Prostaglandin E₂ Increases Growth and Motility of Colorectal Carcinoma Cells*

Hongmiao Sheng‡, Jinyi Shao‡, M. Kay Washington§, and Raymond N. DuBois¶** ‡‡

From the Departments of ‡Medicine, §Pathology, and ¶Cell Biology, the Vanderbilt-Ingram Cancer Center, Vanderbilt University Medical Center and the **Department of Veterans Affairs Medical Center, Nashville, Tennessee 37232-2279.

Received for publication, October 23, 2000, and in revised form, March 12, 2001

There is a 40–50% reduction in the relative risk of colorectal cancer and colorectal cancer-associated mortality in individuals taking nonsteroidal anti-inflammatory drugs (NSAIDs)1 (1–3). Inhibition of cyclooxygenase-2 (COX-2) activity is thought to represent one of the mechanisms by which NSAIDs exert their anti-neoplastic effects (Refs. 4 and 5; reviewed in Ref. 6). In support of this hypothesis, lack of the COX-2 (prostaglandin endoperoxide synthase-2) gene results in a reduction of the number of tumors which develop in mice heterozygous for an APC516 mutation by more than 7-fold (7). Additionally, COX-2 expression in colorectal carcinoma cells provides a growth and survival advantage (5, 8), and increases tumor cell invasiveness (9). Treatment with selective COX-2 inhibitors significantly reduces the adenoma burden in humans (10) and in animals (11). There are two isoforms of prostaglandin endoperoxide synthase, which are commonly referred to as COX-1 and COX-2. COX-1 is produced constitutively in many different cell types and tissues (12), but its expression can be regulated under some circumstances (13). COX-2 is induced by cytokines, growth factors, and tumor promoters (reviewed in Ref. 14). In studies of human colorectal cancer, COX-2 levels are increased in about 90% of cancers and ~50% of pre-malignant colorectal adenomas, but the enzyme is not usually detected in adult intestinal tissues (15, 16). Cyclooxygenase catalyzes the conversion of arachidonic acid to prostaglandins, such as prostaglandin E₂ (PGE₂). Here we demonstrate that PGE₂ treatment of LS-174 human colorectal carcinoma cells leads to increased motility and changes in cell shape. The prostaglandin EP₄ receptor signaling pathway appears to play a role in transducing signals which regulate these effects. PGE₂ treatment results in an activation of phosphatidylinositol 3-kinase/protein kinase B pathway that is required for the PGE₂-induced changes in carcinoma cell motility and colony morphology. Our results suggest that PGE₂ might enhance the invasive potential of colorectal carcinoma cells via activation of major intracellular signal transduction pathways not previously reported to be regulated by prostaglandins.

* This work was supported in part by United States Public Health Services Grants RO1DK 47297, P01CA77839, and P30CA-68485 (all to R. N. D.); the T. J. Martell Foundation; and Katie Couric. 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.

** ‡‡ Recipient of a Veterans Administration Research Merit Grant. To whom reprint requests should be addressed: Dept. of Medicine/GI, MCN C-2104, Vanderbilt University Medical Center, 1161 21st Ave. S., Nashville, TN 37232-2279. Tel.: 615-322-5200; Fax: 615-343-6229; E-mail: raymond.dubois@mcmail.vanderbilt.edu.

†† The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; EP, E-prostanoid; PG, prostaglandin; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; PI3K, phosphatidylinositol 3-kinase; RT, reverse transcription; PCR, polymerase chain reaction; COX, cyclooxygenase; PBS, phosphate-buffered saline; FAK, focal adhesion kinase; PKB, protein kinase B.

1 The precise contribution of increased biosynthesis of prostaglandins by COX-2 to the progression of neoplasia is currently under evaluation. For example, PGE₂ generated in colorectal carcinomas may enhance cell survival and/or may affect other aspects of epithelial cell behavior such as cell-cell or cell-substrate adhesion (5). A link between the neoplastic effect of carcinogen treatment and prostaglandin signaling was recently made by the observation that genetic disruption of the E-prostanoid receptor subtype 1 (EP₁) results in a reduction in the number of aberrant crypt foci that develop in mice following carcinogen treatment (18). Based on these findings, we sought to determine the effects of PGE₂ on the biology of colorectal carcinoma cells. We found that PGE₂ stimulated an increase in the proliferation and motility of colorectal carcinoma cells.

EXPERIMENTAL PROCEDURES

Cell Culture—LS-174 cells were purchased from ATCC (Manassas, VA). The cells were maintained in McCoy’s 5A medium containing 10% fetal bovine serum. LY 294002 and wortmannin were purchased from Calbiochem (La Jolla, CA). PGE₂, butaprost, sulprostone, and PGE₁ alcohol were purchased from Cayman Chemical (Ann Arbor, MI).

Immunoblot Analysis—Immunoblot analysis was performed as described previously (19). Cells were lysed for 30 min in radioimmunoprecipitation assay buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 mM sodium orthovanadate) and then clarified cell lysates were denatured and fractionated by SDS-polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred to nitrocellulose membranes and the filters were incubated with the antibodies indicated and developed by the enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech). The anti-phosphorylated Akt antibody was purchased from New England Biolabs (Beverly, MA).

This paper is available on line at http://www.jbc.org
Prostaglandin E2 and Epithelial Cell Motility

Cell Growth in Matrigel®—1 × 10⁴ LS-174 cells were suspended in 0.5 ml of 1:2 diluted Matrigel® (serum-free). PGE₂ (50 nM) in fresh medium was replaced every 2 days. DMSO, Me₂SO. After the plates were incubated for 15 days, colonies were photographed (magnification, ×40). B, concentration-dependent stimulation of colony growth by PGE₂. 1 × 10⁴ LS-174 cells were suspended in 0.5 ml of 1:2 diluted Matrigel® (serum-free) and treated with indicated concentration of PGE₂. After the plates were incubated for 15 days, relative colony diameter was determined and compared with controls. The mean ± S.E. of assays performed in triplicate are plotted. The results were similar in three separate experiments.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—RT-PCR was carried out using the RNA PCR kit from PerkinElmer Life Sciences.

A set of specific PCR primers for EP receptor subtypes (GenBank® accession numbers NM000955, NM000956, NM000957, and NM000958 for EP₁, EP₂, EP₃, and EP₄, respectively) have been designed as follows: EP₁, fragment, forward (5'-ACGGACCTGGCGCCGCACTGTA-3'; 321–342) and reverse (5'-GCTTGACCCCGCTTGTGACAC-3'; 750–729); EP₂, fragment, forward (5'-TCCAATGACTCCCGTGTCAGAG-3'; 169–190) and reverse (5'-TGGCATAGTACGCGCAGCAG-3'; 642–621); EP₃, fragment, forward (5'-GATCCACATGTCGCTAACGTG-3'; 396–415) and reverse (5'-AGTTATGCGAAGCTATGTC-3'; 904–881); EP₄, fragment, forward (5'-GGGACTGCGTCTGACCGAGCTTG-3'; 665–585) and reverse (5'-GGTGGCGGGCATAGCGGG-3'; 1600–1630).

One μg of total RNA was reverse-transcribed and amplified with 35 PCR cycles. The amplified products were visualized on 1.5% agarose gels.

RESULTS

Alterations in the Phenotype of LS-174 Cells following PGE₂ Treatment—Constitutive expression of COX-2 has been reported in 85–90% of colorectal carcinomas (15, 16). COX-2 is expressed in both carcinoma and stromal cells (21). Therefore, it is possible that carcinoma cells that do not express COX-2 could receive paracrine signals from PGE₂ produced by neighboring stromal cells. In order to elucidate whether PGE₂ might exert any effect on the phenotype of colon cancer cells, LS-174 human colon cancer cells were treated with PGE₂. LS-174 cells do not generate detectable prostaglandins, although COX-2 protein is detected in this cell line (22). LS-174 cells are able to form “crypt-like” aggregates when they are cultured in Matrigel®. To our surprise, treatment with PGE₂ exerted a growth-stimulatory effect on LS-174 cells (Fig. 1A). The size of LS-174 colonies in Matrigel® increased following PGE₂ treatment in a dose-dependent manner (Fig. 1B). Treatment with 10 nM PGE₂ resulted in optimal stimulation of LS-174 cell growth, causing a 2-fold increase in colony diameter.

To our surprise, treatment with PGE₂ caused a dramatic change in the morphology of the LS-174 colonies. When grown in extracellular matrix components (Matrigel®), LS-174 cells formed well organized structures consisting of an outside layer of cells with an acellular center (Fig. 2A, panel a). Positive Alcian Blue staining indicated that the LS-174 colonies were filled with colonic type mucin (data not shown). In contrast, the LS-174 cells exposed to PGE₂ formed irregular solid clumps of cells with a poorly organized structure (Fig. 2A, panel b). When grown on plastic culture dishes, LS-174 cells formed in “non-spreadng” round clumps (Fig. 2A, panel c). Addition of 10 nM ME2SO. After the plates were incubated for 15 days, colonies were photographed (magnification, ×40).
PGE₂ led to a rapid change in phenotype, which included increased spreading of cells within 2–4 h (Fig. 2A). Fluorescent staining with rhodamine-phalloidin demonstrated that PGE₂ treatment for 24 h resulted in protruding actin filaments from the cell periphery in the form of microspikes (Fig. 2B, panel b, white arrows), and an increase in the number of stress fibers (panel b, marked by black arrows). PGE₂ treatment also increased focal adhesion complexes as determined by immunostaining for FAK (panel d, marked by white arrows) and paxillin (panel f, marked by white arrows).

To further examine the spreading behavior induced by PGE₂, we carried out experiments using a modified Boyden chamber. The results are shown in Fig. 3A. Addition of 0.1 µM PGE₂ also promoted the movement of LS-174 cells through a Matrigel®-coated polycarbonate membrane by 2–3-fold (Fig. 3B). Therefore, PGE₂ altered the behavior of LS-174 cells by stimulating an increase in their motility, which could explain, in part, their change in cellular organization when grown as multicellular colonies.

Evaluation of EP Receptor Subtypes—Next, we determined if LS-174 cells express EP receptors, which are known to bind PGE₂ with a high affinity (reviewed in Ref. 23). The expression of EP receptors in LS-174 cells was determined by RT-PCR using specific oligonucleotide primers. EP₂, EP₃, and EP₄ were clearly expressed in LS-174 cells (Fig. 4A), but mRNA for the EP₁ receptor was barely detectable. To elucidate the functional role of EP receptor subtypes in LS-174 cells, we treated the cells with butaprost (1 µM, a selective EP₂ receptor agonist), sulprostone (5 µM, a selective EP₃ receptor agonist), and PGE₁ alcohol (10 nM, a selective EP₄ receptor agonist). Treatment with butaprost or sulprostone did not cause significant changes in cell morphology (data not shown). However, treatment with the PGE₁ alcohol (10 nM) resulted in more rapid and significant cell spreading when compared with the effect of PGE₂ alone. Alterations in LS-174 cell spreading were seen within 1 h following addition of the PGE₁ alcohol (Fig. 4B). Thus, LS-174 cell spreading and migration, stimulated by PGE₂, may be predominantly mediated through the EP₄ signaling pathway.

Regulation of ERK and Akt Activity by PGE₂—A number of signaling pathways is known to regulate cell growth and motility. The MAP kinase/ERK kinase/extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways were evaluated following PGE₂ treatment. Treatment of LS-174 cells with PGE₂ (100 nM) only had a modest effect on the activity of ERK1/2. PGE₂ treatment slightly increased the levels of phosphorylated ERK1/2 as determined by Western blotting analysis (Fig. 5A, upper panel). The results of an ERK kinase assay confirmed this finding (Fig. 5A, lower panel).

On the other hand, treatment with PGE₂ led to a marked activation of the PI3K/Akt pathway. The levels of phosphorylated (Ser-473) Akt/PKB were elevated following treatment.
LS-174 cells were serum-deprived for 48 h and treated with 0.1 mM PGE2 (48 h prior to the treatment with vehicle). LS-174 cells were serum-starved for 48 h prior to the treatment with vehicle (Control) or PGE2 alcohol (0.1 μM). The pictures were taken 24 h after initiation of treatment. The morphology of cell clumps is shown (original magnification, ×200).

FIG. 4. Evaluation of the role of EP receptors. A, expression of PGE2 receptors. One μg of total RNA extracted from LS-174 was reverse-transcribed by using random hexamers. The fragment was amplified by specific primers for EP1, EP2, EP3, and EP4 for 35 PCR cycles. The amplified products were visualized on 1.5% agarose gels. M, molecular weight marker. B, the effect of EP receptor agonists. LS-174 cells were serum-starved for 48 h prior to the treatment with vehicle (Control) or PGE2 (1 μM). The arrows indicate the expression of PGE2 and the effect of EP receptor agonists (Fig. 4, B).

FIG. 5. Effect of PGE2 on ERK and Akt activities in LS-174 cells. A, the activation of ERK1/2. LS-174 cells were serum-starved for 48 h prior to PGE2 (0.1 μM) treatment. Cellular protein was collected at the indicated time points for determination of the levels of phosphorylated ERK1/2 (upper panel). For ERK kinase assays, LS-174 cells were serum-starved for 48 h and cell lysates were prepared at the indicated time points. ERK kinase activity was measured using the Biotrak system. B, the activation of Akt. LS-174 cells were serum-deprived for 48 h prior to PGE2 (0.1 μM) treatment. Cellular protein was collected at the indicated time points for determining the levels of phosphorylated Akt (upper panel). To determine PGE2 regulation of Akt kinase activity, LS-174 cells were serum-deprived for 48 h and treated with 0.1 μM PGE2. Akt was immunoprecipitated using a monospecific Akt antibody. The immunoprecipitate was then incubated with a GSK-3 fusion protein in the presence of ATP. Phosphorylation of GSK-3 was measured by Western blotting using an anti-phospho-GSK-3α/β (Ser21/9) antibody (lower panel). C, P13K assay. LS-174 cells were lysed and immunoprecipitated with anti-Tyr(P) monoclonal antibody. P13K activity in immunoprecipitates was determined by incubating the beads with reaction buffer containing 20 μCi of [γ-32P]ATP and 10 μg of PI3K inhibitor-4-phosphate. Phosphorylated products were separated by thin layer chromatography and visualized by autoradiography. PIP2, phosphatidylinositol-4-phosphate. D, inhibition of P13K. LS-174 cells were serum-deprived for 48 h. Wortmannin (Wort., 0.1 μM) or LY 294002 (LY, 10 μM) were added 1 h prior to the PGE2 (0.1 μM) treatment. Cell lysates were collected after a 2-h incubation, and the levels of pAkt were analyzed by Western blotting.

PGE2 resulted in rapid induction of PI3K activity, as determined by the conversion of phosphatidylinositol 4-phosphate to phosphatidylinositol 3,4-biphosphate (Fig. 5C). To confirm the involvement of PI3K in PGE2 activation of Akt/PKB, we evaluated two inhibitors of this pathway (wortmannin (0.1 μM) and LY 294002 (10 μM)) and found that they both completely blocked PGE2-induced phosphorylation of Akt/PKB (Fig. 5D).

The Role of PI3K/Akt in PGE2-induced Pro-neoplastic Effects—To determine whether the activation of Akt/PKB by PGE2 altered the phenotype of LS-174 cells, the cells were treated with PGE2 in the presence of specific PI3K inhibitors, LY 294002 (5 μM) and wortmannin (0.1 μM). Both LY 294002 and wortmannin, at low concentrations, have been demonstrated to specifically target PI3K activity (24). LS-174 cells were grown on plastic dishes and subjected to serum deprivation for 48 h. The cells were then treated with PGE2 (0.1 μM) in the presence or absence of LY 294002 or wortmannin for 24 h. DNA synthesis was evaluated by [3H]thymidine incorporation assays. PGE2 treatment resulted in a 70% increase in [3H]thymidine incorporation in LS-174 cells, and addition of 5 μM LY 294002 completely blocked the PGE2-induced increase in DNA synthesis. The presence of 0.1 μM wortmannin also abolished the PGE2 effect on DNA synthesis (Fig. 6A). Although inhibition of PI3K/Akt activity blocked PGE2-induced growth effects, LY 294002 and wortmannin (0.1 μM) also prevented the PGE2-induced cell spreading in LS-174 cells (Fig. 6B and data not shown). Next, we evaluated the role of PI3K/Akt activity on cell motility. Modified Boyden chamber assays demonstrated that PGE2 treatment resulted in a 2–2.5-fold increase in cell motility, and 5 μM LY 294002 or 0.1 μM wortmannin completely abolished this effect (Fig. 6, C and D).

Since PGE2 treatment dramatically altered the growth and morphology of LS-174 colonies in Matrigel®, it was of interest to determine the effects of PI3K/Akt activity on LS-174 cells grown in Matrigel®. As demonstrated in Fig. 7A, LY 294002 impaired the ability of LS-174 cells to grow in Matrigel® whereas PGE2 significantly increased the size and altered the morphology of LS-174 colonies. Interestingly, addition of LY 294002 completely blocked the PGE2 effects on cells grown in Matrigel® by inhibiting colony growth and the invasive morphology. Wortmannin exerted similar effects but to a lesser degree on LS-174 cells grown in Matrigel® (Fig. 7B and data not shown).

DISCUSSION

It is now clear that COX-2 plays a role in the promotion of colorectal cancer (6). However, the effects of prostaglandins generated by COX-2 have largely been unexplored. Here we provide evidence that prostaglandin-mediated signaling affects cell proliferation, motility, and morphogenesis and that activa-
tion of the PI3K/Akt pathway is essential for the PGE2-induced changes in neoplastic potential.

To evaluate the effect of prostaglandins on the behavior of colorectal carcinoma cells, we employed several approaches. Treatment with PGE2 stimulated DNA synthesis and cell spreading in LS-174 cells grown on plastic cultures. LS-174 cells form well differentiated multicellular colonies in Matrigel®, mimicking tumor growth in animals. Treatment of LS-174 colonies with PGE2 led to a significant disruption of their cellular organization with increased motility. The stimulation of cell migration by PGE2 has been observed previously in mesangial, endothelial, and T cells (25–27). Forced expression of COX-2 in colon carcinoma cells results in increased invasiveness compared with the parental cells (9). These findings suggest that a prostaglandin product, such as PGE2, might stimulate cell motility and invasiveness under certain circumstances. In the present study, we show that addition of PGE2 to serum-deprived LS-174 cells results in increased cell spreading accompanied by polymerization of actin and assembly of stress fibers, indicating that PGE2 induced cytoskeletal reorganiza-
tion. A role for the actin cytoskeleton has been implicated in many cellular functions, including motility, chemotaxis, cell division, endocytosis, and secretion (28–30). Our data also demonstrate that PGE2 treatment caused aggregation of FAK and paxillin, promoting the formation of focal adhesion complexes, which are known to be essential for cell migration (31, 32).

PGE2 acts via specific transmembrane G protein-coupled receptors (EP receptors) (23). Four EP receptor subtypes have been identified and are designated EP1, EP2, EP3, and EP4. EP1, EP4, signals via increased Ca2+, which leads to vasoconstriction. EP3 can also serve to stimulate vasoconstriction and inhibits the generation of cAMP, whereas EP2 and EP4 are known to mediate vasorelaxation by stimulating an increase in cAMP levels. Our results show that both sulprostone (EP3 agonist) and butaprost (EP2 agonist) (33, 34) did not mimic the effect of PGE2 to increase cell spreading. However, both the PGE2 alcohol and misoprostol (relatively selective EP4 agonist, data not shown) (33–35) induced significant cell spreading. These findings suggest that signaling via the EP1 receptor is, at least in part, responsible for the PGE2-induced changes in LS-174 cell behavior.

Evidence suggests that the PI3K/Akt pathway promotes growth factor-mediated cell survival and inhibits apoptosis (36). PI3K/Akt also plays a key role in the regulation of cell adhesion and actin rearrangement (37, 38). These observations suggest that the PI3K/Akt pathway is oncogenic and involved in the neoplastic transformation of mammalian cells. PI3K can be activated by growth factors, oncogenes, and is involved in the transmission of signals from certain G protein-coupled receptors (39–41). Akt is stimulated by a variety of agonists acting on G protein-coupled receptors (42–44). Murga et al. (41) recently reported that PI3Kα is necessary and sufficient to transmit signals from G proteins to Akt/PKB. Akt/PKB may also be activated by cyclic AMP-dependent protein kinase in a wortmannin-insensitive manner (42, 45). Here, we found that treatment with PGE2 rapidly increased the kinase activity of Akt/PKB and that wortmannin and LY 294002 blocked PGE2-induced phosphorylation of Akt/PKB, suggesting the involvement of PI3K. Thus far, the mechanism by which prostaglandin activates Akt/PKB is not clear, and, to our knowledge, this represents the first report of Akt/PKB modulation by PGE2. However, we have not established a direct link between the EP receptor and PI3K activation in the present study.

Our data further demonstrate the involvement of Akt/PKB activity in the PGE2-induced increase in cell proliferation and motility. LY 294002, at low concentrations (5–20 μM), specifically targets PI3K activity (24). The observation that both LY 294002 and wortmannin (structurally unrelated PI3K inhibitors) exerted similar effects on LS-174 cells indicates that PI3K is the likely target of these compounds (reviewed inRefs. 46 and 47). We found that the growth of LS-174 cells (either on plastic or in Matrigel®) was significantly impaired by LY 294002 (5 μM), suggesting that basal levels of PI3K/Akt activity are required for continuous growth of LS-174 cells. Specific inhibitors of PI3K that blocked the activation of Akt/PKB did inhibit PGE2-induced changes in cell behavior, suggesting that both PGE2-induced growth stimulation and cytoskeletal reorganization involve the activation of the PI3K/Akt pathway. Several studies demonstrated that Akt/PKB pathway plays an extremely important role in cell cycle progression via modifying the expression of cell cycle proteins, such as cyclin D1 and p27kip1 (48–51). Activation of the PI3K/Akt pathway is thought to be essential for cytoskeletal reorganization under certain circumstances (37). These previous studies strongly support our findings that the PI3K/Akt activity is required for PGE2-induced increases in the growth and invasiveness of LS-174 cells.

Although COX enzyme activity is proposed to play a pro-neoplastic role in colorectal carcinogenesis, the downstream signaling that mediates these effects is poorly understood. Our results demonstrate that PGE2 can induce significant phenotypic alterations in colorectal carcinoma cells. These changes include increased motility, changes in cell shape, and stimulation of cell growth. We found the PI3K/Akt signaling pathway to be involved in the regulation of morphogenic and proliferative changes. This work establishes a role for PGE2 in the stimulation of tumor cell motility and reveals an additional cellular target, the EP3 receptor, which appears to be involved in this process.
| Reference                                                                 | Journal/Volume/Issue/Pages                                                                 |
|--------------------------------------------------------------------------|-------------------------------------------------------------------------------------------|
| 37. Rodriguez-Viciana, P., Warne, P. H., Khwaja, A., Marte, B. M., Pappin, D., Das, P., Waterfield, M. D., Ridley, A., and Downward, J. (1997) | *Cell* **89**, 457–467                                                                    |
| 38. Osada, M., Tolkacheva, T., Li, W., Chan, T. O., Tsichlis, P. N., Saez, R., Kimmelman, A. C., and Chan, A. M. (1999) | *Mol. Cell. Biol.* **19**, 6333–6344                                                       |
| 39. Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. H., and Tsichlis, P. N. (1995) | *Cell* **81**, 727–736                                                                    |
| 40. Downward, J. (1997) | *Adv. Second Messenger Phosphoprotein Res.* **31**, 1–10                                     |
| 41. Murga, C., Fukuhara, S., and Gutkind, J. S. (2000) | *J. Biol. Chem.* **275**, 12069–12073                                                      |
| 42. Sable, C. L., Filippa, N., Hemmings, B., and Van Obberghen, E. (1997) | *FEBS Lett.* **409**, 253–257                                                             |
| 43. Stephens, L. R., Eguinoa, A., Erdjument-Bromage, H., Lui, M., Cooke, F., Coadwell, J., Smrcka, A. S., Thelen, M., Cadwallader, K., Tempst, P., and Hawkins, P. T. (1997) | *Cell* **89**, 105–114                                                                   |
| 44. Murga, C., Laguinge, L., Wetzker, R., Cuadrado, A., and Gutkind, J. S. (1998) | *J. Biol. Chem.* **273**, 19080–19085                                                     |
| 45. Filippa, N., Sable, C. L., Filloux, C., Hemmings, B., and Van Obberghen, E. (1999) | *Mol. Cell. Biol.* **19**, 4989–5000                                                       |
| 46. Ward, S. G., June, C. H., and Olive, D. (1996) | *Immunol. Today* **17**, 187–197                                                          |
| 47. Vanhaesebroeck, R., and Waterfield, M. D. (1999) | *Exp. Cell Res.* **253**, 239–254                                                           |
| 48. Gille, H., and Downward, J. (1999) | *J. Biol. Chem.* **274**, 22033–22040                                                      |
| 49. Muise-Helmericks, R. C., Grimes, H. L., Bellacosa, A., Malstrom, S. E., Tsichlis, P. N., and Rosen, N. (1998) | *J. Biol. Chem.* **273**, 28864–28872                                                     |
| 50. Diehl, J. A., Cheng, M., Roussel, M. F., and Sherr, C. J. (1998) | *Genes Dev.* **12**, 3499–3511                                                            |
| 51. Medema, R. H., Kops, G. J., Bos, J. L., and Burgering, B. M. (2000) | *Nature* **404**, 782–787                                                                  |