Differential MAPK Pathways Utilized for HGF- and EGF-dependent Renal Epithelial Morphogenesis*

Anil Karihaloo‡§, Dawn A. O'Rourke§, Christian Nickel‡, Katherine Spokes**, and Lloyd G. Cantley‡

From the ‡School of Medicine, Yale University, New Haven, Connecticut 06520, ¶ACLARA Biosciences, Mountain View, California 94043, and **Beth Israel Deaconess Medical Center, Boston, Massachusetts 02215

** This work was supported by National Institutes of Health Grant DK54911 (to L. G. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Both authors contributed equally to this work.

§ Both authors contributed equally to this work.

Cells derived from the inner medullary collecting duct undergo in vitro branching tubulogenesis to both the c-met receptor ligand hepatocyte growth factor (HGF) as well as epidermal growth factor (EGF) receptor ligands. In contrast, many other cultured renal epithelial cells respond in this manner only to HGF, suggesting that these two receptors may use independent signaling pathways during morphogenesis. We have therefore compared the signaling pathways for mIMCD-3 cell morphogenesis in response to EGF and HGF. Inhibition of the p42/44 mitogen-activated protein kinase (MAPK) pathway with the mitogen-activated protein kinase kinase (MKK1) inhibitor PD98059 (50 μM) markedly inhibits HGF-induced cell migration with only partial inhibition of EGF-induced cell motility. Similarly, HGF-dependent, but not EGF-dependent, branching morphogenesis was more greatly inhibited by the MKK1 inhibitor. Examination of EGFR-stimulated cells demonstrated that extracellular-regulated kinase 5 (ERK5) was activated in response to EGF but not HGF, and that activation of ERK5 was only 60% inhibited by 50 μM PD98059. In contrast, the MKK inhibitor U0126 markedly inhibited both ERK1/2 and ERK5 activation and completely prevented HGF- and EGF-dependent migration and branching process formation. Expression of dominant negative ERK5 (dnERK5) likewise inhibited EGF-dependent branching process formation, but did not affect HGF-dependent branching process formation. Our results indicate that activation of the ERK1/ERK2 signaling pathway is critical for HGF-induced cell motility/morphogenesis in mIMCD-3 cells, whereas ERK5 appears to be required for EGF-dependent morphogenesis.

In renal epithelial cells, cell morphogenesis is an important process in embryonic development and repair of injured tubules. Two cell types commonly utilized to examine renal epithelial morphogenesis are murine mIMCD-3 collecting duct cells and canine MDCK tubular cells. In MDCK-3 cells, both the c-met receptor ligand hepatocyte growth factor (HGF) and the epidermal growth factor receptor (EGFR) ligands, transforming growth factor-α and epidermal growth factor (EGF), are capable of mediating branching morphogenesis (1, 2). In contrast, despite the presence of the EGFR in both cell types, only HGF is capable of mediating branching morphogenesis in MDCK cells, suggesting that these two receptors initiate morphogenesis utilizing similar, but not identical, signaling pathways. Upon activation, both the c-met receptor and the EGFR are tyrosine-phosphorylated and subsequently interact with GRB-2 and GAB1 (GRB-2-associated binding protein) (3, 4). These adapter molecules allow the activated receptor to initiate signaling through phosphoinositide 3-kinase (PI3K), phospholipase C-γ (PLC-γ), SHPTP and mitogen-activated protein kinase (MAPK) (3, 5–10). We have found that activation of the PI3K results in an increase in receptor-mediated activation of PLC-γ and that these signaling pathways then mediate PKC activation as an important component of the cell migratory response (11, 12). However, the role of MAPK signaling in epithelial cell motility and tubulogenesis, independent of its role in cell proliferation, is not as well defined.

Studies examining the importance of MAPK activation in cell motility in mammalian cells have had conflicting results. Some researchers conclude that cell motility involves only pathways independent of the MAPK cascade (13–16), whereas other studies indicate that MAPK activity is required for cell motility (17–20). This discrepancy may be due to differences in the cell types studied and/or growth factors utilized. For example, Anand-Apte et al. (21) examined PDGF- and fibronectin-stimulated migration in fibroblasts and determined that they were differentially regulated by Rac and MAPK. Their results indicated that the fibronectin pathway requires ERK1/2 activity for cell motility but the PDGF pathway does not. They concluded that different receptor classes in response to different ligands differentially utilize Rac and ERK pathways in cell migration.

There have been few studies to date examining the role of MAPK activation specifically in HGF- and/or EGF-dependent cell migration and tubulogenesis. One study of MAPK signaling in EGF-induced cell motility argues that MAPK activation may not be critical for this response in fibroblasts (22). These investigators found that two truncation mutants of the EGFR (lacking the tyrosine residues C-terminal to the kinase domain), which activate ERK1/2 do not support normal EGF-dependent cell migration, suggesting that activation of the MAPK pathway is not sufficient for cell migration. In a three-dimensional assay, Wang et al. (23) found that T4-2 breast carcinoma cells

*This paper is available on line at http://www.jbc.org
expressing abnormally high levels of the EGFR had an exaggerated MAPK response to EGF associated with an overtly proliferative, invasive morphology (23). This abnormal morphogenesis was completely suppressed by the MEK inhibitor PD98059. In contrast, Polk et al. (24) found no effect of MAPK inhibition on EGF-induced epithelial cell motility. An investigation of the role of MAPK signaling in HGF-stimulated MDCK cells recently demonstrated that the scattering response (a phenotype unique to HGF/c-met, which requires dissolution of existing cell-cell interactions followed by random cell migration) and tubulogenesis (a prolonged assay dependent on both cell morphogenesis and division) was prevented by the MEK inhibitor PD98059, suggesting that MAPK activation plays an important role in morphogenesis in these cells (25).

One possible explanation for these conflicting results is that activation of MAPK may be a ubiquitous requirement for cell morphogenesis, but that different growth factor receptors utilize different MAPK pathways. For example, it has been recently demonstrated that the EGFR utilizes ERK5 (also called Big MAPK (BMK1)) for mitogenic signaling rather than ERK1/2 (26). In the present study, we utilized mIMCD-3 cells to compare signaling pathways downstream of the c-met and EGFR in cell migration and early branching morphogenesis assays. Both receptors were found to require activation of the PI3K and PLCγ pathways in a normal motility response, whereas the c-met receptor appears to utilize the ERK1/2 signaling pathways during cell migration and branching morphogenesis and EGFR-dependent cell morphogenesis is primarily dependent on ERK5 activation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**Immortalized mIMCD-3 (27) cells were grown in DMEM/F12 media supplemented with 10% fetal calf serum. The response of these cells to HGF and EGF has been extensively studied in both cell migration assays and three-dimensional matrix assays (1, 8, 11, 28). All chemicals were purchased from Sigma Chemical Co. unless otherwise noted.

**Protein Analysis—**Subconfluent mIMCD-3 cells were serum-starved for 24 h prior to stimulation with either HGF (40 ng/ml, Sigma) or EGF (20 ng/ml, Upstate Biotechnology Inc.) for 10 min at 37 °C. The cells were then washed twice with ice-cold phosphate-buffered saline, scraped in ice-cold 0.5% Igepal lysis buffer (137 mM NaCl, 20 mM Tris base, 1 mM MgCl2, 1 mM CaCl2, 10 mM sodium orthovanadate, 10% glycerol, 0.5% Igepal CA-630, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin A, and 25 μg/ml antipain) and vortexed vigorously. Nonsolubilized debris was removed by microcentrifugation at 13,000 × g for 10 min at 4 °C, and the supernatant was collected. In the MEK inhibitor experiments, the cells were preincubated with the appropriate concentration of PD98059 (Calbiochem) or U0126 (Promega) for 20 min prior to stimulation with growth factor.

**Kinase Assay—**For kinase assays of endogenous ERK5, near confluent mIMCD-3 cells were serum-starved for 8 h and then treated with or without stimuli (HGF, 40 ng/ml, or EGF, 20 ng/ml, for 10 min), in the presence or absence of 50 μM PD98059 or 20 μM U0126. The cells were then washed once in ice-cold phosphate-buffered saline, collected in 500 μl of lysis buffer (20 mM Tris-CI (pH 7.5), 5 mM EGTA, 25 mM β-glycerophosphate, 1% Triton X-100, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin A, and 25 mM sodium fluoride), vortexed, and incubated on ice for 10 min and centrifuged at 14,000 rpm for 10 min. Protein assay was performed on the supernatant, and 1 μg of protein was incubated with 40 μl of Protein G-Sepharose beads (WVR Scientific Products) and 3 μg of goat-anti ERK5 antibody (Santa Cruz Biotechnology) for 3 h at 4 °C. The immune complex was then washed twice with the lysis buffer followed by two incubations in reaction buffer (20 mM Tris-CI, pH 7.5, 2 mM EGTA, 2 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 0.5 μg/ml pepstatin A, and 25 mM sodium fluoride) and incubated for 30 min at 30 °C with 20 μg of myelin basic protein (Sigma) in a buffer containing 20 mM Tris-CI (pH 7.5), 10 mM MgCl2, 100 μM ATP, and 10 μM γ-[32P]ATP. The supernatant was separated by SDS-PAGE, and phosphorylation of MBP was analyzed via autoradiography.

**RESULTS**

**Inhibition of ERK1/2 Activation Differentially Inhibits HGF- and EGF-dependent Cell Migration—**To determine whether HGF- and EGF-dependent morphogenesis required activation of the extracellular signal-regulated kinase (ERK) pathway, we utilized the MEK1 inhibitor PD98059 in both cell migration and branching morphogenesis assays. A dose-response curve for inhibition of ERK1/2 phosphorylation was performed in the presence of either HGF or EGF stimulation (Fig. 1A). At 50 μM, PD98059 inhibited HGF-induced phosphorylation of ERK2 by...
100% (the small degree of basal ERK2 phosphorylation seen in these cells was inhibited as well) and EGF-induced ERK2 phosphorylation by 92%. This dose was then chosen for further experiments. In later experiments, the more potent MEK1/2 inhibitor U0126 was utilized (31). A similar dose-response curve demonstrated 100% inhibition of ERK1/2 phosphorylation at a dose between 10 and 50 μM U0126 (Fig. 1B). Based on this result, 20 μM U0126 was used in subsequent experiments.

To examine the importance of ERK1/2 activation in the cell migratory response to HGF and EGF, a modified Boyden chamber assay was performed. Inhibition of the p42/44 ERK pathway with 50 μM PD98059 blocked HGF-induced cell migration by 75% (Fig. 2A) while causing only 37% inhibition of EGF-induced cell migration (control: 10.2 ± 1.3 cells/mm²; 50 μM PD98059: 20.2 ± 2.4; HGF: 114.9 ± 8.5; HGF + 50 μM PD98059: 36.0 ± 4.2; EGF: 228 ± 12.5; EGF + 50 μM PD98059: 145.7 ± 5.7). Of note, in different experiments, the inhibitory effect of PD98059 on EGF-dependent cell migration varied between 0 and 50% depending on the supplier.

In prior studies, we and others have demonstrated that activation of the phosphoinositide 3-kinase (PI3K) and phospholipase Cγ are required for PDGF-induced cell motility (8, 9). To determine whether these pathways were also differentially required in HGF- and EGF-dependent cell migration, we utilized the PI3K inhibitor wortmannin and the PLC inhibitor U73122. As compared with inhibition of MEK1, inhibition of phospholipase C equally inhibited HGF- and EGF-induced cell motility (control, 6.33 ± 0.84; U73122, 10.6 ± 3.3; HGF, 211.8; HGF + U73122, 13.5 ± 1.88; EGF, 180.4 ± 20.1; EGF + U73122, 9.67 ± 1.63) (Fig. 2B). Similarly, inhibition of the PI3K equally inhibited both HGF- and EGF-induced motility by ~60% (control, 10.2 ± 1.3 cells/mm²; wortmannin, 9.17 ± 0.91; HGF, 309 ± 24; HGF + wortmannin, 127 ± 12.6; EGF, 327 ± 26.9; EGF + wortmannin, 128.7 ± 6.12) (Fig. 2C). This partial inhibition of cell motility with wortmannin is comparable to that seen in PDGF-dependent migration (8). These results indicate that, although activation of the PI3K and PLCγ are equally important for HGF- and EGF-mediated cell migration, ERK1/2 activation appears to be differentially required for HGF- and EGF-dependent motility.

**EGF Activates ERK5 in mIMCD-3 Cells**—The recent observation that activation of the EGFR results in activation of ERK5 and that ERK5 plays a critical role in EGF-dependent cell proliferation (26) led us to test whether EGF activates ERK5 in mIMCD-3 cells and whether this alternate MAPK member might be important in EGF-dependent cell migration. The activation of ERK5 occurs following MEK5 phosphorylation of ERK5 on both threonine and tyrosine, similar to that seen for MEK1 phosphorylation of ERK1/2 (30, 32, 33). To investigate the ability of either HGF and/or EGF to activate ERK5, we initially utilized a gel shift assay. We detected a marked gel shift of a fraction of the immunoprecipitated ERK5 following EGF but not HGF stimulation (Fig. 3A). This reduced gel mobility is consistent with the classically described decrease in gel mobility of ERK1/2 following their threonine/tyrosine dual site phosphorylation by MEK1 and with the results of Kato et al. (26) examining ERK5 activation by EGF. With prolonged exposure of the immunoblot, we could observe a modest level of phosphorylated ERK5 detected in the HGF-stimulated cells (data not shown). The EGF-induced gel shift was not prevented by pretreatment with 50 μM PD98059 but was completely inhibited by the more potent MEK inhibitor U0126. We further investigated this differential activation of ERK5 by HGF and EGF using an antibody specific for the threonine-tyrosine dual phosphorylation site on ERK5 and detected phospho-ERK5 in mIMCD-3 lysates following EGF stimulation that was completely inhibited by U0126 and partially inhibited by PD98059 (Fig. 3B). In contrast, HGF failed to stimulate ERK5 phosphorylation (Fig. 3B).

Examination of ERK5 kinase activity revealed a significant activation of ERK5 by EGF and not by HGF (Fig. 3C). When the data from four kinase assays was quantified, we observed a 3-fold increase in ERK5 activation by EGF and no significant activation by HGF (Fig. 3D). EGF-induced activation was 62% inhibited by PD98059, whereas U0126 blocked EGF-dependent activation of ERK5 by 97%. As noted for the effects on cell migration, inhibition of ERK5 activation by PD98059 varied greatly in these assays. Thus, ERK1 and -2 were activated by both HGF and EGF, whereas only EGF significantly activates ERK5. Furthermore, U0126 markedly inhibits the activation of both MAPK signaling pathways, whereas PD98059, used at doses that completely inhibit ERK1/2 activation, is a less potent inhibitor of ERK5 activation in mIMCD-3 cells.

**ERK5 Inhibition Prevents EGF-dependent Cell Migration and Branching Process Formation**—We next examined HGF- and EGF-dependent cell migration using the U0126 compound to inhibit both ERK1/2 and ERK5 activation. As compared with the previously demonstrated 37% inhibition by PD98059, preincubation with U0126 inhibited EGF-dependent cell migration by 92% and HGF mediated cell migration by 97% (control, 2.82 ± 0.35; U0126, 2.80 ± 0.57; HGF, 30.65 ± 2.46; HGF + U0126, 3.78 ± 0.46; EGF, 54.06 ± 3.52; EGF + U0126, 11.94 ± 0.42) (Fig. 4A). These results suggest that EGF-dependent cell migration may be largely dependent on ERK5 activation. mIMCD3 cells grown in a three-dimensional collagen matrix in the presence of HGF or EGF transforming growth factor-α (TGF-α) exhibit elongated branching process formation as an early precursor to multicellular tubulogenesis (1, 28). To determine if MAPK activation is necessary for this process, mIMCD3 cells were suspended in a type 1 collagen and treated with HGF or EGF in the presence of the MEK inhibitors PD98059 or U0126 and the number of processes/cell was then determined 24 h following matrix polymerization. Similar to the results observed with cell migration, HGF-induced branching process...
formation was inhibited 76% by the MEK1 inhibitor PD98059, whereas EGF receptor-induced morphogenesis was inhibited only 23% (Fig. 4B). In contrast, U0126 entirely inhibited both HGF- and EGF-induced branching process formation (control, 0.72 ± 0.13 processes/cell; PD98059, 0.91 ± 0.08; U0126, 0.12 ± 0.14; HGF, 3.63 ± 0.25; HGF + PD98059, 1.41 ± 0.27; HGF + U0126, 0.25 ± 0.06; EGF, 2.59 ± 0.13; EGF + PD98059, 2.15 ± 0.49; EGF + U0126, 0.35 ± 0.05). Again, this result suggests that HGF-dependent morphogenesis is primarily dependent on ERK1/2 activation whereas EGF-induced branching process formation is ERK5-dependent.

Expression of Dominant Negative ERK5 Prevents EGF-in-
EGF-induced Morphogenesis Requires ERK5 Activation

**DISCUSSION**

There is a rapidly enlarging literature in regards to a role for MAPK activation in cell morphogenesis. In yeast, the MAPK pathway has been found to be critical for the morphogenic events necessary for hyphae formation and budding, specifically mediated by the G-protein-coupled Ste20/MEKK upstream activators of MEK and MAPK (34). In mammalian endothelial cells, proliferin can induce cell motility and angiogenesis (an event similar to epithelial tubulogenesis) via the G-protein-linked insulin-like growth factor-II receptor, and Groskopf et al. (18) have found that proliferin-mediated chemotaxis is blocked by the MEK inhibitor PD98059. Similarly, Graf et al. (17) found that either inhibition of MEK with PD98059 or down-regulation of MAPK expression with antisense oligodeoxynucleotides resulted in a 70–80% inhibition of PDGF-mediated cell migration in vascular smooth muscle cells.

It has been recently demonstrated that HGF-dependent tubulogenesis in MDCK cells (a 7- to 10-day assay of multicellular tubule formation requiring cell division) was inhibited by the MEK1 inhibitor PD98059 (25). Because cell proliferation requires MAPK activation, we chose to utilize a 4-h cell migration assay and a 24-h single cell branching morphogenesis assay to eliminate the role of cell division in this process and thereby specifically examine regulation of cell morphology. In the present study, we find that hepatocyte growth factor-de-
pendent cell migration and branching process formation are blocked by PD98059, demonstrating that these morphogenic responses are dependent on activation of the MEK1 substrates ERK1 and/or ERK2.

In contrast to the results observed with HGF, we found that EGF-dependent morphogenesis in mIMCD-3 cells was only modestly inhibited by doses of PD98059 that resulted in >90% inhibition of ERK1/2 activation. Thus it appears that the classical ERK1/2 MAPK pathway is critical for HGF- but not EGF-dependent cell migration and branching process formation. Of note, the dependence of EGF-induced cell migration on activation of the phosphoinositide 3-kinase and phospholipase C was nearly indistinguishable from that demonstrated for HGF. To determine a mechanism for the differential MAPK dependence, we tested the ability of HGF and EGF to activate the MEK5/ERK5 MAPK pathway that has been found to be critical for EGF-dependent mitogenesis (26). In mIMCD-3 cells, EGF was found to activate ERK5 kinase activity 3-fold, whereas HGF failed to activate ERK5.

In work by Kamakura et al. (33) using COS cells transfected with tagged ERK5, EGF activation of ERK5 was found to be fully inhibited by both PD98059 and U0126. In the present study examining the kinase activity of native ERK5 in mIMCD-3 cells, we found that concentrations of PD98059 that are sufficient to fully inhibit EGF-dependent ERK1/2 activation only inhibited ERK5 activation by 60%. In contrast, the alternate MEK inhibitor U0126 fully inhibited both pathways. The difference in our result as compared with the work by Kamakura and coworkers may be due to differences in cell type, differences in the native versus overexpressed protein, or differences in the PD98059 itself. It has been our experience that the apparent $k_i$ for MEK varies with different lots of PD98059. Of note, in one series of experiments, ERK5 activation was more fully inhibited in our cells by $100 \mu M$ PD98059 (data not shown).

Our observations, i.e. that EGF (but not HGF) activates ERK5 and that 50 $\mu M$ PD98059 (which inhibits ERK1/2 activation fully but ERK5 activation by only 60%) inhibited HGF-dependent morphogenesis in a much greater fashion than EGF-dependent morphogenesis, suggested to us that EGF utilizes the MEK5/ERK5 MAPK pathway for initiation of morphogenesis. To test this possibility, we expressed dominant negative ERK5 in mIMCD-3 cells. In our experience, LipofectAMINE transfection of mIMCD-3 or MDCK cells typically results in 25–35% transfection efficiency. Therefore, to identify those cells that were expressing the dnBMK1 construct, we performed cotransfection with a plasmid expressing GFP, allowing us to quickly identify and count transfected cells based on fluorescence. By utilizing one-fourth of the amount of cDNA for GFP as compared with dnERK5, cells expressing GFP are expected to be cotransfected with the dnBMK1 construct. We found that, although expression of enhanced GFP (EGFP) with
In fibroblasts, Slack et al. (35) have recently described that EGF-mediated migration was blocked by either PD98059 or U0126, suggesting that inhibition of ERK1/2 alone may be sufficient to prevent migration in these cells. Interestingly, examination of their results reveals that, although PD98059 inhibited their EGF-dependent cell migration index from 1.9 to 1.2, U0126 produced a 2-fold greater inhibition from 1.9 to 0.2, again consistent with a role for a non-ERK1/2 MAPK family member in EGF-dependent fibroblast migration. Their detection of significant inhibition of EGF-mediated fibroblast migration by the MEK1 inhibitor PD98059 may reflect relative differences in the dependence of the EGFR on ERK1/2 versus ERK5 in fibroblasts as compared with epithelial cells or may reflect the ability of PD98059 to significantly inhibit ERK5 activation in their cells at the doses utilized.

The requirement of these fairly rapid cell morphologic changes for ERK activation suggests that there may be ERK phosphorylation targets involved other than the classically described nuclear transcriptional regulatory proteins. We have recently found that, following HGF or EGF stimulation of mIMCD-3 cells, activated ERK1/2 associates with and phosphorylates the receptor docking protein GAB1 (36). This protein is of particular interest, because it has been found to associate with both the activated EGFR (4) and c-met (3) and mediate signaling interactions with the PI3K, PLCγ, and SHPTP. Overexpression of either the GAB1 c-met receptor binding domain (3) or a truncated GAB1 construct missing the PH1 domain that mediates membrane localization (37) results in a dominant negative phenotype for HGF-induced epithelial tubulogenesis. Thus, this docking protein appears to be a critical signaling intermediate in induction of the tubulogenic phenotype. Examination of the GAB1 sequence reveals multiple potential ERK phosphorylation sites (P(S/T)P), including sites immediately adjacent to the potential SH2 interaction sites of the PI3K, suggesting that ERK phosphorylation of GAB1 might regulate the degree or duration of signaling activation following receptor stimulation. In addition, activated ERK may play a direct role in regulating the cellular machinery required for morphogenesis. For example, Xie et al. (16) found that EGF-mediated disassembly of focal adhesions was dependent on ERK activation and Klemke et al. (38) have found that activated ERK can associate with and phosphorylate myosin light chain kinase, thereby increasing its kinase activity and enhancing cell migration. More recently, Chen et al. (39) have found that the assembly of the tight junctional proteins claudin-1 and ZO-1 was regulated by MAPK activation. Whether ERK5 can mediate these functions downstream of EGFR activation remains to be explored.

In conclusion, HGF-mediated cell migration and branching process formation requires the coordinate activation of the PI3K, PLCγ, and ERK1/2. Although EGF-dependent morphogenesis appears to have similar requirements for PI3K and PLCγ activation, this receptor pathway utilizes the alternative MEK5/ERK5 MAPK pathway for cell morphogenesis. This differential signaling in growth factor-induced morphogenesis may help to further explain how cell types expressing identical growth factor receptors can demonstrate divergent morphogenic responses.

Acknowledgments—We thank Dr. J. D. Lee for his kind gift of the BMK1 constructs, and we thank Fiona Watson and Eiisuke Nishida for their helpful comments.

REFERENCES

1. Barros, E. J., Santos, O. F., Matsumoto, K., Nakamura, T., and Nigam, S. K. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4412–4416
2. Cantley, L. G. (1996) Am. J. Physiol. 271, F1103–F1113
3. Weidner, K. M., Di Cesare, S., Sachs, M., Brinkmann, V., Behrens, J., and Birchmeier, W. (1996) Nature 384, 173–176
4. Holgado-Madruga, M., Emlet, D. R., Moscatello, D. K., Godwin, A. K., and Wong, A. J. (1996) *Nature* **379**, 560–564
5. Graziani, A., Gramaglia, D., Cantley, L. C., and Comoglio, P. M. (1993) *J. Biol. Chem.* **268**, 9165–9168
6. Okano, Y., Mizuno, K., Osada, S., Nakamura, T., and Nishida, E. (1993) *Biochem. Biophys. Res. Commun.* **190**, 842–848
7. Derman, M. P., Cunha, M. J., Barros, E. J. G., Nigam, S., and Cantley, L. G. (1995) *Am. J. Physiol.* **269**, F1211–F1217
8. Kato, Y., Tapping, R. I., Huang, S., Watson, M. H., Ulevitch, R. J., and Lee, J. D. (1996) *Nature* **384**, 713–716
9. Rameh, L., Rhee, S., Spokes, K., Kazlauskas, A., Cantley, L., and Cantley, L. G. (1998) *J. Biol. Chem.* **273**, 18623–18632
10. Chen, Y., Lu, Q., Schneeberger, E. E., and Goodenough, D. A. (2000) *Mol. Biol. Cell* **11**, 849–862