATP binding site of DAGKc (Topham and Prescott, 1999). Of note, a lysine residue downstream of the glycine-rich region which is conserved in the ATP binding sites of protein kinases (Hanks et al., 1988) and absent in DAGKc, is also present in SphKc and in this new lipid kinase (Fig. 1A). However, Clustal W alignment revealed that hSphK1 and hSphK2 are more closely related to each other than to this new putative lipid kinase. Pairwise comparisons of the conserved subdomains of SphK1/SphK2, new lipid kinase/SphK1, new lipid kinase/SphK2, new lipid kinase/CERK, and new lipid kinase/DAGK, indicated sequence identities of 53, 29, 23, 26 and

24 percent, 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 7q34, while SphK1 and SphK2 have been localized to chromosomes 17q25.2 and 19q13.2, respectively.

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

Although this new kinase was cloned based on its homology to SphKs, it displayed only barely detectable phosphorylating activity with sphingosine as substrate when compared to cells transfected with SphK1 or SphK2 (Fig. 1B). Moreover, unlike SphKs, neither D-*erythro*-dihydrosphingosine nor phytosphingosine were substrates for this kinase. In addition, there were no detectable changes in the levels of the sphingolipid metabolites, ceramide, sphingosine or S1P, in cells overexpressing this lipid kinase. Furthermore, when AGK transfectants were labeled with [^{3}H]sphingosine, there were no significant increases detected in the formation of [^{3}H]S1P compared to vector transfected cells (data not shown).

We next examined *in vitro* kinase activity with an array of lipid substrates, including different ceramide species and glycerolipids, such as 1,2-dioleoyl-*sn*-glycerol (DAG), glycerol-3-phosphate, anandamide, phosphatidylinositol, phosphatidylglycerol, cardiolipin, and the monoacylglycerol, 1-oleoyl-2-*sn*-glycerol (MOG). Significant phosphorylated products were only detected with monoacylglycerols and diacylglycerols as substrates, but not with any other lipid tested, including ceramide and sphingosine (Fig. 1C) and thus, we have designated this lipid kinase as an acylglycerol kinase (AGK).

Surprisingly, although AGK contains a DAGKc (Fig. 1), it did not significantly phosphorylate diacylglycerol (1,2-dioleoyl-*sn*-glycerol or 1-palmitoyl-2-oleoyl-*sn*-glycerol) when activity was measured in the presence of the detergent octyl-β-glucopopyranoside, as

usually used for DAGK activity measurements (Bunting et al., 1996) (Fig. 1C), suggesting that AGK is distinct from other known DAGKs.

Previously, a partially purified bovine brain monoacylglycerol kinase (MAGK) was reported to prefer substrates containing unsaturated fatty acid esters (Shim et al., 1989; Simpson et al., 1991). Interestingly, AGK has higher activity with substrates containing a C18 fatty acid with one double bond, as monoacylglycerol with an oleoyl (18:1) substitution in the *sn1* position was phosphorylated to a greater extent than 1-palmitoyl-2-*sn*-glycerol (16:0), which was a better substrate than 1-stearoyl-2-*sn*-glycerol (18:0) (Fig. 1C). Moreover, 1-*sn*-2-arachidonoyl-glycerol, an endogenous cannabinoid receptor ligand (Sugiura et al., 2000), was also a reasonably good substrate (Fig. 1C). Like the crude bovine brain MAGK activity (Shim et al., 1989), AGK required magnesium for maximal activity, whereas other divalent cations, including Ca^{2+} and Zn^{2+}, inhibited phosphorylation of 1-oleoyl-2-sn-glycerol. 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. 1C and data not shown).

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 pulldown of AGK was determined. Phosphorylation of MOG was 6-fold greater in immunoprecipitates from V5-AGK transfectants than vector transfectants (Fig. 1D,E) and neither had significant phosphorylating activity with other lipid substrates (data not shown).

Subcellular localization of AGK

SMART search did not reveal any other identifiable motifs in AGK except for the DAGKc. A Kyte-Doolittle hydropathy plot indicated a hydrophobic membrane-spanning domain

at the N-terminus of this new lipid kinase and the program TMpred also predicted one transmembrane region from amino acid 11 to 30. Unexpectedly, confocal immunofluorescence microscopy revealed that AGK was distributed in a punctate, reticular pattern in NIH 3T3 cells (Fig. 2A), reminiscent of a mitochondrial localization. There was no significant colocalization with the ER marker calnexin, as demonstrated by the absence of yellow color in the merged images (Fig. 2A). On the other hand, AGK expression clearly co-localized with mitochondria stained with MitoTracker (Fig. 2A). Similar mitochondrial localization of AGK was also observed in HEK 293 and PC-3 cells, 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.

To further substantiate the localization of AGK, protein expression and enzymatic activity were examined in subcellular fractions prepared by differential centrifugation. V5-Epitope-tagged AGK with the predicted MW of 46.4 kD was highly enriched in the P2 mitochondria fraction that was characterized by the presence of cytochrome c oxidase (Fig. 2B). Much less AGK was present in the P3 fraction containing intracellular membranes of the ER and Golgi, as determined with the ER marker PDI, or in the P4 plasma membrane fraction, characterized by the presence of α_V-integrin (Fig. 2B). In concordance with the protein expression pattern, the highest AGK specific activity was in P2 (Fig. 2C).

AGK regulates LPA and PA *in vivo*

To identify the phosphorylated lipids produced by AGK *in vivo*, vector and AGK PC-3 transfectants were incubated with ^{32}P-labeled orthophosphate and labeled phospholipids in

isolated mitochondria examined (Fig. 2D). Expression of AGK resulted in 80% increase of ^{32}P-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; Vancura and Haldar, 1992) or be rapidly metabolized to PA, changes in total cellular phospholipids were also examined (Fig. 3A-C). There were no obvious differences in labeling of the major known cellular phospholipids in AGK expressing cells compared to the vector cells. However, two-dimensional HPTLC analysis revealed that a labeled phospholipid that co-migrated with authentic LPA (Fig. 3A,B), while barely detectable in vector cells, was increased three-fold 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 phosphatidic acid was also increased in these transfectants (Fig. 3C), albeit much less than LPA. Of note, in these cells, AGK mRNA levels relative to 18S RNA were increased by almost 2-fold 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 ^{32}P-labeled LPA was significantly increased three-fold by overexpression of AGK (Fig. 3F), 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 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 and its expression had no discernable effects on ^{32}P-labeled LPA, PA, or other phospholipids (data not shown).

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 upregulated in prostate cancers compared to 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 to the normal tissues, while 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. 4C-E)

and NIH 3T3 fibroblasts (Fig. S1A). The growth promoting effect of AGK was observed even in the presence of sub-optimal serum concentrations (Fig. 4C and Fig. S1A). Although AGK stimulates growth, it had no cytoprotective effects on apoptosis induced by serum deprivation or the anti-cancer drug doxorubicin (Fig. S1D). As expected, exogenous LPA increased proliferation of both AGK and vector transfectants to the same extent (Fig. 4D). 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. 4E). This is probably due to rapid metabolism and degradation of MOG and thus, only in AGK expressing cells is sufficient LPA produced and secreted (Fig. 3F) to stimulate proliferation.

The growth promoting effects of AGK were also examined by DNA flow cytometry. FACS analysis revealed that 83 ± 0.4 % of the vector transfectants were in Go/G1 phase and 7 ± 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 ± 0.1% and increased the proportion in the S phase by more than 2-fold (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 (Hooks et al., 2001), possibly as an agonist of the nuclear transcription factor PPARγ (Zhang et al., 2004). To examine the potential involvement of intracellular actions of LPA generated by expression of AGK, its mitogenic effects were determined in rat hepatoma RH7777 cells that do not express LPA_{1-4} and do not respond to LPA (Fukushima et al., 1998). However, in contrast to PC-3 (Fig. 4C-E) and NIH 3T3 (Fig. S1A) 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. 4F). Moreover, although GW9662, a selective antagonist of PPARγ, inhibited proliferation of vector transfected PC-3 cells, it did not abrogate the mitogenic effect of AGK (Fig. 5A). Consistent with previous results (Kue and Daaka, 2000; Kue et al., 2002), we found that LPA_1, LPA_2, and LPA_3 are expressed in PC-3 cells (data not shown). It is known that LPA_1 couples to pertussis toxin (PTX)-sensitive Gi, whereas LPA_2 and LPA_3 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; Kue and Daaka, 2000). In agreement, PTX pretreatment not only inhibited growth of vector transfectants, it also markedly decreased the growth promoting effects of AGK (Fig. 5A).

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 (Daub et al., 1996; Mills and Moolenaar, 2003; Prenzel et al., 1999). 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. 5B). 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. S2). Because LPA stimulates tyrosine phosphorylation of the EGFR in PC-3 cells (Kue et al., 2002), it was important to substantiate that the enhanced tyrosine phosphorylation of the 170 kD protein represented activation of the EGFR. Indeed, anti-

phosphotyrosine immunoblotting of anti-EGFR immunoprecipitates revealed enhanced EGFR tyrosine phosphorylation in cells overexpressing AGK, even in the absence of serum (Fig. 5C).

AGK-induced ERK1/2 activation requires EGFR

Previously, it has been suggested that EGFR activation is required for signal relay from LPA receptors to ERK1/2 activaton in prostate cancer cells (Guo et al., 2000; Kue et al., 2002; Prenzel et al., 1999; Raj et al., 2002). AGK expression markedly increased activation of ERK1/2, as determined with a phospho-specific antibody, which was further enhanced by serum (Fig. 5B) and EGF (Fig. 5D and Fig. S1C). To further confirm that activation of the EGFR was necessary for AGK-stimulated ERK activation, we utilized the specific EGFR tyrosine kinase inhibitor, tyrphostin AG1478. As expected, AG1478 abolished EGFR-induced tyrosine phosphorylation (Fig. S2B). AG1478 blocked AGK-mediated ERK1/2 phosphorylation (Fig. 5D) and decreased its mitogenic effect (Fig. 5E) and also inhibited MOG-stimulated proliferation by 35 ± 4%. Nonetheless, prolonged treatment with AG1478 did not affect AGK protein levels (Fig. 5E, insert).

Taken together, these results suggest that the tyrosine kinase activity of EGFR contributes to AGK-induced activation of the ERK cascade and that stimulation of DNA synthesis is mediated not only by transactivation of EGFR, but also by direct activation of Gi-coupled LPA receptors.

Involvement of AGK in motility

Transactivation of the EGFR has also been implicated in motility of cancer cells (Gschwind et al., 2002). In agreement, AGK overexpression enhanced migration of PC-3 cells towards EGF, which was blocked by the EGFR inhibitor AG1478 (Fig. 6A). AGK also enhanced migration of NIH 3T3 fibroblasts towards serum (Fig. S1B).

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. 6B,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 upregulates 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 significantly further increased by addition of MOG, the precursor of LPA (Fig. 6D). Although EGF alone can stimulate IL-8 release, albeit to a lesser extent than LPA, 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 cell cycle progression

Surprisingly, we noticed that serum and EGF induced significant increases in AGK expression as determined by quantitative real-time PCR (Fig. 7A). It has previously been shown that stimulated PC-3 cells produce high levels of LPA and also respond to LPA (Xie et al., 2002). Moreover, in some cancer cells, LPA itself is sufficient to increase its own production, indicating the presence of an autocrine network (Qi et al., 1998). Consistent with an autocrine function for LPA, we found that LPA also increased expression of AGK by 3 fold in naïve PC-3 cells (Fig. 7A). Since AGK expression was stimulated by these potent growth factors, it was of interest to examine the physiological function of AGK by knocking down its expression with

small interfering RNA (siRNA). siAGK, but not control siRNA, markedly reduced AGK mRNA in PC-3 cells, as determined by quantitative real-time PCR, without influencing expression of SphK1 (Fig. 7B). Consistent with its role in synthesis of LPA and PA, the most striking effect of downregulating AGK was reduction of mitochondrial PA and LPA by approximately 30% (Fig. 7C). siAGK completely blocked stimulation of ERK induced by EGF (Fig. 7D) and also markedly reduced migration towards EGF (Fig. 7E). siAGK not only inhibited basal secretion of IL-8, it also reduced secretion of IL-8 induced by EGF and LPA (Fig. 7F). This was due to specific downregulation of AGK as treatment with control siRNA did not influence IL-8 secretion.

We next examined the role of endogenous AGK in cell growth regulation. Remarkably, treatment with siRNA targeted to AGK markedly decreased DNA synthesis as measured by incorporation of BrdU into nascent DNA (Fig. 8A,B). In contrast, treatment with non-specific siRNA did not alter BrdU incorporation. In agreement, cell cycle analysis revealed that after one day in serum free medium, more than 75% of PC-3 cells transfected with non-specific RNA were in G_o/G_1 phase and only a small fraction were in S and G_2/M phases, which was similar to untreated cells (Fig. 8C and data not shown). Transfection with siAGK increased the fraction of cells in G_o/G_1 and decreased the proportion in the S phase and, to a lesser extent, in the G_2/M phase. Even in the presence of 10% serum, which markedly increased the proportion of cells in the S phase and G_2/M phase, siAGK, but not control siRNA, further reduced cells in S phase (Fig. 8C).

DISCUSSION

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 downregulated 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 counter-regulatory lipids that have been shown to play opposing roles in growth and survival of prostate cancers. MOG, the best substrate for AGK, is phosphorylated to form 18:1 LPA, and LPA species with unsaturated fatty acids, in particular, 18:1 and 18:2 LPAs, are much more potent than their saturated counterparts in stimulating growth of ovarian (Xu et al., 1995) and prostate cancer cells (Xie et al., 2002). In this regard, it has been suggested that increased LPA species with unsaturated fatty acid chains may be associated with late-stage or recurrent ovarian cancer (Shen et al., 2001). LPA with unsaturated fatty acids preferentially stimulates LPA_3, whereas LPA_1 and LPA_2 show broader ligand specificities (Bandoh et al., 2000). Of note, LPA_3 was originally cloned from prostate cancer cells (Im et al., 2000), concordant with the ability of LPA to induce autocrine proliferation of these cells (Xie et al., 2002). Moreover, PC-3 cells

express LPA_{1-3} 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 LPA_{1-4} independent (Hooks et al., 2001). However, in prostate cancer cells, LPA transduces mitogenic signals via activation of $G_{\alpha i}$ and $G_{\beta\gamma}$ subunits (Bookout et al., 2003; Kue and Daaka, 2000). PTX inhibited serum and LPA-mediated growth of PC-3 cells as well as activation of ERK1/2 (Kue and Daaka, 2000) and reduced local progression and metastasis after orthotopic implantation in nude mice (Bex et al., 1999). In addition, expression of a $G_{\beta\gamma}$-sequestering peptide, GRK2ct, abrogated the LPA-mediated activation of ERK1/2 (Kue and Daaka, 2000). 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. First, In RH7777 cells, which are devoid of LPA_{1-4}, expression of AGK had no effect on DNA synthesis. Second, PTX pretreatment decreased the growth promoting effects of AGK. Third, although GW9662, a selective antagonist of PPARγ, inhibited proliferation of vector transfected PC-3 cells, it did not abrogate the mitogenic effect of AGK. Thus, our results are consistent with the notion that production and secretion of LPA by AGK induces Gi-dependent proliferation, likely through LPA receptors.

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, be secreted and/or converted to PA (Chakraborty et al., 1999; Haldar and Lipfert, 1990; Hiroyama and Takenawa, 1999; Vancura and Haldar, 1992). In addition, LPA generated by prostate cancer cells in response to mitogenic stimuli can be secreted (Gibbs et al., 2000; Guo et al., 2000; Kue and Daaka, 2000; Kue et al., 2002; Qi et al., 1998; 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 LPA_{1-3}, it was suggested that 18:1 LPA can act as an autocrine mediator (Kue et al., 2002; Prenzel et al., 1999; 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 AGK

AGK was cloned and the catalytically inactive mutant was prepared as described in the online supplemental materials.

Cell culture and transfection

Human PC-3 prostate cancer cells, NIH 3T3 fibroblasts, HEK 293, and rat hepatoma RH7777 cells were cultured and transfected as described in the online supplemental materials.

Lipid kinase activity

Lipids (100 nmol) were dried under N_2 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). After brief sonication, 10 µl lysates (10 µg) and 10 µl [γ-^{32}P]ATP (10 µCi, 1 mM) containing $MgCl_2$ (10 mM) were added and reactions were carried out for 30 min at 37 °C. [^{32}P]-Labeled lipids produced were extracted into 0.8 ml $CHCl_3$/MeOH/conc. HCl (100:200:1, v/v), and phase separation effected by adding 0.25 ml 2 M KCl and 0.25 ml $CHCl_3$. Aliquots of the organic phases were analyzed by TLC on silica gel G60 with $CHCl_3$/acetone/methanol/acetic acid/water (10:4:3:2:1, v/v) as solvent and the radioactive spots corresponding to migration of standards were quantified with a FX Molecular Imager (Bio-Rad, Hercules, CA). In some experiments, SphK1 (Liu et al., 2000), SphK2 (Liu et al., 2000), ceramide kinase (Sugiura et al., 2002), and diacylglycerol kinase (Bajjalieh et al., 1989) activities were measured exactly as described. AGK activity in immunoprecipitates was also determined as described in the online supplemental materials.

^{32}P 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 ^{32}Pi in phosphate-free DMEM at 37 °C, washed and incubated for a further 2 h in 4 ml phosphate-free DMEM. Medium was removed and after brief centrifugation, lipids were extracted from a 3 ml aliquot of the medium by addition of 10.8 ml chloroform/methanol/conc. HCl (100:200:1, v/v), followed by 3.6 ml each of chloroform and 2 M KCl. Lipids were also extracted from the cells after washing and scraping into 1.2 ml cold methanol/conc. HCl (100:1), followed by addition of 0.6 ml chloroform. After vigorous vortexing, 0.6 ml $CHCl_3$ and 0.5 ml H_2O was added. Phases were separated by addition of 0.6 ml 2 M KCl. The organic phases were transferred to siliconized glass tubes and the aqueous phases re-extracted with 0.6 ml $CHCl_3$. Aliquots containing 50,000 cpm were separated by two-dimensional TLC (Yokoyama et al., 2000). Radioactive spots were identified by comparison to standard phospholipids and quantified with a phosphoimager. In some experiments, phospholipids were separated by one-dimensional TLC using $CHCl_3$/methanol/water/ ammonium hydroxide (120:75:6:2, v/v) (Liu et al., 2003).

Northern analysis and matched tumor/normal expression array

Poly(A)$^+$ RNA blot of multiple adult human tissues (Clontech, Palo Alto, CA) was used for Northern blotting analysis of AGK expression. The blot was hybridized with a probe prepared by labeling the PCR product with [γ-^{32}P]dCTP in ExpressHyb buffer (Clontech) at 65 °C overnight. A matched tumor/normal expression array (Clontech) was similarly probed with radiolabeled full length AGK.

IL-8 secretion

PC-3 cells were serum-starved overnight and after stimulation in serum-free DMEM for

16 h, media was collected and briefly centrifuged to remove cells. Secreted IL-8 was determined with the Quantikine IL-8 ELISA kit (R&D Systems, Minneapolis, MN).

Chemotactic motility

Chemotaxis was measured in a modified Boyden chamber using collagen coated polycarbonate filters (25 x 80mm, 8 μM pore size) as previously described (Wang et al., 1999).

***In vitro* wound closure assay**

Confluent monolayers of PC-3 cells were serum-starved for 24 h, wounded by making a uniform scratch with a pipet tip, and washed to remove detached cells. Wound closure was monitored after 24 h in serum-free medium by determining the number of cells migrating into the wound using Image-Pro Plus software to analyze digital images from an inverted phase microscope.

Cell proliferation assays

PC-3 cell proliferation was determined with crystal violet (Olivera et al., 1999). In some experiments, cell growth was measured by adding WST-1 reagent (Roche, Rockford Ill) and incubating at 37 °C for 3 h. Absorbance was measured at 450 nm with background subtraction at 650 nm. BrdU incorporation, and analysis of cell cycle profile by flow cytometry were carried out exactly as described (Olivera et al., 1999).

Online supplemental material

Details about cell culture and transfection, siRNA transfection, real-time PCR, subcellular fractionation, immunoprecipitation, immunofluorescence and confocal microscopy can be found as supplemental material. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.xxxxxxxx/DC1.

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Abbreviations used in this paper: AGK, acylglycerol kinase; DAGK, diacylglycerol kinase; ERK, extracellular signal related kinase; GPRC, G protein-coupled receptor; LPA, lysophosphatidic acid; MAGK, monoacylglycerol kinase; MOG, 1-oleoyl-2-sn-glycerol; PA, phosphatidic acid; PPAR-γ, peroxisome proliferators-activated receptor-gamma; siRNA, small interfering RNA; SphK, sphingosine kinase.

Figure legends

Figure 1. Predicted amino acid sequence of human AGK. (**A**) The amino acid sequence of human AGK is shown with a ClustalW alignment of the catalytic domains of other human lipid kinases, including type 1 and type 2 sphingosine kinases (SphK1, SphK2), ceramide kinase (CerK), and diacylglycerol kinase alpha (DAGKα). Putative ATP binding sites are indicated by the solid lines. Identical residues are shaded in dark gray and highly conserved residues are shaded in light gray. The dashes represent gaps in sequences and numbers on the right refer to the amino acid sequence of hAGK. **(B) AGK is not a sphingosine kinase.** 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 (Liu et al., 2000). **(C) Lipid kinase activity of recombinant AGK.** Lipid phosphorylating activity was determined in cell lysates from NIH 3T3 cells transiently transfected with vector (open bars) or hAGK (filled bars). The following lipids were tested: Sph, C_6-cer; 1-oleoyl-2-*sn*-glycerol (18:1), 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-β-glucopyranoside detergent was added to a final concentration of 1.5 %. The data are expressed as pmol phosphorylated product formed/min/mg ± S.D. Similar results were obtained in four additional experiments. *$P < 0.05$ by *t*-test. (**D**) shows TLC separation of products formed with MOG as substrate visualized with a phosphoimager. (E) AGK activity was measured with MOG as substrate after immunoprecipitation with anti-V5 antibody from HEK 293 cells transiently transfected with vector (open bars) or V5-hAGK (filled bars). Data are expressed as pmol LPA formed in 30 min.

Figure 2. Subcellular localization of AGK. (**A**) NIH 3T3 fibroblasts were transiently transfected with V5-tagged hAGK and mitochondria stained with MitoTracker Red. The endoplasmic reticulum was visualized with anti-calnexin antibody followed by FITC-conjugated anti-rabbit as the secondary antibody. AGK was stained with monoclonal anti-V5 antibody followed by secondary FITC-conjugated or Texas Red-condugated anti-mouse antibody. Cells were visualized by dual wavelength confocal microscopy. Superimposed merged pictures are shown in the lower panels, yellow color indicates colocalization. (**B,C**) **Activity and expression of AGK in subcellular fractions.** Lysates from HEK 293 cells transfected with vector or V5-AGK were fractionated into P1 (nuclei and unbroken cells), P2 (mitochondria), P3 (ER, and Golgi), P4 (plasma membrane) and cytosol. Proteins (25 μg) were resolved by SDS-PAGE and immunoblotted with anti-V5 antibody or with antibodies to the specific organelle markers anti-cytochrome c oxidase, anti-phosphodisulfide isomerase (PDI), and anti-α_v-integrin. AGK activity was also determined in each subcellular fraction with MOG as substrate. Results are means ± S.D. of triplicate determinations. Similar results were obtained in two additional experiments. (**D**) PC-3 cells stably transfected with vector or AGK were labeled with ^{32}P-orthophosphate for 2 h. Phospholipids were then extracted from mitochondria isolated by differential centrifugation. After separation of equal amounts of ^{32}P-labeled phospholipids by one-dimensional TLC, radioactive spots were visualized with a phosphoimager and the indicated lipids identified based on co-migration with authentic standards. The ratio of ^{32}P-PA to ^{32}P-PC in vector and AGK transfectants was 0.38 ± 0.02 and 0.68 ± 0.03, respectively. * $P < 0.05$ by Student *t*-test.

Figure 3. LPA production and secretion induced by expression of AGK. PC-3 cells stably transfected with vector or AGK were prelabeled with ^{32}P-orthophosphate for 2 h, washed, and

incubated for 2 h in chemically defined medium. Lipids were then extracted from cells (**A-C**) and media (**D-F**). Equal amounts of ^{32}P-phospholipids were separated by 2-dimensional HPTLC, first in chloroform-methanol-formic acid-water (60:30:7:3, v/v), followed by chloroform-methanol-ammonium hydroxide-water (50:40:8:2, v/v). Radioactive spots were visualized with a phosphoimager and the indicated lipids identified based on co-migration with authentic standards. (**C,F**) ^{32}P incorporation into the indicated phospholipids (LPA, PA, PC, and unidentified phospholipid X) was quantified by phosphoimager. Similiar results were obtained in two additional experiments.

Figure 4. Expression of hAGK. (**A**) Northern blot analysis of hAGK expression in human tissues. Random labeled probe was hybridized to poly(A)$^+$ RNA blots from the indicated human tissues. β-Actin expression was used to confirm equal loading. (**B**) **Matched tumor/normal array analysis of hAGK expression.** An array containing cDNA samples from multiple tissues and tumor types as well as nine cancer cell lines was probed with ^{32}P-labeled AGK probe. Each pair of tumor and normal samples came from the same patient. Human cancer cell lines: 1. HeLa; 2. Burkitt's lymphoma, Daudi; 3. chronic myelogenous leukemia; 4. promyelocytic leukemia HL-60; 5. melanoma; 6. lung carcinoma; 7. lymphoblastic leukemia, MOLT-4; 8. colorectal adenocarcinoma, SW480; 9. Burkitt's lymphoma, Raji. There was no specific hybridization to the control nucleic acids, which included ubiquitin cDNA, yeast total RNA, yeast tRNA, *E. coli* DNA, poly(A), human C_0t-1 DNA, and human genomic DNA. **(C-E) AGK stimulates proliferation.** PC-3 cells stably transfected with vector (open symbols) or AGK (filled symbols) were cultured in serum-free medium supplemented with 0.5% serum (C), 10 μM LPA (D), or 10 μM MOG (E), and cell numbers determined at the indicated days. Data are expressed as fold increase relative to day 0 and are means ± S.D. Similar results were obtained in two additional

experiments. Asterisks denote significant differences ($P < 0.05$, Student *t*-test). **(F) AGK does not enhance proliferation of RH7777 cells.** RH7777 cells were co-transfected with vector (open bars) or AGK (filled bars) together with GFP at a ratio of 1:4. After 24 h, cells were cultured in serum-free medium or in the presence of 0.5% serum. BrdU was then added 16 h later for an additional 3 h. Double immunofluorescence was used to visualize transfected cells and BrdU incorporation into nascent DNA. The proportion of cells incorporating BrdU among total GFP transfected cells was determined. Data are means ± S.D. of triplicate cultures from a representative experiment. At least three different fields with a minimum of 100 cells/field were scored.

Figure 5. (A) Effect of pertussis toxin and PPAR-γ antagonist GW9662 on AGK-induced proliferation. PC-3 cells stably transfected with vector (open bars) or AGK (filled bars) 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 determined after 6 days with WST-1. Asterisks denote significant differences compared to untreated controls ($P < 0.05$, Student *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-phospho-tyrosine, anti-V5 antibody, or phospho-specific anti-ERK1/2 antibodies. Blots were then stripped and re-probed with ERK 2 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 the immunoprecipitates analyzed by western blotting using anti-phosphotyrosine or anti-EGFR antibody. **(D,E) Blockage 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 AG1478 (200 nM), then treated with EGF for 10 min. Cell lysate proteins were analyzed by immunoblotting with phospho-specific ERK1/2 antibody. Blots were stripped and re-probed with ERK2 antibody to demonstrate equal loading. (**E**) PC-3 cells stably transfected with vector or AGK were cultured in medium supplemented with 1% or 10% serum with or without AG1478 (200 nM) and cell proliferation determined after 6 days with crystal violet. Similar results were obtained in two additional experiments. Asterisks denote significant differences ($P < 0.05$, Student *t*-test). Insert: PC-3 cells stably transfected V5-AGK were incubated for 6 days without (None) or with AG1478 and AGK expression determined by immunoblotting with anti-V5 antibody. The blot was stripped and re-probed with anti-tubulin as a loading control.

Figure 6. EGFR is required for AGK-stimulated cell migration towards EGF and wound closure. (**A**) PC-3 cells transfected with vector (open bars) or AGK (filled bars) were pretreated without or with AG1478 (200 nM) for 20 min and then allowed to migrate for 3 h towards EGF (10 ng/ml). The data are means ± S.D. of two determinations. Similar results were obtained in two independent experiments. (**B,C**) Monolayers of vector (open bars) or AGK (filled bars) PC-3 transfectants were wounded and treated with vehicle, MOG (10 μM), LPA (10 μM), or EGF (10 ng/ml). Migration of cells into the wound was determined after 24 h by processing digital photographs with Image Pro Plus. Where indicated, cells were also treated with AG1478 (200 nM). (**B**) Representative images of a wound healing assay with vector and AGK transfected PC-3 cells before and 24 h after treatment with MOG. **(D) AGK induces IL-8 secretion.** PC-3 cells transfected with vector (open bars) or AGK (filled bars) were serum-starved for 24 h and treated in serum-free DMEM with or without MOG (10 μM) or LPA (1 μM) for 16 h and IL-8 secretion

was measured by ELISA. Where indicated, cells were also treated with AG1478 (200 nM). * $P < 0.05$ by Student *t*-test.

Figure 7. Effectiveness and specificity of siAGK. (A) Expression of endogenous AGK. Naïve PC-3 cells were serum-starved for 24 h and treated in DMEM with or without 10% serum, LPA (10 μM), or EGF (100 ng/ml) for 16 h and AGK mRNA determined by quantitative real time PCR. Data were normalized to expression of 18S RNA and are means ± S.D. of triplicate determinations. * $P < 0.05$ by Student *t*-test. **(B)** PC-3 cells were transfected with control siRNA (open bars) or siRNA specific for AGK (gray bars) and mRNA levels of AGK and SphK1, and 18S RNA determined by quantitative real-time PCR. **(C)** Duplicate cultures were labeled with ^{32}Pi for 12 h. Phospholipids were then extracted from mitochondria isolated by differential centrifugation. Equal amounts of ^{32}P-phospholipids were analyzed by TLC and quantified with a phosphoimager. The data are expressed as percent of total ^{32}P-labeled phospholipids and are means ± S.D. of duplicate determinations. * $P < 0.05$ by Student *t*-test. **(D) Downregulation of AGK with siRNA blocks EGF-induced ERK1/2.** PC-3 cells were transfected with control siRNA or siRNA specific for AGK and treated with EGF (10 ng/ml) 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-ERK2 as a loading control. **(E) Downregulation of AGK decreases migration towards EGF.** PC-3 cells were transfected with control siRNA (open bars) or siRNA specific for AGK (hatched bars) and then allowed to migrate for 3 h towards EGF (10 ng/ml). The data are means ± S.D. of two determinations. * $P < 0.05$ by Student *t*-test. **(F) Downregulation of AGK with siRNA decreases IL-8 secretion.** PC-3 cells were transfected with control siRNA (open bars) or siRNA specific for AGK (hatched bars), treated in serum-free DMEM without 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 Student *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 medium supplemented with 10% serum, BrdU was added for 3 h and the fraction of cells incorporating BrdU was determined. Data are means ± S.D. 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 siControl and siAGK transfected cells. **(C) Cell cycle analysis.** PC-3 cells transfected with control siRNA or siAGK were cultured in serum-free medium or medium supplemented with 10 % serum. After 24 h, cellular DNA was stained with propidium idodide and cell cycle analysis was performed with an Epics XL-MCL flow cytofluorometer (Coulter, Hialeah, FL). Asterisks indicate significant differences from vector-transfected values as determined by Student's *t*-test ($P \leq 0.05$).

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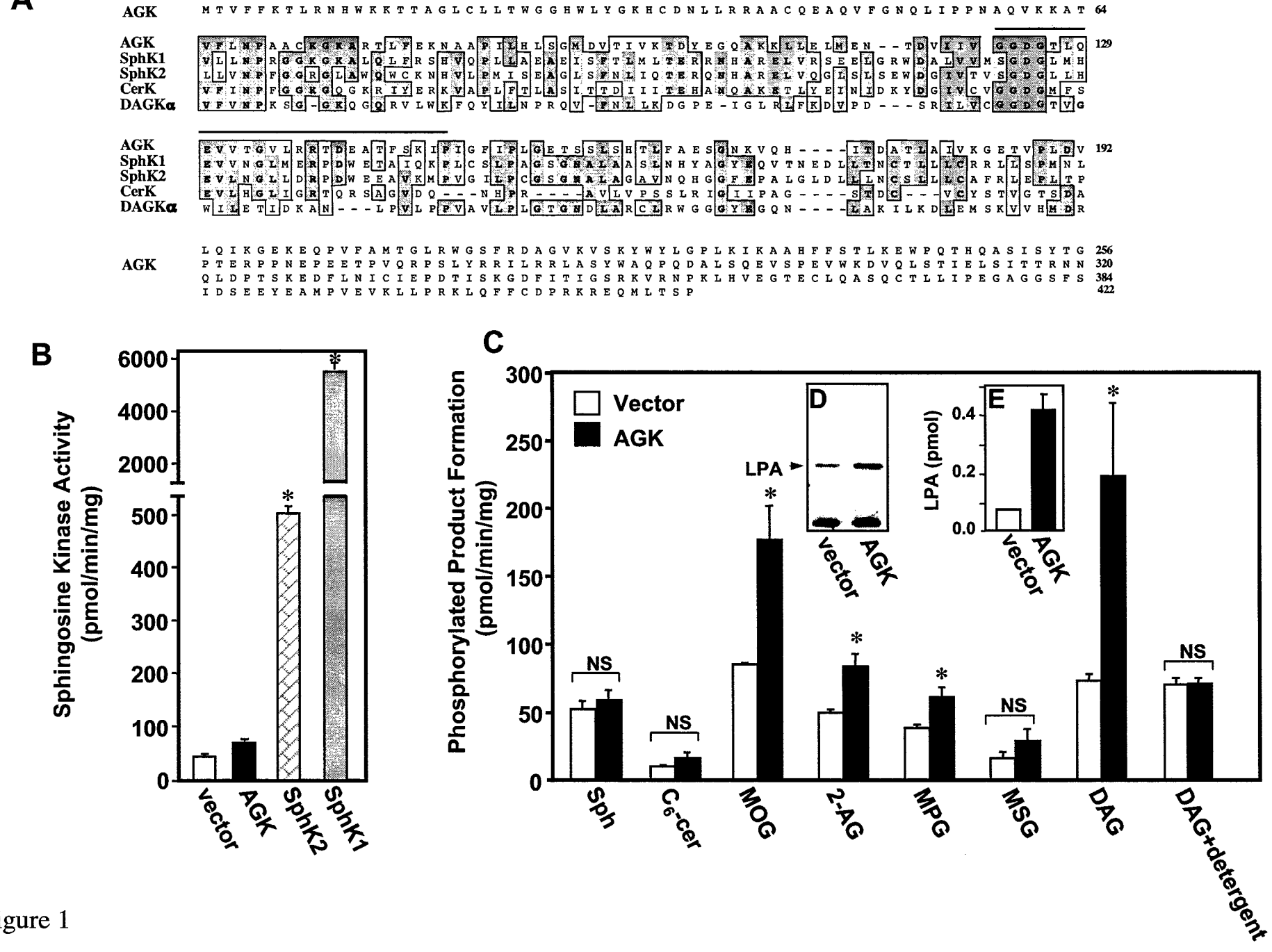
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vector
AGK
NS
Sph
C6-cer
MOG
2-AG
MPG
MSG
DAG
DAG+detergent

Figure 1

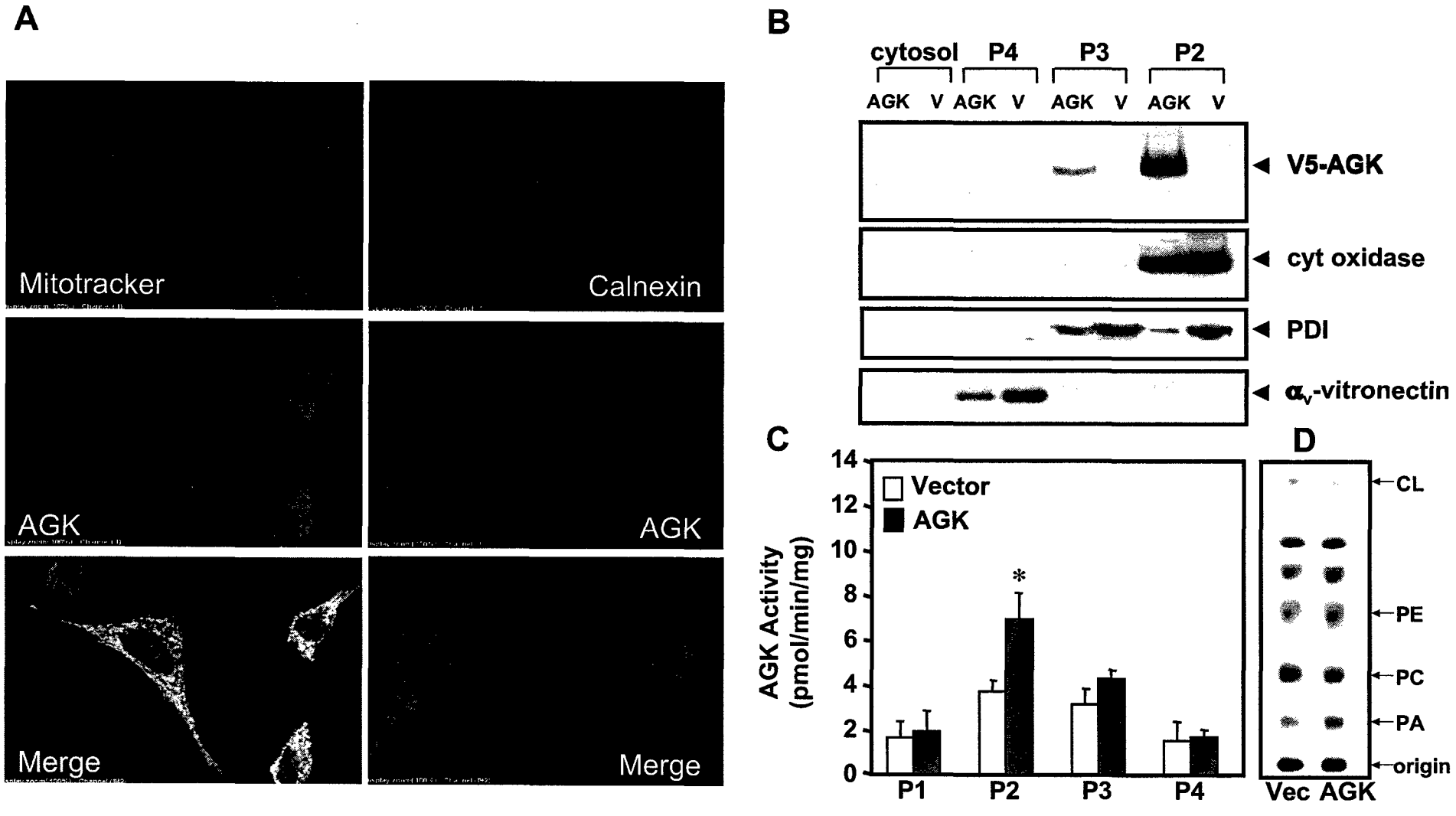
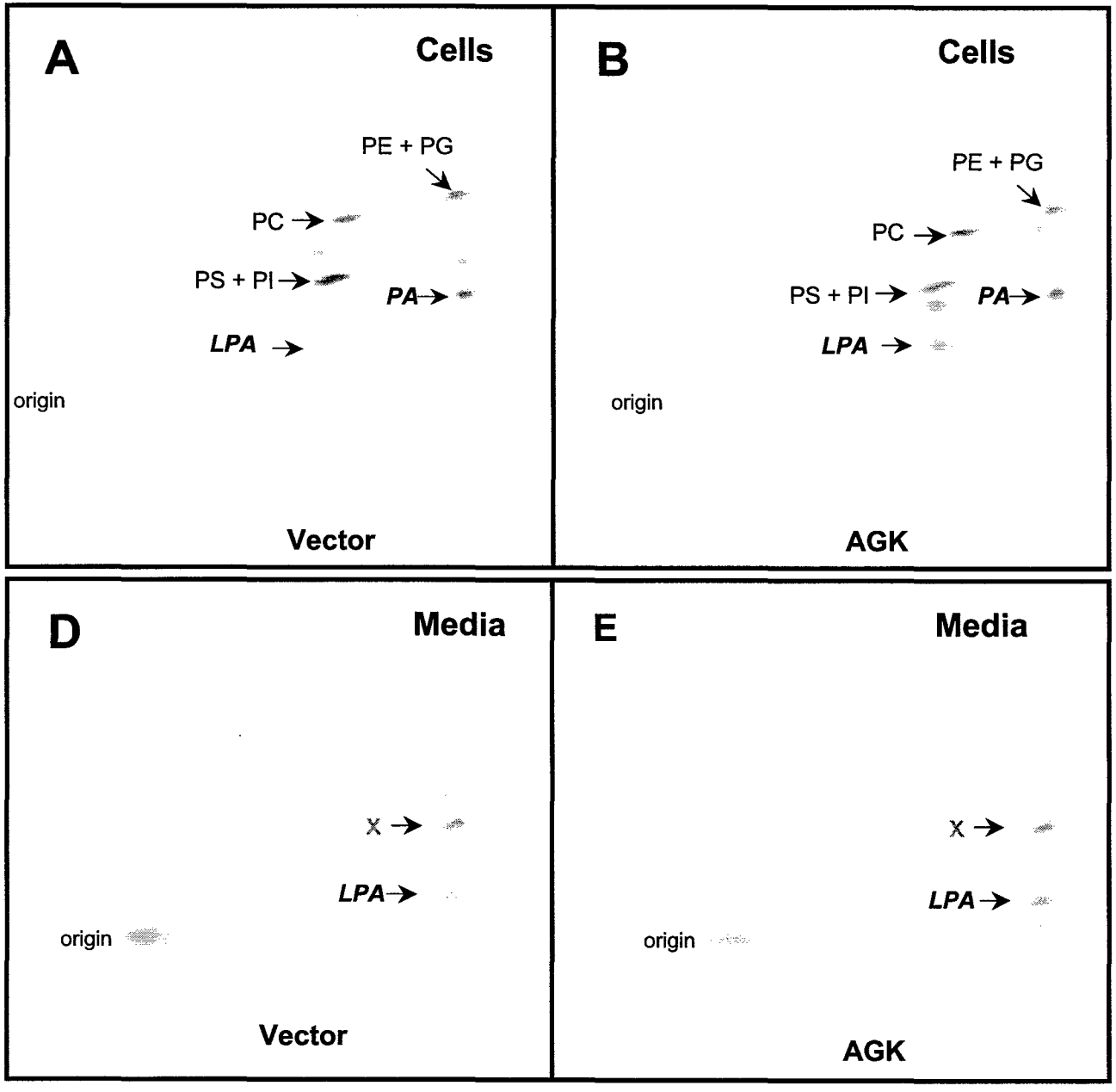
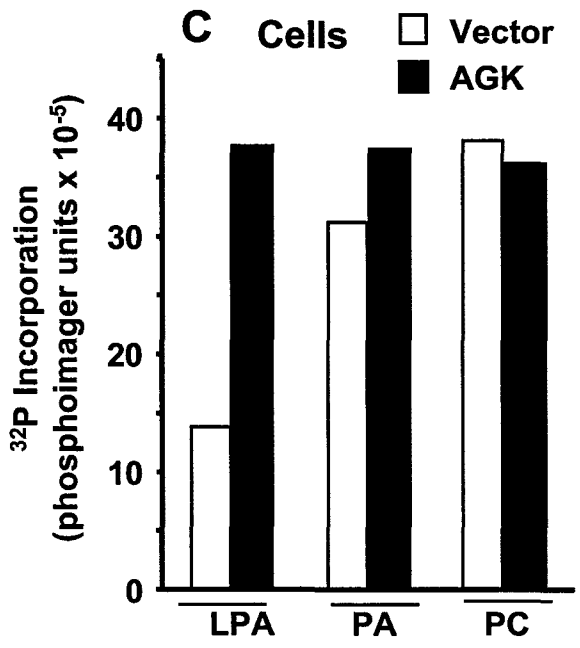

A
Mitotracker
Calnexin
AGK
AGK
Merge
Merge
B
cytosol
P4
P3
P2
AGK V AGK V AGK V AGK V
V5-AGK
cyt oxidase
PDI
αv-vitronectin
C
AGK Activity (pmol/min/mg)
Vector
AGK
*
P1
P2
P3
P4
D
CL
PE
PC
PA
origin
Vec AGK

Figure 2


A
Cells
PE + PG
PC
PS + PI
PA
LPA
origin
Vector
B
Cells
PE + PG
PC
PS + PI
PA
LPA
origin
AGK
D
Media
X
LPA
origin
Vector
E
Media
X
LPA
origin
AGK


C
Cells
Vector
AGK
32P Incorporation (phosphoimager units x 10-5)
0
10
20
30
40
LPA
PA
PC

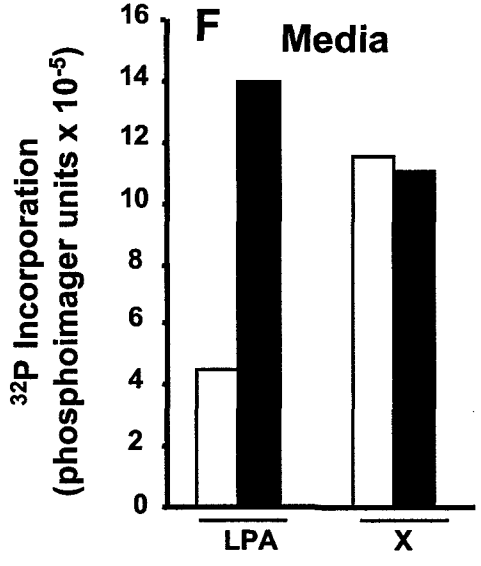

F
Media
32P Incorporation (phosphoimager units x 10-5)
0
2
4
6
8
10
12
14
16
LPA
X

Figure 3

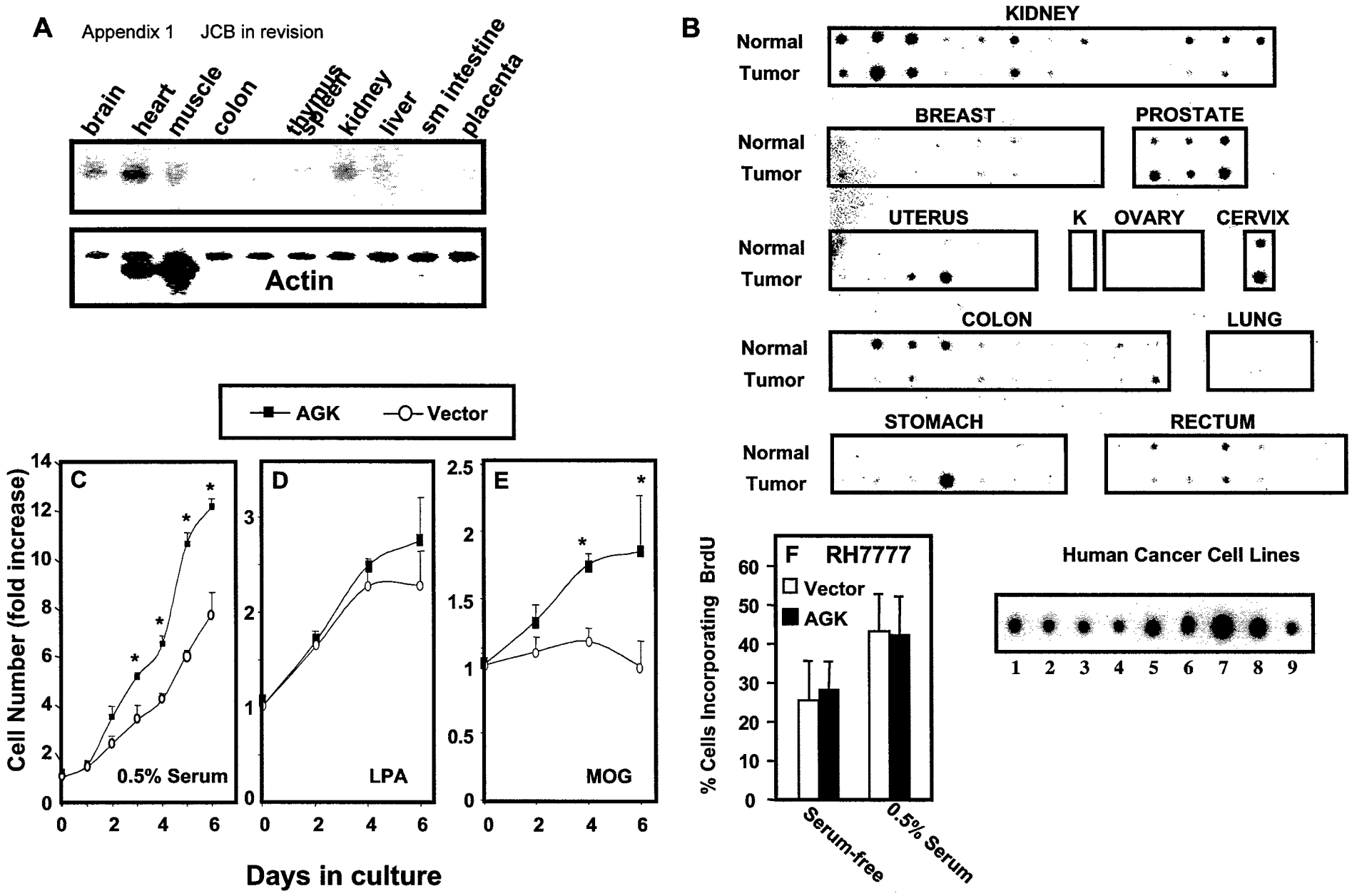

A
Appendix 1 JCB in revision
brain
heart
muscle
colon
thymus
spleen
kidney
liver
sm intestine
placenta
Actin
B
KIDNEY
Normal
Tumor
BREAST
PROSTATE
UTERUS
K
OVARY
CERVIX
COLON
LUNG
STOMACH
RECTUM
AGK
Vector
C
D
E
Cell Number (fold increase)
0.5% Serum
LPA
MOG
Days in culture
F RH7777
Vector
AGK
% Cells Incorporating BrdU
Serum-free
0.5% Serum
Human Cancer Cell Lines
1 2 3 4 5 6 7 8 9

Figure 4

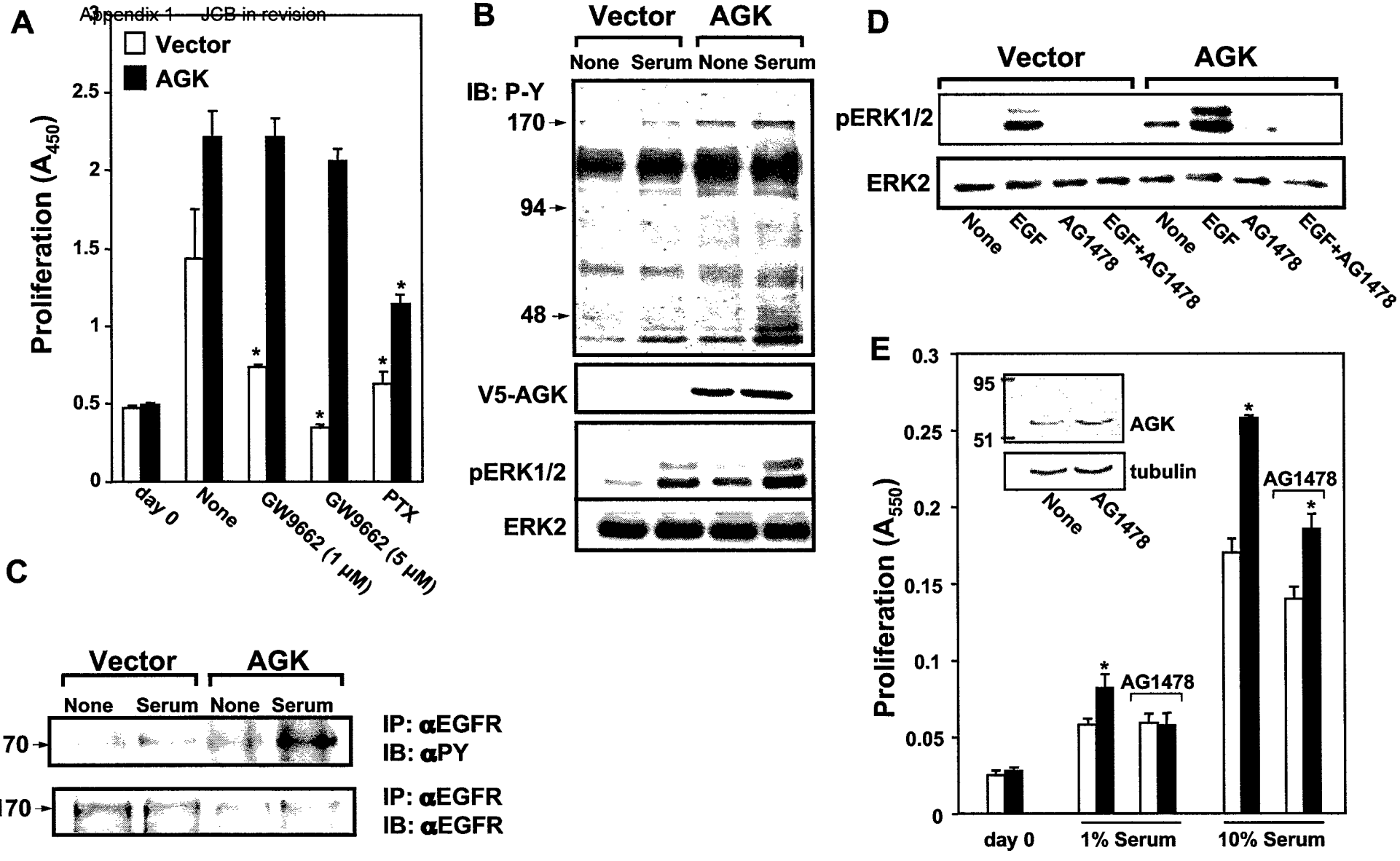
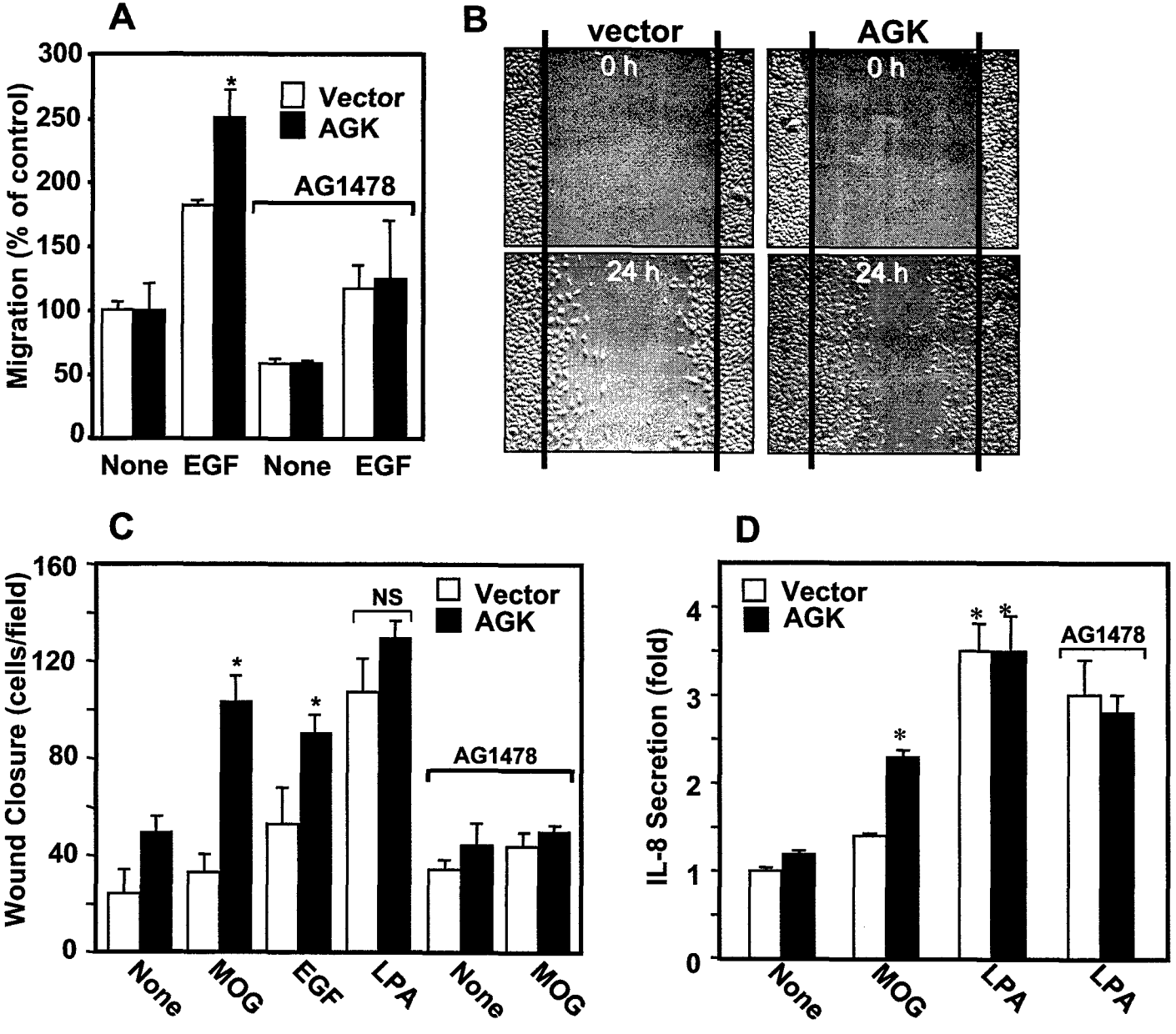
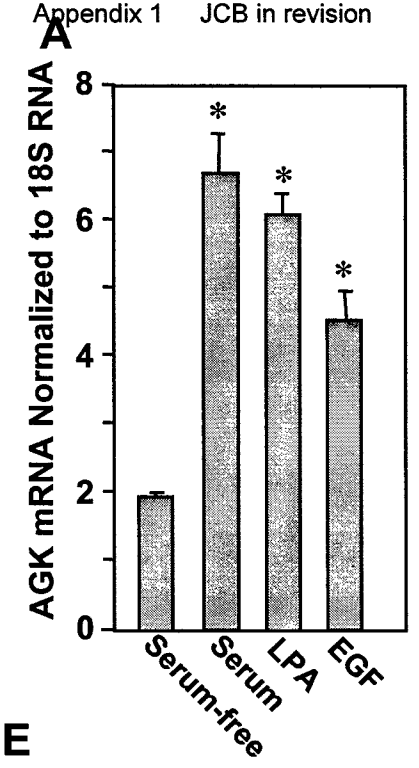
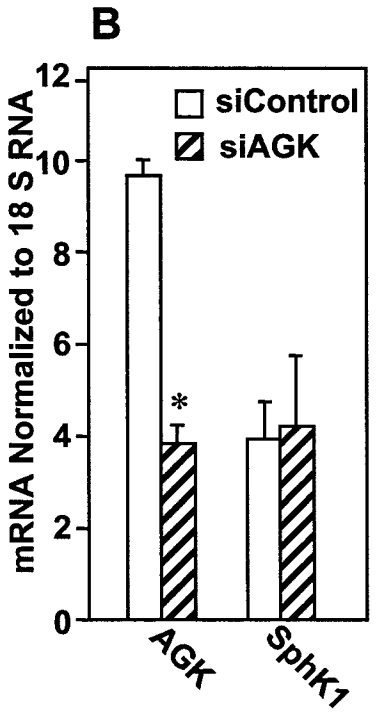

A
Appendix 1 JCB in revision
Vector
AGK
Proliferation (A_{450})
2.5
2
1.5
1
0.5
0
day 0
None
GW9662 (1 μM)
GW9662 (5 μM)
PTX
B
Vector
AGK
None Serum None Serum
IB: P-Y
170
94
48
V5-AGK
pERK1/2
ERK2
C
Vector
AGK
None Serum None Serum
170
IP: αEGFR
IB: αPY
170
IP: αEGFR
IB: αEGFR
D
Vector
AGK
pERK1/2
ERK2
None
EGF
AG1478
EGF+AG1478
None
EGF
AG1478
EGF+AG1478
E
Proliferation (A_{550})
0.3
0.25
0.2
0.15
0.1
0.05
0
95
51
AGK
tubulin
None
AG1478
AG1478
AG1478
day 0
1% Serum
10% Serum

Figure 5

Figure 6

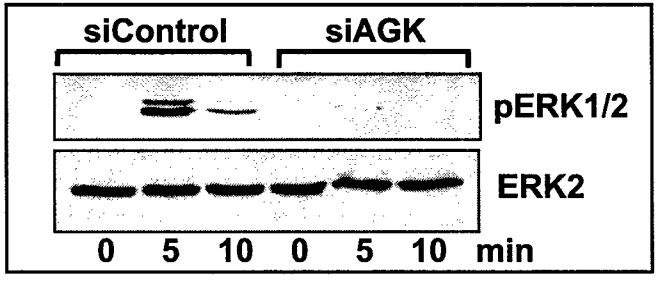

A
Migration (% of control)
300
250
200
150
100
50
0
Vector
AGK
AG1478
None EGF None EGF
B
vector
AGK
0 h
0 h
24 h
24 h
C
Wound Closure (cells/field)
160
120
80
40
0
NS
Vector
AGK
AG1478
None MOG EGF LPA None MOG
D
IL-8 Secretion (fold)
4
3
2
1
0
Vector
AGK
AG1478
None MOG LPA LPA

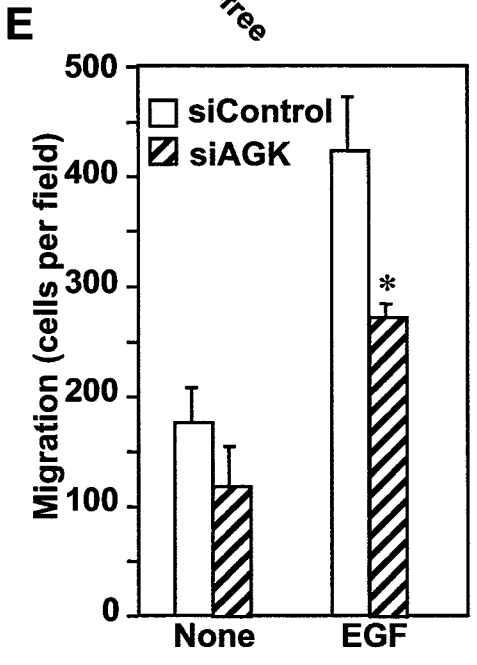




C

PL	siControl	siAGK
LPA	1.2 ± 0.02	0.8 ± 0.05*
PA	4.4 ± 0.2	2.9 ± 0.02*
PC	53.5 ± 0.32	48.6 ± 0.13
PI	9.7 ± 0.7	9.7 ± 0.02
PE	15.7 ± 0.9	18.3 ± 0.26
CL	1.7 ± 0.04	1.5 ± 0.02





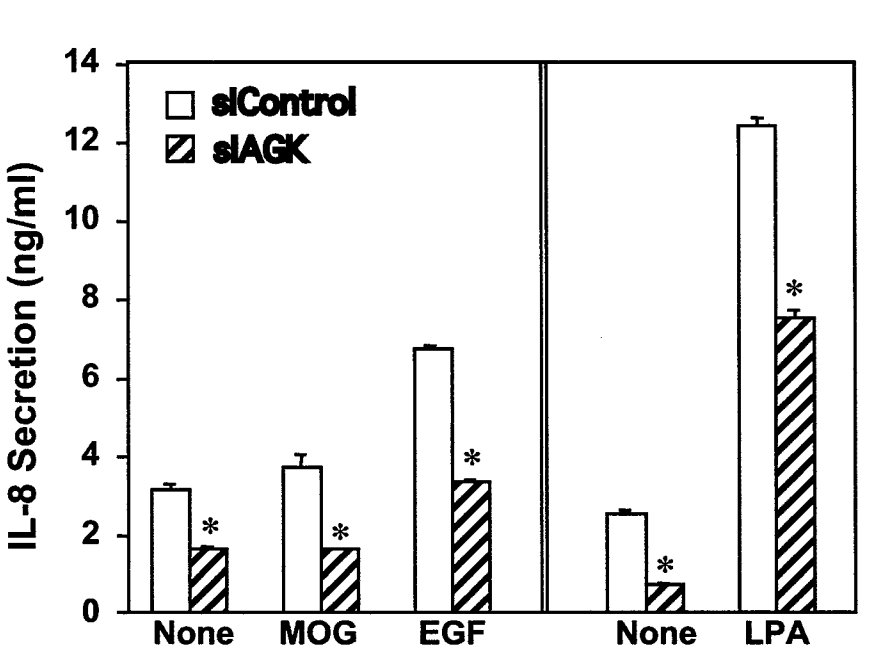
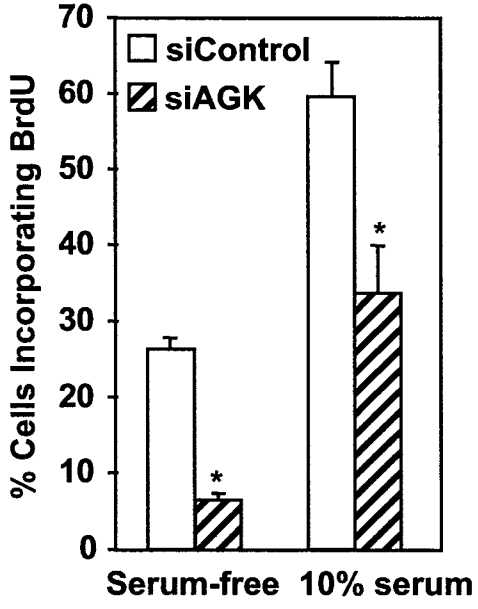
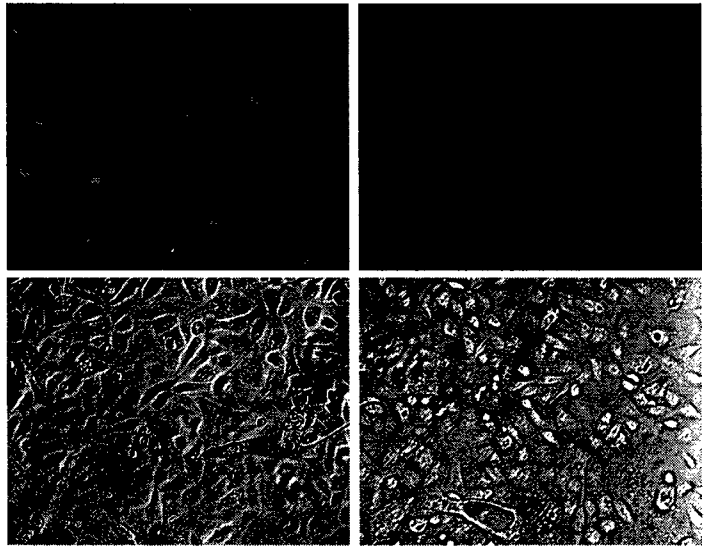


Figure 7

A



B



C

	Cells	G_o/G_1	S	G_2/M
Serum-free	siControl	75.1 ± 2.3	12.5 ± 2.0	12.4 ± 1.3
	siAGK	**83.8 ± 1.9**	**6.8 ± 1.4 ***	**9.4 ± 0.6***
10% Serum	siControl	54.8 ± 0.6	22.9 ± 0.5	22.3 ± 0.1
	siAGK	**64.3 ± 0.8**	**16.3 ± 0.6 ***	**19.4 ± 0.5***

Figure 8

ONLINE SUPPLEMENTAL MATERIALS

Bektas et al.

Cloning of AGK

An EST (AW321722) was identified that contained an open reading frame with 25% identity and 50% similarity to hSphK2 from amino acids 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 (Fig. 1). A nearly identical mouse homologue (CAC06108) was also identified. 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'-TTGGAGGAGGAGATGAGACACTGCAGGAGGTT-3', and reverse primer, 5'-AACCTCCTGCAGTGTCTCATCTCCTCCTGCAA-3'). The mutation was confirmed by sequencing.

Cell culture and transfection

Human PC-3 prostate cancer cells (ATCC CRL-1435), NIH 3T3 fibroblasts (ATCC CRL-1658), rat hepatoma RH7777 cells (kindly provided by Dr. X. Fang). and human embryonic kidney cells (HEK 293, ATCC CRL-1573) were seeded at 4 - 5 x 10^5 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, Gaithersburg, MD).

siRNA transfection

AGK expression was downregulated with sequence specific siRNA. siRNA target sequence for AGK (5'-AACAGATGAGGCTACCTTCAG-3') and control siRNA (5'-TTCTCCGAACGTGTCACGT-3') were obtained from Xeragon-Qiagen. In some experiments, cells were transfected with control and AGK siRNAs from Dharmacon. Cells (3×10^5) were transfected in 6-well dishes for 3-4 h with the RNA duplexes (300 nM) using Oligofectamine (LifeTechnologies) according to manufacturer's protocol. $90 \pm 2\%$ of the cells were transfected as determined with siGLO RISC-Free siRNA (Dharmacon).

Real-time PCR

Quantitative real-time PCR was performed on an ABI 7900 (Taqman; PE Applied Biosystems) with the following primers/probes: AGK forward primer 5'-CGAAGGCTTGCGTCCTACTG-3', reverse primer 5'-TGGTGGACAGCTGCACATCT-3', probe CACAACCACAGGATGCCCTTTCCC (Integrated DNA Technologies); pre-mixed primer-probe set for hSphK1 was purchased from ABI (cat. # Hs00184211_m1). Ribosomal RNA (18S rRNA) measured using TaqMan®Assay Reagents served as endogenous control.

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 800 μg protein in 500 μl was incubated with 2 μg anti-V5 (Invitrogen) for 4 h at 4 °C. Protein A/G PLUS-Agarose beads (20 μl, Santa Cruz Biotechnology) were added and incubated for an

additional 1 h. The beads were washed 4 times with the same buffer, resuspended in 20 µl and AKG activity was determined as described in Materials and Methods.

Immunofluorescence and confocal microscopy

Cells were grown on glass coverslips and transfected with vector or V5-tagged AGK. 24 h later, cells were incubated with 200 nM MitoTracker Red CMXRos (Molecular Probes, Eugene, OR) to stain mitochondria, fixed in 3% paraformaldehyde in PBS containing 0.1% Triton X-100. ER was visualized with polyclonal rabbit anti-calnexin antibody followed by anti-rabbit-IgG-FITC. Transfected cells were visualized simultaneously with anti-V5 antibody (1:500, Invitrogen) followed by a secondary anti-mouse antibody conjugated with FITC or Texas red (Molecular Probes), respectively. Coverslips were mounted on glass slides using an Anti-Fade kit (Molecular Probes) and examined by confocal microscopy. Images were collected with an Olympus IX70 laser scanning microscope equipped with argon (488 nm) and krypton (568 nm and 647 nm) lasers and a 60 X/1.4 NA PlanApo lens. Quantitative image analysis was performed using Metamorph image processing software.

Subcellular fractionation

Cells transfected with vector or AGK were Dounce homogenized in buffer containing 20 mM Hepes (pH 7.4), 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 250 mM sucrose, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM PMSF. Subcellular fractionation was carried out by differential centrifugation at 4 °C. Briefly, lysates were centrifuged at 1000 x g for 5 min to remove unbroken cells and nuclei (P1, nuclei and unbroken cells); postnuclear supernatants were centrifuged at 5000 x g for10 min (P2, mitochondria); followed by 17,000 x g for 15 min (P3, intracellular membrane fraction containing ER and Golgi). The remaining supernatant was centrifuged at 100,000 x g 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 $MgCl_2$, 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 dithiothreitol, 1 mM PMSF and 10 μg/ml leupeptin. Lysates were cleared by centrifugation at 10,000 x g for 10 min and incubated with 2 μg anti-EGFR antibody for 2 h at 4 °C. Protein A/G Sepharose beads (20 μl, Santa Cruz Biotechnology) were added and incubated for an additional 1 h at 4 °C. Sepharose beads were washed, boiled in SDS sample buffer, and bound proteins analyzed by Western blotting.

Supplemental Figure Legends

Figure 1S. Effect of AGK on NIH 3T3 fibroblasts. (A) NIH 3T3 fibroblasts stably transfected with vector (open bars) or AGK (filled bars) were cultured in the presence of 0.5% or 10% serum for 6 days and cell growth determined by the WST-1 assay. Data are means ± S.D. of triplicate determinations. (B) Duplicate cultures were allowed to migrate towards medium (None) or 20% serum for 8 h in modified Boyden chambers. (* $P < 0.05$ by Student *t*-test) (C) Duplicate cultures were stimulated with EGF (10 ng/ml) for the indicated times, lysed, and equal amounts of proteins analyzed by immunoblotting with ERK1/2 phosphospecific antibodies. Blots were stripped and re-probed with ERK2 antibody to show equal loading. (D) NIH 3T3 fibroblasts were transfected with vector or AGK together with GFP at a 4:1 ratio. Cells were cultured in serum-free medium or in 10% serum without or with doxorubicin (1 μg/ml) for 24 h. Cells were then fixed and nuclei stained with Hoechst. Total GFP-expressing cells and GFP-expressing cells

displaying condensed, fragmented nuclei indicative of apoptosis were scored. At least 300 cells were scored in a double-blind manner. Data are means ± S.D. of triplicate determinations.

Figure 2S. AGK expression enhances activation of EGFR. (A) Serum-starved PC-3 cells transfected with vector or AGK were treated with 10% serum for the indicated times. Equal amounts of cell lysates were separated by SDS-PAGE and immunoblotted with anti-phospho-tyrosine antibody. Blots were stripped and re-probed with anti-tubulin antibody as a loading control. (B) Serum-starved PC-3 cells transfected with AGK were preincubated for 60 min in the absence or presence of AG1478 (200 nM), then treated with the indicated concentrations of EGF for 10 min. Cell lysate proteins were analyzed by immunoblotting with anti-phospho-tyrosine antibody. Blots were stripped and re-probed with anti-ERK2 to demonstrate equal loading.

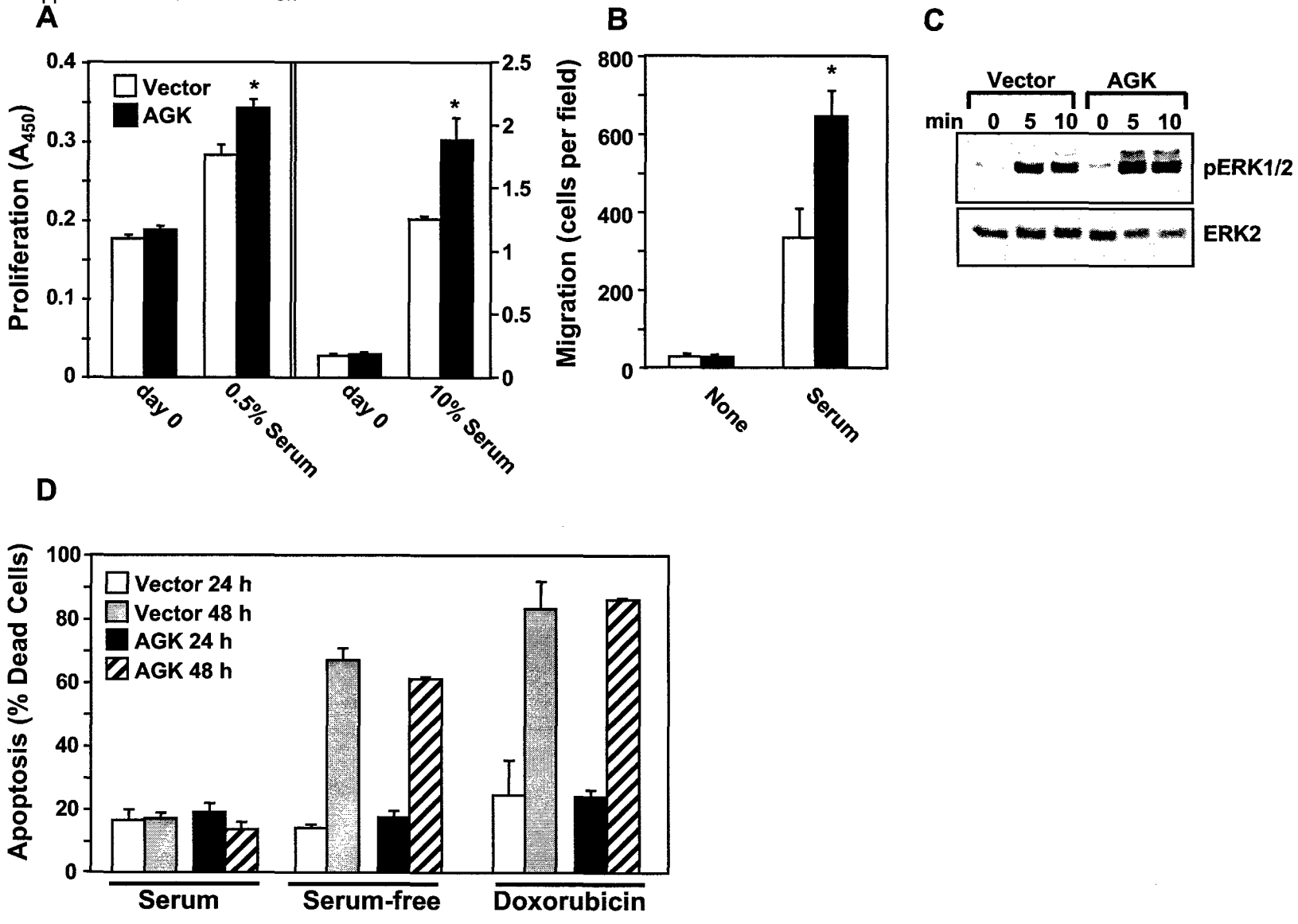


Figure 1 supplement

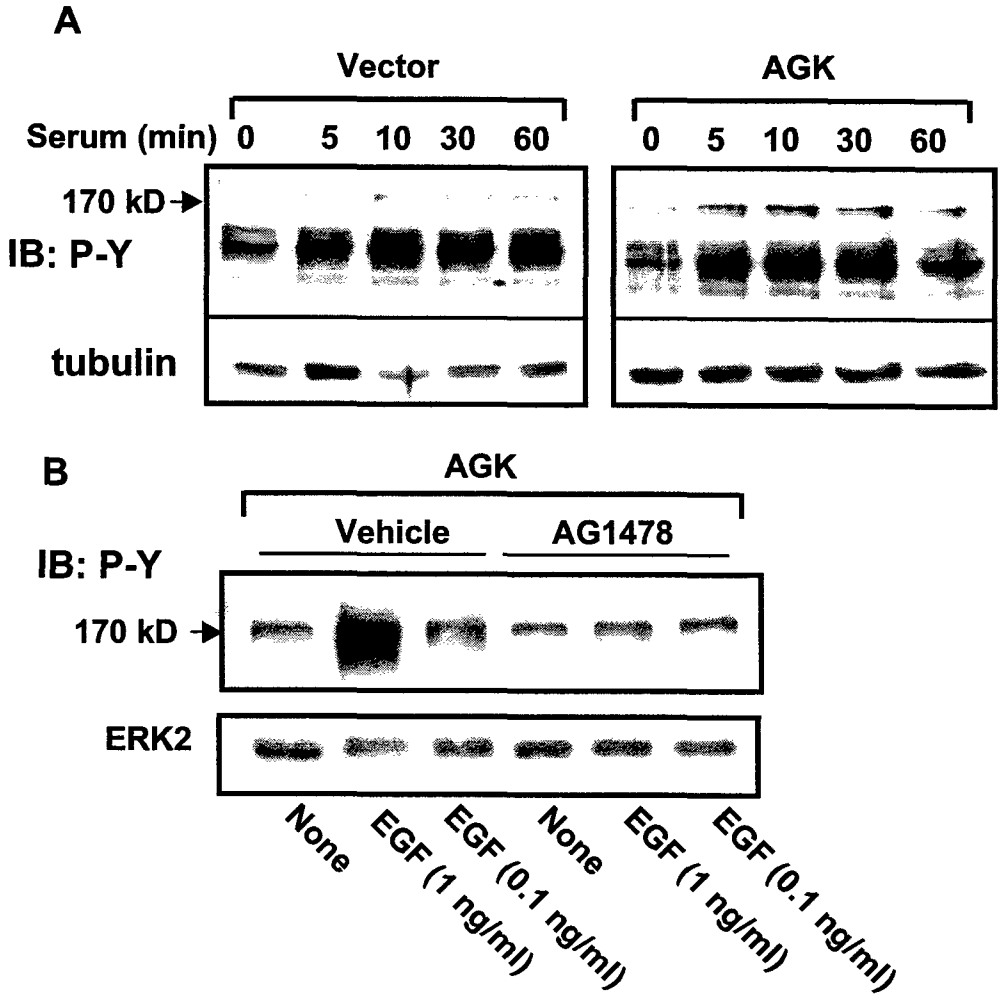


Figure 2 supplement

Appendix 2

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BIOLOGY

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Modulation of adpative immune responses by sphingosine-1-phosphate

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Abstract

Sphingosine-1-phosphate (S1P) has long been recognized as a mediator of a variety of cell functions. A growing body of evidence has accumulated demonstrating its role in cell migration and as a mediator of growth factor-induced events. In recent years, it has become apparent that S1P also mediates many cytokine and chemokine functions. Cells of the immune system function and migrate in response to a complex network of cytokines and chemokines, and the outcome is determined by the interplay of the effects of these molecules on the target cell. S1P may be a bona fide component of these networks and influence the responses of cells to these immune modulators.

Keywords: Cytokines; Chemokines; GPCR; FTY720; Sphingosine-1-phosphate

1. Introduction

Sphingosine-1-phosphate (S1P), a bioactive sphingolipid metabolite formed by the phosphorylation of sphingosine catalyzed by two isoforms of sphingosine kinase (SphK), is a multifunctional mediator of a variety of cell processes important for immune system functions, including cell growth regulation, inhibition of apoptosis, vascular development, and migration. S1P is an extracellular component of plasma and tissue fluids and functions by binding to a family of five specific cell surface G protein-coupled receptors. It is synthesized by many types of cells in response to a variety of stimuli and may also act as an intracellular second messenger. The extracellular and intracellular functions of S1P have been the subject of recent reviews [1–3] and will not be discussed extensively here. Interesting recent findings have begun to define a pivotal role of S1P in immunology as a modulator of cytokine synthesis as well as cellular responses to some chemokines. S1P may also function as a downstream mediator of cytokine signaling. Because cytokines and chemokines play central roles in homeostasis and activation and regulation of the immune system, S1P is emerging as an important player in the control of immune responses. The recent findings that the immunomodulatory drug, FTY720, when phosphorylated, is a S1P mimetic that acts by binding to S1P receptors [4,5], thereby mediating profound effects on the immune system, has highlighted the role of S1P and its receptors in chemokine and cytokine functions in the immune system.

2. Cytokine signaling through S1P

Over the past 15 years, a mass of evidence has accumulated that demonstrates that sphingolipid metabolites, particularly ceramide, act as second messengers and mediate many of the biological effects induced by TNF-α [6–9]. During inflammatory and other immune responses, TNF-α activates endothelial cells by inducing the expression of adhesion molecules, such as E-selectin and vascular cell adhesion molecule-1 (VCAM-1), and stimulates secretion of various cytokines. In human umbilical vein endothelial cells (HUVEC), the SphK inhibitor, *N,N*-dimethylsphingosine (DMS), inhibited TNF-α-induced upregulated expression of adhesion molecules, as well as activation of ERK and NFκB, leading to the conclusion that formation of S1P was critical for these TNF-α-mediated events [10]. Similarly, increased S1P levels in human neutrophils induced by TNF-α has been linked to TNF-α-induced priming of these cells [11,12].

TRAF2, a component of the TNF receptor complex that is important for NFκB activation and anti-apoptosis [13,14], may be the link between TNF-α and SphK activation [15]. Co-immunoprecipitation studies demonstrated physical interaction between TRAF2 and SphK1, and transfection with dominant-negative TRAF2 blocked TNF-α-stimulated SphK activity, suggesting that TRAF2 is a scaffolding protein that brings the TNF-α receptor and SphK together [15]. Several

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groups have reported that inhibition of apoptosis by TNF-α is mediated by SphK activation and S1P generation [16,17] and it has been found that a catalytically inactive SphK abolishes the cytoprotective effects of TRAF2, suggesting that SphK activity is required for TRAF2 to function, and therefore also for the anti-apoptotic effects of TNF-α [15]. In human hepatocytes, inhibition of SphK with DMS indicates that not only SphK but also NF-κB and PI3 kinase/Akt are involved in signaling pathways for protection from the apoptotic actions of TNF-α [17]. However, it is still not clear in this case whether S1P acts intracellularly to activate PI3 kinase or extracellularly via S1P receptors.

Because DMS and other pharmacological SphK inhibitors inhibit both SphK1 and SphK2 and also may have other non-specific effects, it was important to confirm the importance of SphK by more specific approaches. To further address the role of SphK and S1P formation in TNF-α-mediated inflammatory events, Pettus et al. [18] used a more specific molecular approach to downregulate SphK1 and SphK2 expression. They found that in L-929 fibroblasts, small interfering RNA (siRNA) targeted to SphK1, but not SphK2, decreased SphK activity and concomitantly inhibited the stimulatory effects of TNF-α on COX-2 expression and secretion of the inflammatory mediator prostaglandin E_2. These studies clearly place SphK1 and S1P formation in the TNF-α signaling repertoire (Fig. 1). However, links between the TNF receptors and SphK, as well as the downstream targets of S1P, are not yet well-characterized.

Interestingly, a physical association between Sphk2 and the β chain of the IL-12 receptor has recently been demonstrated [19] and Sphk2 augments IL-12 receptor signaling, further indicating a role for S1P in Th1 responses and inflammation.

Another protein that has been gaining more attention and has recently been linked to S1P is the tumor-associated cytokine, autotaxin, a protein factor that increases tumorigenicity and metastasis [20,21]. Autotaxin is an ectoenzyme originally shown to be a phosphodiesterase with important functions in non-transformed cells, such as oligodendrocytes during myelination [22]. Autotaxin has recently been discovered to have lysophospholipase D (lyso-PLD) activity that can generate lysophosphatidic acid (LPA) from lysophosphatidylcholine (LPC) [23,24], as well as S1P from sphingosylphosphorylcholine (SPC) [25]. It is therefore possible that some of the biological effects of autotaxin are mediated through the generation of S1P and/or LPA in the extracellular environment. Indeed, treatment of cells with SPC and autotaxin mimics some of the effects of S1P, such as increased motility and angiogenesis [25].

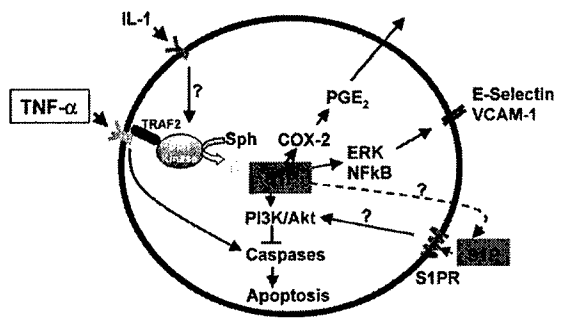

Fig. 1. The role of S1P as a mediator of TNF-α receptor signaling.

3. S1P Effects on cytokine production

Although there is substantial evidence establishing S1P as an extracellular mediator of cell migration and differentiation, the role of S1P in the control of cytokine and chemokine production and as a mediator of their effects is an emerging area of research. Cytokines and chemokines play central roles in activation, differentiation into effector states, function and migration of immune cells, including T- and B-lymphocytes, monocytes and dendritic cells [26,27]. T cells express S1P receptors, particularly $S1P_1$ and $S1P_4$, and nanomolar concentrations of S1P stimulate chemotaxis of Jurkat and splenic T cells [28]. Given that S1P and cytokines/chemokines have similar roles in different cell types, it might be expected that in immune cells, their signaling pathways would communicate and influence each other.

There are conflicting reports of the effects of S1P on the production of IL-2 and IFN-γ in activated T cells. Splenic CD4 T cells secrete IL-2, IFN-γ and IL-4 when stimulated with anti-CD3 together with anti-CD28. Co-treatment with low concentrations of S1P decreased IFN-γ and IL-4 secretion, but not IL-2 [28]. A similar pattern of inhibition of cytokine secretion by S1P was seen when the cells were stimulated with anti-CD3 and IL-7, but in this case, S1P marginally inhibited IL-2, although nanomolar concentrations were not effective. Similar doses of LPA also inhibited IL-2 secretion, in agreement with previous reports [29,30]. On the other hand, S1P enhanced IFN-γ and IL-2 secretion in CD3/CD28-stimulated peripheral blood lymphocytes [31]. There were several differences between these studies that may account for the discrepancy. The former study used purified CD4-positive splenic T cells and S1P concentration less than 1 μM, whereas the latter study utilized peripheral blood T cells, a mixture of both CD4 and CD8 cells, and S1P at much higher concentrations. In addition, it is possible that the effects of low concentrations of S1P are mediated through distinct cell surface S1P receptors while S1P may act intracellularly at higher concentrations. Nevertheless, S1P can alter the cytokine profile in activated T cells (Fig. 2).

The cytokine and chemokine expression profiles of activated T cells are determined by the interactions with antigen presenting cells (dendritic cells (DC), in particular) that sense environmental "cues" and then secrete cytokines that determine whether Th1 (cell mediated) or Th2 (antibody) responses occur. DC are specialized antigen-sensing and presentation cells that are important in the activation of T and

Appendix 2

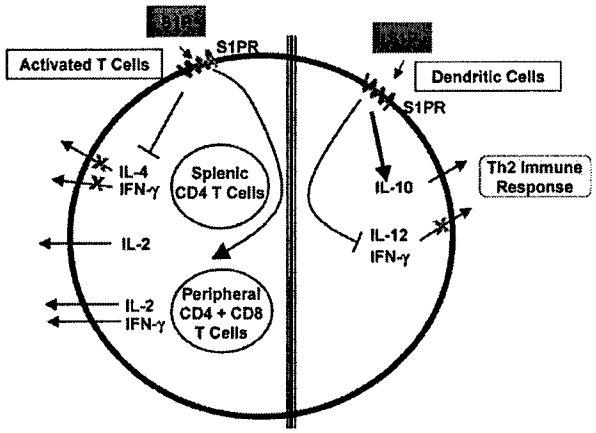

Fig. 2. The effect of extracellular S1P on secretion of cytokines by dendritic cells and activated T cells.

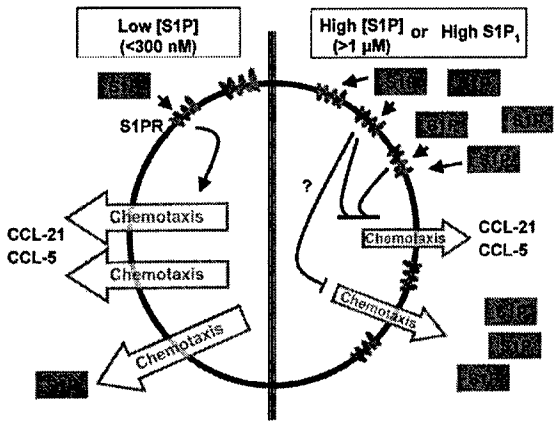

Fig. 3. The effect of varying S1P levels and $S1P_1$ expression on the chemotactic response of T cells to the chemokines CCL-21 and CCL-5.

B cells [32,33]. Immature DC reside in peripheral tissues where they capture and process antigen. This initiates their maturation and migration to secondary lymphoid tissues where they stimulate T and B cells to initiate the immune response to the antigen. DC maturation, as well as lymphocyte stimulation, requires a variety of cytokines and the composition of the cytokine environment during maturation and stimulation can determine the nature of the immune response. Although S1P does not affect basal cytokine expression in immature DC, it does alter the cytokine profiles of mature DCs [34]. In DC matured by treatment with lipopolysaccharide, S1P inhibited secretion of IL-12 and IFN-γ, but enhanced secretion of IL-10 (Fig. 2). IL-10 promotes a Th2 type immune response whereas it inhibits a Th1 response. Indeed, when S1P-treated DC were used to prime naive T cells, the T cells displayed an enhanced Th2 response, characterized by enhanced IL-4 and decreased IFN-γ production. In contrast, naive T cells that were primed with untreated DC displayed a predominantly Th1 profile, characterized by enhanced IFN-γ production [34].

Chemokines are a large group of secreted, small chemotactic cytokines that control leukocyte (T cells, B cells, DC, macrophages/monocytes, granulocytes) traffic throughout the body [35]. Chemokines are loosely grouped into two categories: "homeostatic" chemokines, which control leukocyte movement during hematopoieis, their navigation through the lymph nodes, and thymic maturation and immune surveillance; and "inflammatory" chemokines, which recruit leukocytes to sites of inflammation and tissue injury.

Nanomolar concentrations of S1P stimulate chemotactic responses of CD4 and CD8 T cells, as well as immature dendritic cells [28,34]. Activation of CD4 T cells by TcR activation (anti-CD3 + anti-CD28) downregulates expression of $S1P_1$ and the cells lose their chemotactic responses to S1P, whereas their chemotactic responses to the chemokine CCL-21 are increased. CCL-21 is a homeostatic, secondary lymphoid tissue chemokine involved in splenic and lymph node T cell homing. Treatment of CD4 T cells with S1P concentrations within the normal plasma range, enhanced their chemotactic responses to CCL-21 and CCL-5 in transwell chamber motility assays [36–38]. However, treatment with micromolar concentrations of S1P inhibited chemokine-induced chemotaxis (Fig. 3). These studies were also carried over into an in vivo migration model in which a chemoattractant was injected into a dorsal subcutaneous air pouch in a mouse and fluorescent-labeled cells were injected intraperitoneally. The number of fluorescent cells that migrate into the air pouch is a measure of the chemotactic activity of the chemoattractant. Migration of T cells that were pretreated with micromolar concentrations of S1P into the air pouch was significantly lower into CCL-21-treated air pouches than migration of untreated T cells. In contrast to the effects of S1P on cytokines, S1P does not appear to affect CCL-21 and CCL-5 production, but only modulates responsiveness to these chemokines.

S1P is nearly as effective a chemoattractant for DC as are C5a and CCL19 [34]. Like T cells, DC express CCR7 and migrate in response to its ligands CCL19 and CCL21 [39,40]. Although S1P induces actin polymerization and chemotaxis in immature DC, these effects are not observed in LPS-induced mature DC [34]. Similarly, prion protein fragment 106-126 is a chemoattractant for monocyte-derived immature but not mature DC [41]. This chemotaxis was inhibited by blockade of SphK, suggesting that transactivation of S1P receptors may be involved in prion protein-induced motility of DC [41]. These observations could have implications for mediation of neuroinvasion in transmissible spongiform encephalopathies by circulating DC.

Interleukin-8 (IL-8) is an important chemoattractant that recruits neutrophils to sites of inflammation and is also a proangiogenic factor [42]. S1P increases IL-8 expression and secretion in a variety of ovarian cancer cell lines [42]. Bronchial epithelial cells also secrete IL-8 in response to stimulation by S1P, a process that is mediated by S1P-stimulated activation of phospholipase D (PLD)

and subsequent generation of phosphatidic acid and ERK activation [43,44].

Our lab has recently demonstrated that S1P induces production of chemokines by bone-marrow-derived mast cells (BMMC) [45]. Mast cells are key players in both immediate-type and inflammatory allergic reactions and nanomolar concentrations of S1P upregulated both mRNA and secretion of CCL-4 (M1P1β) and CCL-2 (MCP-1). CCL-4 and CCL-2 are inflammatory chemokines that induce the recruitment of inflammatory cells, such as monocytes and eosinophils, to sites of inflammation. Our results suggest that S1P may have a more significant role in the production of inflammatory chemokines than in regulating homeostatic chemokines, such as CCL-21. This enhancement of mast cell-mediated inflammation by S1P is consistent with its effects on DC, as S1P-treated DC promote Th2 responses that result in IgE production [34].

Our results also suggest that activation of SphKs, production of S1P, and consequently activation of S1PRs by FcεR1 triggering plays a crucial role in mast cell functions and might be involved in movement of mast cells to sites of inflammation [45]. We found that whereas transactivation of $S1P_1$ and Gi signaling are important for cytoskeletal rearrangements and migration of mast cells towards antigen, $S1P_1$ is dispensable for FcεRI-triggered degranulation. In contrast, $S1P_2$, whose expression is upregulated by FcεRI crosslinking, was required for degranulation and inhibited migration towards antigen [45].

S1P may also have a more indirect effect on functions of chemokines. Matrix metalloproteinases (MMPs) are a family of enzymes involved in extracellular matrix (ECM) remodeling during cell migration. S1P and MMPs appear to play complementary roles during cell migration, as S1P has been shown to upregulate MMP expression [46,47] and MMPs can also cleave and inactivate chemokines in the ECM. However, direct evidence for this interaction is lacking. Moreover, it has been reported that S1P induces MMP expression in endothelial cells and it also paradoxically antagonizes the cell surface proteolytic cleavage of pro-MMP, which is required for its activation [46].

$S1P_1$ and $S1P_4$ are the two predominantly expressed S1PRs on T cells [28]. During T-cell activation, $S1P_1$ expression decreases whereas chemotactic responses to CCL-21 increase. T-cell activation also reduced both the S1P enhancing (at low concentrations) and inhibitory (at high concentrations) effects on chemokine-induced chemotaxis [36]. Similarly, chemotactic responses to the chemokine CXCL-4 of rat hepatoma cells overexpressing $S1P_1$ were enhanced at low S1P concentrations and inhibited at micromolar S1P concentrations, while overexpression of $S1P_4$ did not influence these responses. In agreement, overexpressing $S1P_1$ in T cells produced stronger migratory response to low S1P concentrations [38]. However, nanomolar concentrations of S1P, which in naive T cells enhanced CCL-5 and CCL-21-induced chemotaxis, inhibited chemotaxis in the $S1P_1$ overexpressing cells (Fig. 3). These results suggest that increased expression of $S1P_1$ may have the same effects as high S1P levels on chemotactic responses to CCL-5 and CCL-21. As well, high $S1P_1$ expression may affect different chemokine responses differently; e.g. increased chemotaxis to CXCL-4 but decreased chemotaxis to CCL-21. Thus, the $S1P_1$ receptor appears to be the most important determinant of cytokine and chemokine functions that are regulated by S1P.

T-cell responses to S1P-induced chemokine effects are also determined by prior S1P exposure [37]. Pre-incubation for 1 h with 100 nM S1P abolished the subsequent inhibition by micromolar concentrations of S1P on CCL-21 chemotaxis and the enhancement by low S1P concentrations. However, by 24 h post pre-incubation, the cells recovered the inhibitory and enhancement effects of S1P. This may be a result of $S1P_1$ downregulation during the pretreatment and subsequent recovery of $S1P_1$ expression.

4. Phosphorylated FTY720 is a S1P receptor agonist

FTY720 is an immunosuppressive agent that is highly effective in the prevention of transplant rejection [48]. Its apparent mode of action is to sequester lymphocytes in secondary lymphoid organs, such as lymph nodes, without affecting T-cell activation and expansion [49,50]. As a result, FTY720 prevents lymphocyte invasion into the transplant, and therefore prolongs its survival, without the induction of general immunosuppression. More recently, it was shown that FTY720 can also prevent egress of mature T cells from the thymus [51]. FTY720 has structural homology to sphingosine, and there are now several reports demonstrating that FTY720 is phosphorylated in vivo and in vitro is a substrate for SphK2 [4,52,53]. The phosphorylated form of FTY720 is biologically active and binds to all of the S1P receptors except $S1P_2$ [4].

The mechanisms by which FTY720 (or phospho-FTY720) induces lymphocyte sequestration and inhibits egress from the thymus are not well-understood. Given that S1P affects lymphocyte migration, either directly or indirectly by altering cellular responses to chemokines and cytokines, and chemokines are important mediators of lymphocyte trafficking, it follows that FTY720 may function in a similar manner. Indeed, lymphocytes from mice treated with FTY720 showed enhanced chemotaxis to a number of chemokines, including CCL-21 and CCL-5 [54]. However, FTY720 did not induce significant differences in chemokine or chemokine receptor expression.

CCR7 is the receptor for CCL-21, and both are required for lymphocyte homing to lymph nodes. CCR7-null mice have decreased numbers of T cells in their lymph nodes, as do CCL-21-knockout (*plt*) mice. If CCL-21 is required for FTY720-induced homing and sequestration, it might be expected that a defect in CCL-21 production would antagonize the FTY720-induced accumulation of lymphocytes in secondary lymphoid tissues. Indeed, *plt* mice treated with

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FTY720 for 6 or 12 h have higher numbers of lymphocytes in the periphery than wild-type mice, suggesting that CCL-21 is essential for homing in response to FTY720 [54]. However, in similar studies with CCR-7-deficient and *plt* mice, it was found that CCL19 and CCL21 chemokines do not play significant roles in FTY720-induced lymphocyte homing [55]. Treatment of CCR-7$^{-/-}$ and *plt* mice with FTY720 resulted in loss of lymphocytes from the peripheral blood with concomitant increased numbers in lymph nodes and Peyer's patches, albeit with a slower kinetics than those of wild type mice. The differences between these two studies may be that the time of exposure to FTY720 was not the same. Moreover, adoptively transferred CCR-7-deficient lymphocytes that were treated in vitro with FTY720 migrated into lymph nodes whereas CCR-7-deficient, untreated cells did not [55]. Although CCL-21 and CCR-7 do not appear to be required for FTY720 homing, CCL-21 does play some role in the accelerated lymphocyte homing.

The control of egress of mature T cells from the thymus is an important step in controlling the number of T cells in peripheral blood and secondary lymphoid organs. Migration of T cells through the thymus and subsequent egress is a complex process that is highly dependent upon chemokines. $S1P_1$-deficient thymocytes fail to leave the thymus, yet remain responsive to CCL-21. In addition, mature thymocytes from FTY720-treated wild-type mice fail to leave the thymus, yet remain responsive to CCL-21 [51]. It would therefore appear that chemokine-responsiveness of mature thymocytes is not affected by FTY720 or S1P in the same way as are peripheral blood and secondary lymphoid T cells. However, maturation and egress from the thymus involves interplay of multiple chemokines (reviewed in [35]) and FTY720 and/or S1P may influence the action of other chemokines involved in this process.

To date, FTY720, unlike S1P, has not been linked to any significant alterations in cytokine profiles that may specifically affect Th1 or Th2-type immune responses. In a Th1- versus Th2-mediated airway inflammation mouse model, it was shown that FTY720 effectively suppresses both responses, as well as suppressing both Th1 and Th2-associated cytokines in bronchial alveolar lavage fluid [56]. However, there is no evidence that FTY720 directly affected cytokine secretion and reduced cytokine levels in the alveolar lavage fluid could be a reflection of the reduced lymphocyte infiltration into the bronchial tissue.

5. Conclusion

From the studies cited in this review, it is clear that S1P plays a role in TNF-α signaling and that it can also act as an extracellular mediator to affect chemokine and cytokine functions. Cytokine and chemokine production, as well as the subsequent cellular responses, orchestrate a complex network of events that in the immune system result in the inflammatory responses of the innate immune system, selection of functional lymphocytes, deletion of self-reactive lymphocytes, cell activation and expansion in response to foreign antigen, and in dampening of the response after antigen clearance. Moreover, these events occur in different locations around the body and the movement of immune cells must be controlled so the proper cells will be in the correct site at the right time.

The data to date demonstrate that S1P may influence the direction of an immune response by modulating cytokine secretion patterns, as well as by modulating chemotactic responsiveness of DC and lymphocytes to chemokines. These latter events may enhance or inhibit immune cell migration into, or egress from tissues and organs. Lymphocytes express multiple S1P receptors that appear to have both unique and overlapping functions. Given that S1P receptor levels on lymphocytes vary depending on state of activation, and S1P levels may vary between plasma and tissues, the net effects of S1P on chemokine-responsiveness may be determined by the location and level of activation of the cell. For example, a naive T cell in the periphery may be responsive to the effects of S1P but once activated in a secondary lymphoid organ, it may lose its responsiveness not only to S1P but also to chemokines. However, this is an oversimplified view given the enormous complexity of chemokine and cytokine networks and the fact that studies to date have only investigated functions of S1P in the context of a few cytokines and chemokines. It is apparent that we have only barely scratched the surface of S1P and cytokine/chemokine networks.

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