Akt/PKB Activity Is Required for Ha-Ras-mediated Transformation of Intestinal Epithelial Cells*

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Phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB/Akt) is thought to serve as an oncogenic signaling pathway which can be activated by Ras. The role of PI3K/Akt in Ras-mediated transformation of intestinal epithelial cells is currently not clear. Here we demonstrate that inducible expression of oncogenic Ha-Ras results in activation of PKB/Akt in rat intestinal epithelial cells (RIE-iHa-Ras), which was blocked by treatment with inhibitors of PI3K activity. The PI3K inhibitor, LY-294002, partially reversed the morphological transformation induced by Ha-Ras and resulted in a modest stimulation of apoptosis. The most pronounced phenotypic alteration following inhibition of PI3K was induction of G1 phase cell cycle arrest. LY-294002 blocked the Ha-Ras-induced expression of cyclin D1, cyclin-dependent kinase (CDK) 2, and increased the levels of p27kip1. Both LY-294002 and wortmannin significantly reduced anchorage-independent growth of RIE-iHa-Ras cells. Forced expression of both the constitutively active forms of Raf (ΔRaf-22W or Raf BBX) and Akt (Akt-myr) resulted in transformation of RIE cells that was not achieved by transfection with either the Raf mutant construct or Akt-myr alone. These findings delineate an important role for PI3K/Akt in Ras-mediated transformation of intestinal epithelial cells.

The serine/threonine kinase Akt, or protein kinase B (Akt/PKB)1 is a direct downstream effector of phosphatidylinositol 3-kinase (PI3K) (1, 2). Akt/PKB lies in the crossroads of multiple cellular signaling pathways and acts as a transducer of input initiated by growth factor receptors that activate PI3K (reviewed in Ref. 3). Akt/PKB regulates gene transcription by direct phosphorylation of some of the Forkhead transcription factors such as FKHR, FKHRL, and AFX (4–6) or indirectly by modifying the CAM-responsive element-binding protein (7, 8), E2F (9), or nuclear factor-κB (10). Evidence suggests that the PI3K/Akt/PKB pathway promotes growth factor-mediated cell survival and inhibits apoptosis (11) by modifying the anti-apoptotic and pro-apoptotic activities in the bcl-2 gene family (12, 13).

Activation of the PI3K/Akt pathway is important for Ras transformation of mammalian cells and essential in Ras-induced cytoskeletal reorganization (14). The PI3K/Akt signaling pathway plays a critical role in R-Ras-mediated transformation, adhesion, and cell survival (15). Additionally, transformation of hematopoietic cells by BCR/ABL requires activation of the PI3K/Akt signaling pathway (16). Ectopic expression of active Akt/PKB leads to transformation of NIH 3T3 and chicken embryo fibroblasts (17, 18). These observations strongly suggest that the PI3K/Akt pathway is oncogenic and widely involved in the neoplastic transformation of mammalian cells. Understanding the role of Akt/PKB in malignant transformation has been greatly enhanced by recent work on the tumor suppressor gene, PTEN/MMAC1. A large body of evidence demonstrates that PTEN (phosphatase and tensin homologue deleted on chromosome ten) suppresses tumor formation by restraining the PI3K/Akt pathway (Refs. 19–22; reviewed in Ref. 23). PTEN has been shown to dephosphorylate the 3-position of both phosphatidylinositol-3,4,5-P3 and phosphatidylinositol-3,4-P2 to reverse the reaction catalyzed by PI3K, which leads to activation of Akt/PKB (23). Interestingly, a high incidence of epithelial dysplasia and colonic carcinoma has been observed in PTEN+/− heterozygous mice (24, 25), suggesting that Akt activity may be important in colorectal carcinogenesis.

Colorectal cancer is the second leading cause of death related to cancer in the United States. About 50% of colorectal carcinomas contain Ki-ras mutations (26) and the Ki-ras oncogene plays a key role during the adenoma to carcinoma sequence of events involved in the neoplastic transformation of colonic epithelial cells (reviewed in Ref. 27). The activated form of Ras can directly bind to p110, the catalytic subunit of PI3K (28), and ectopic expression of oncocogenic Ras in mammalian cells results in the activation of the PI3K pathway. We have established a rat intestinal epithelial cell line (RIE-iHa-Ras) in which oncocogenic RasVal12 can be induced upon addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG), which results in morphological transformation (29). In the present study, we sought to determine the role of the PI3K/Akt in Ha-RasVal12-mediated transformation of intestinal epithelial cells to further evaluate the oncogenic potential of this signaling pathway.

EXPERIMENTAL PROCEDURES

Cell Culture—The RIE-iHa-Ras cell line with an inducible Ha-RasVal12 cDNA was generated by using LacSwitch eukaryotic expression system (Stratagene, La Jolla, CA) and described previously (29). The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 400 μg/ml G418.
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(Akt/Life Technologies, Inc.), and 150 µg/ml hygromycin B (Calbiochem, San Diego, CA). The Ha-RasVal12 cDNA is under the transcriptional control of the Lac operator. IPTG (Life Technologies, Inc.) at a concentration of 5 mM was used to induce the expression of mutated Ha-Ras. RIE/iHa-Ras cell line was a kind gift of Dr. Robert Coffey (Vanderbilt University Medical Center, Nashville, TN). Akt-myr was described previously (30). PD-155092, PD-98059, wortmannin, and LY-294002 were purchased from Calbiochem (San Diego, CA).

Northern Analysis—Total cellular RNA was extracted as previously described (31). RNA samples were separated on formaldehyde-agarose gels and blotted onto nitrocellulose membranes. The blots were hybridized with RNA probes labeled with [α-32P]UTP using MAXIscript™ kit (Ambion, Austin, TX). After hybridization and washes, the blots were subjected to autoradiography.

Immunoblot Analysis and Antibodies—Immunoblot analysis was performed as previously described (32). 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-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose membrane. The filters were then probed with the indicated antibodies and developed by the enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech). The anti-pan Ras antibody was purchased from Calbiochem. The anti-cyclin D1 antibody was purchased from Transduction Laboratories, (Lexington, KY) and the anti-cyclin D1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-phosphorylated Akt antibody was obtained from New England Biolabs (Beverly, MA), and the anti-active ERK1/2 antibody was from Upstate Biotechnology (Lake Placid, NY). Anti-cyclin D1, anti-CDK2, CDK4, Anti-phosphorylated Akt, Anti-cyclin D1, and Anti-pan Ras antibodies were purchased from Calbiochem. The anti-cyclin D1 antibody was obtained from Calbiochem. The anti-cyclin D1 antibody was purchased from Upstate Biotechnology.

RESULTS

Activation of Akt/PKB by the Induction of Ha-RasVal12—Oncogenic Ras is known to activate Akt/PKB through direct binding and activation of the p110 catalytic subunit of PI3K (33). In order to elucidate whether expression of mutated Ha-Ras activates Akt/PKB in intestinal epithelial cells, we evaluated the effect of inducible expression of Ha-RasVal12 in RIE cells. As shown in Fig. 1A, the expression of Ha-RasVal12 in RIE-iHa-Ras cells is detectable 4 h following treatment with IPTG and markedly increased within 24 h. The levels of phosphorylated extracellular signaling-regulated kinase (ERK) and Akt were coincidentally induced following induction of Ras expression. Following Ras induction, RIE cells acquired a transformed appearance characterized by growth in overlapping clusters and formation of colonies in soft agarose (Fig. 1B). To block the Ras-induced activation of Akt/PKB a specific inhibitor for PI3K, LY-294002 (20 µM) (34) was added to the RIE-iHa-Ras cells prior to IPTG treatment. The Ras-induced phosphorylation of ERKs was not affected by the treatment with LY-294002. In contrast, treatment with LY-294002 almost completely blocked the activation of Akt/PKB following induction of oncogenic Ha-Ras, but pAkt was maintained at a basal level (Fig. 1C).

Inhibition of Akt/PKB and Apoptosis—Akt/PKB has been shown to promote cell survival in a variety of different cell lines (12, 13). Therefore, we evaluated the role of Akt/PKB in programmed cell death of Ras-transformed intestinal epithelial cells. As demonstrated in Fig. 2A, RIE-iHa-Ras cells underwent apoptosis when they reached confluence as indicated by their DNA banding pattern. In contrast, programmed cell death was almost completely inhibited following induction of Ha-RasVal12 and DNA fragmentation was barely detected in IPTG-treated RIE-iHa-Ras cells. Inhibition of mitogen-activated protein kinase/ERK kinase (MEK) with PD-98059 (50 µM) greatly increased DNA fragmentation despite the induction of oncogenic Ha-Ras. Surprisingly, inhibition of Akt/PKB activation by LY-294002 only partially blocked the Ras-induced increase in cell survival. Treatment with PD-98059 resulted in an increased fraction of apoptotic floating cells (Fig. 2B, left panel) as determined by fluorochrome staining (Fig. 2B, right panel).

Akt/PKB and Cell Morphology—Non-induced RIE-iHa-Ras cells displayed the same non-transformed morphology as the parental RIE-1 cells, which grew as monolayer cultures with contact inhibition (Fig. 3A, panel a). Morphological transformation of the RIE-iHa-Ras cells was observed between 24 and 48 h after IPTG treatment (Fig. 3A, panel b). Inhibition of MEK/ERK activity by PD-98059 completely blocked the Ras-induced transformation in RIE-iHa-Ras cells (Fig. 3A, panel c). Inhibition of Akt/PKB activity resulted in a partial suppression of Ras-mediated transformation. The spindly appearance of cells and overlapping growth was still observed in some areas (Fig. 3A, panel d). Fluorescent staining with rhodamine-phalloidin clearly demonstrated stress fibers in unperturbed RIE-Ras cells. Induction of Ha-RasVal12 resulted in a more prominent formation of stress fibers compared with Fig. 3B, panels a and b. Treatment with LY-294002 arrested the organization of stress fibers (Fig. 3B, panel c). Induction of oncogenic Ha-Ras also increased focal adhesion complexes, as determined by immunostaining for focal adhesion kinase (FAK). Normally, FAK was localized at the ends of stress fibers (Fig. 3B, panel d), but, following induction of Ras, the proteins diffusely accumulated in the cytoplasm (Fig. 3B, panel e). Treatment with LY-294002 restored the FAK expression.

The expression of Akt-myr was verified by detection of tagged hemagglutinin protein. To establish the RIE/Akt-myr/Raf BXB cell line, RIE-1 cells were first transfected with pZeOSV2/Akt-myr and then co-transfected with pSRα/Raf BXB plus pTK/hyg. Stable clones were selected by growth in media containing hygromycin (100 µg/ml), and expression was verified by measuring increased levels of Raf protein. To establish RIE-RasVal12 cells, RIE parental cells were stably transfected with the pCDNAS/Ha-RasVal12 expression vector and were selected by growth in neomycin (600 µg/ml).
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Fig. 1. Ha-Ras-mediated transformation and induction of ERK and Akt. A, Western analysis for Ras, pERK, and pAkt. RIE-iHa-Ras cells were treated with IPTG and lysed in radioimmunoprecipitation assay buffer at the indicated time points. After electrophoresis, the proteins were transferred to nitrocellulose filters and the filters were blotted with an anti-pan-Ras antibody, active ERK antibody, or active Akt antibody. The relative expression levels were determined by autoradiography using the ECL chemiluminescence system. The results were similar in three independent experiments. B, morphological transformation of RIE-iHa-Ras cells. RIE-iHa-Ras cells were grown on 60-mm tissue culture dishes and treated with vehicle (left panel; original magnification, ×100) or IPTG (central panel; original magnification, ×100). For anchorage-independent growth of RIE-iHa-Ras cells, 1 × 10^4 cells were mixed with Sea plaque agarose at a final concentration of 0.4% in DMEM containing vehicle or IPTG, and overlaid onto a 0.8% agarose layer in 35-mm plates. Colonies were photographed using an inverted microscope (right panel; original magnification, ×40). C, the effect of inhibition of PI3K on the levels of pERK and pAkt. LY-294002 (20 μM) was added to subconfluent RIE-iHa-Ras cells 1 h prior to IPTG treatment. Cellular protein was collected at the indicated time points for Western analysis of pERK and pAkt. Data shown in this figure represent three independent experiments.

Fig. 2. Inhibition of Akt and apoptosis. A, DNA fragmentation assay. RIE-iHa-Ras cells were grown to confluence and subjected to the treatment indicated. PD, PD-98059 (50 μM); LY, LY-294002 (20 μM). Cells were incubated for 48 h following treatment, and then floating and attached cells were collected in lysis buffer and the soluble DNA was isolated. DNA laddering was visualized on 1.6% agarose gel. B, RIE-iHa-Ras cells were treated with 5 mM IPTG and 50 μM PD-98059 for 48 h. Adherent cells were photographed (left panel; original magnification, ×200), and the floating cells were collected, stained with 167 μM Hoechst 33258 and visualized under a fluorescent microscope (right panel; original magnification, ×1000). Data shown in this figure are representative of three independent experiments.

Akt/PKB and Cell Proliferation—The rate of cell turnover is determined by the balance between cell proliferation and cell death. Akt/PKB activity is known to be involved in the regulation of cell proliferation (3). In order to elucidate whether Akt/PKB activity was required for the growth of Ras-transformed RIE cells, their growth rates following different treatments were determined. As demonstrated in Fig. 4A, when uninduced RIE-iHa-Ras cells reached confluence (at day 6), further growth was inhibited by cell-cell contact. Induction of oncogenic Ras significantly increased the cell density, and at the end of the experiment, the number of Ras-induced cells was 2.3-fold greater than the number of uninduced RIE-iHa-Ras cells. Both PD-98059 (50 μM) and LY-294002 (20 μM) completely blocked Ras-induced growth stimulation. LY-294002 inhibited the growth of Ras-transformed RIE-iHa-Ras cells in a concentration-dependent manner and reduced the cell number by 35%, 55%, and 76% at 5, 10, and 20 μM respectively (Fig. 4B). Next, we determined the effect of Akt/PKB on cell cycle regulation using flow cytometry. Induction of Ha-RasVal12 accelerated the G1/S transition (Fig. 4C). Addition of LY-294002 led to an accumulation of Ras-induced RIE-iHa-Ras cells in the G1 phase of the cell cycle. Interestingly, treatment with PD-98059 barely altered cell cycle progression in these cells.

Growth inhibition that resulted from blocking the activation of Akt/PKB was also observed when RIE-iHa-Ras cells were grown in an anchorage-independent manner. Induction of oncogenic Ras led RIE-iHa-Ras cells to form colonies in soft agarose, and both PD-98059 and LY-294002 reduced the colony number by >80% (Fig. 5A). To confirm that the growth-inhibitory effect of LY-294002 was mediated through inhibition of Akt/PKB activity, a structurally unrelated PI3K inhibitor, wortmannin, was employed. Both 0.1 and 1 μM wortmannin reduced the number of Ras-induced RIE-iHa-Ras colonies by >50%.

To further elucidate the mechanism by which Akt/PKB regulates cell cycle progression, we analyzed the regulation of cell cycle proteins in RIE-iHa-Ras cells. Induction of oncogenic Ras increased levels of cyclin D1 and CDK2, but had less of an effect on expression of CDK4, cyclin E, and p27kip (Fig. 6A). Treat-
ment with LY-294002 inhibited the expression of cyclin D1 and CDK2 in a concentration-dependent manner (Fig. 6B), and 20 \( \mu \)M LY-294002 completely blocked the Ras-induced expression of cyclin D1 and CDK2. LY-294002 also increased the expression of p27\(^{kip}\). In contrast, treatment with PD-98059 blocked Ras-induced expression of cyclin D1 and increased the levels of p27\(^{kip}\) but did not inhibit the Ras-mediated induction of CDK2.

Akt/PKB and Cell Transformation—PI3K/Akt/PKB is thought to represent an oncogenic signaling pathway. In the present study, we demonstrated that pharmacologic inhibition of Akt/PKB significantly inhibited the Ras-mediated transformation of intestinal epithelial cells. Oldham \textit{et al}. (30) demonstrated that ectopic expression of active Raf (\( \Delta \)Raf-22W) was not sufficient to transform RIE cells. We stably transfected the RIE/\( \Delta \)Raf-22W cells with an expression vector containing myristoylated form Akt (Akt-myr). The vector-transfected RIE/\( \Delta \)Raf-22W cells did not form colonies in soft agarose (Fig. 7A). In contrast, the RIE cells that expressed both active Raf kinase and Akt kinase grew in an anchorage-independent fashion. None of the clones that were selected from vector-transfected cells grew in soft agarose, whereas 50% of the clones isolated from Akt-myr-transfected RIE/\( \Delta \)Raf-22W cells formed colonies in soft agarose. To confirm the finding that ectopic expression of both active Raf kinase and Akt kinase was sufficient to cause RIE cell transformation, we first transfected the parental RIE-1 cells with an Akt-myr expression vector and found that this did not result in a significant phenotypic alteration. Interestingly, introduction of a constitutively active Raf mutant, Raf BXB (35), into the Akt-myr-transfected RIE cells resulted in morphological transformation of these cells. The morphological appearance of RIE/Akt-myr/Raf BXB cells was similar to Ha-RasVal12-transformed RIE cells with a spindly appearance and
overlapping growth (Fig. 7B, upper panels). RIE/Akt-myr/Raf BXB cells grew in soft agarose and formed colonies that were relatively smaller than the colonies formed by RIE-Ras<sup>Val12</sup> cells (Fig. 7B, bottom panels).

Previous reports indicate that an epidermal growth factor receptor (EGFR)-dependent autocrine growth loop is required for Ras-mediated transformation (36, 37). TGF<sub>A</sub> levels were increased in Ras-induced RIE-iHa-Ras cells and treatment with LY-294002 abolished Ras induction of TGF<sub>A</sub> (Fig. 7C, upper panel). The expression of TGF<sub>A</sub> was elevated in RIE/AKT cells and co-expression of AKT and Akt-myr further increased the level of TGF<sub>A</sub> in RIE cells (Fig. 7C, lower panel). Treatment with an EGFR inhibitor (1 μM of PD-153035) for 48 h almost completely reversed the transformed appearance of RIE/AKT cells and Akt-myr cells (Fig. 7D). PD-153035 treated RIE/AKT cells acquired a cuboidal appearance, and cell-cell contact inhibition was restored in these cells. As expected, PD-98059 (50 μM) reversed the transformed morphology of RIE/AKT cells by 24 h.

**DISCUSSION**

A previous study by Oldham et al. (36) reports that Ras<sup>12V</sup>/35S and Ras<sup>12V/37G</sup> mutants that have an impaired ability to activate PI3K are able to transform RIE cells, suggesting that activation of PI3K is not required for Ras-mediated transformation. Our data here clearly demonstrate that activation of the PI3K/Akt pathway is involved in the transformation of RIE cells. The PI3K/Akt pathway is known to be oncogenic in other circumstances. Ectopic expression of active Akt/PKB leads to transformation of NIH 3T3 and chicken embryo fibroblasts (17, 18). Transformation of hematopoietic cells by BCR/ABL requires activation of the PI3K/Akt signaling pathway (16). It has been demonstrated that the tumor suppressor gene, PTEN, suppresses tumor formation by restraining the PI3K/Akt pathway (Refs. 19–22; reviewed in Ref. 23). Several reports indicate that expression of wild type PTEN that down-regulates Akt/PKB activity results in G<sub>1</sub> growth arrest (19, 20). Interestingly, a high incidence of aberrant transcripts of the PTEN/MMAC1 gene has been observed in colorectal neoplasia (40), suggesting that Akt activity might play some role in colorectal carcinogenesis.

Mounting evidence suggests that Akt/PKB mediates growth factor-induced cell survival and blocks programmed cell death (11–13). Induction of oncogenic Ras promotes cell survival and resulted in loss of contact inhibition. We sought to determine which Ras effector was responsible for this effect and found that, in Ras-induced RIE-iHa-Ras cells, inhibition of Akt/PKB activity only partially restored the effect on programmed cell death, whereas inhibition of ERK activity completely blocked the Ras-induced increase in cell survival. On the other hand, Akt/PKB appears to play an extremely important role in cell cycle progression. Inhibition of Akt/PKB activity led to an accumulation of G<sub>1</sub>-phase cells that are necessary to prevent cells from uncontrolled proliferation and eventual transformation. Progression through the mid to late G<sub>1</sub> phase of the mammalian cell cycle is dependent upon the cyclin D1-mediated activation of CDK4 or the related CDK6.
were treated with 5 mM IPTG or IPTG growth of RIE/Akt-myr/Raf BXB cells or to determine the anchorage-independent growth of RIE/Akt-myr/Raf BXB cells or RIE-Ha-RasV12 expression. Stable clones were selected and transfected with pSRα-Raf BXB. The morphology of RIE/Akt-myr/Raf BXB cells was compared with the morphology of Ha-RasV12-transfected RIE cells (upper panels; original magnification, ×100). To determine the anchorage-independent growth of RIE/Akt-myr/Raf BXB cells or RIE-Ha-RasV12 cells, they were seeded in 0.4% soft agarose and were incubated for 10 days (lower panels; original magnification, ×40). C, Northern analysis for TGFA. Upper panel, RIE-iHa-Ras cells were treated with 5 mM IPTG or IPTG plus 20 μM LY-294002 for 24 h. The levels of TGFA mRNA were analyzed by Northern blotting. Lower panel, the expression of TGFA mRNA in RIE, RIE-Δα-Raf-22W (Raf), and RIE/Δα-Raf-22W(Akt-myr (Raf/Akt) cells. D, inhibition of EGFR in RIE/Δα-Raf-22W(Akt-myr cells. RIE/Δα-Raf-22W(Akt-myr cells were treated with Me2SO (control), PD-153035 (1 μM) for 48 h, or PD-98059 (50 μM) for 24 h (original magnification, ×100).

Fig. 7. The role of Raf and Akt in the transformation of RIE cells. A, RIE/Δα-Raf-22W cells were transfected with a pZeoSV2/Akt-myr plasmid or the empty vector and selected by growth in medium containing zeocin. 1 × 10^4 cells from the pooled clones were seeded in soft agarose. The plates were incubated for 10 days, and colony numbers were counted. All stable transfection experiments shown in this figure were repeated at least twice. B, transformation of RIE/Akt-myr/Raf BXB cells. RIE parental cells were transfected with pZeoSV2/Akt-myr. Stable clones were selected and transfected with pSRα-Raf BXB. The morphology of RIE/Akt-myr/Raf BXB cells was compared with the morphology of Ha-RasV12-transfected RIE cells (upper panels; original magnification, ×100). To determine the anchorage-independent growth of RIE/Akt-myr/Raf BXB cells or RIE-Ha-RasV12 cells, they were seeded in 0.4% soft agarose and were incubated for 10 days (lower panels; original magnification, ×40). C, Northern analysis for TGFA. Upper panel, RIE-iHa-Ras cells were treated with 5 mM IPTG or IPTG plus 20 μM LY-294002 for 24 h. The levels of TGFA mRNA were analyzed by Northern blotting. Lower panel, the expression of TGFA mRNA in RIE, RIE-Δα-Raf-22W (Raf), and RIE/Δα-Raf-22W(Akt-myr (Raf/Akt) cells. D, inhibition of EGFR in RIE/Δα-Raf-22W(Akt-myr cells. RIE/Δα-Raf-22W(Akt-myr cells were treated with Me2SO (control), PD-153035 (1 μM) for 48 h, or PD-98059 (50 μM) for 24 h (original magnification, ×100).

(41). Previous studies suggest that cyclin D1 is regulated via the Ras signaling pathway. Activation of ERK1 and ERK2 up-regulates cyclin D1 (42). Several studies suggest that cyclin D1 is also regulated by the Akt/PKB pathway at both the transcriptional (43) and post-transcriptional levels (44). Akt/PKB stabilizes cyclin D1 protein through inhibition of glycogen synthase kinase-3β kinase (45). We recently reported that induction of Ras activates Akt, inactivates glycogen synthase kinase-3β, and increases the level of cyclin D1 in RIE-iHa-Ras cells (31). In the present study, we show that inhibition of Akt/PKB completely blocked Ras-induced expression of cyclin D1, indicating that both ERK and Akt/PKB are essential for Ras-mediated induction of cyclin D1. Interestingly, forced expression of a stable mutant of cyclin D1 (T286A) (46) failed to override LY-294002-induced G1 growth arrest in RIE-iHa-Ras cells (data not shown). Treatment with PD-98059 decreased the levels of cyclin D1 to a similar extent but did not cause growth arrest at the G1 phase, suggesting that inhibition of cyclin D1 alone is not sufficient for LY-294002-induced G1 growth arrest.

G1-S transition also requires the activity of cyclin E/CDK2 which accelerates the phosphorylation of retinoblastoma protein initiated by the cyclin D/CDK complex and prevents its inhibition of transcription factors (including the E2Fs). In the present study, we found that induction of oncogenic Ras increased the levels of CDK2, which was dependent on the activation of Akt/PKB but not MEK/ERK activity. p27kip1, which represses the activity of the cyclin E/CDK2 complex, is thought to be directly involved in restriction point control (47, 48). Recently, Medema et al. (49) demonstrated that expression of AFX-like forkhead transcription factors blocks cell cycle progression at the G1 phase through up-regulation of p27kip1. Activation of Akt/PKB results in phosphorylation of AFX and FKHR-L1 and a reduction of p27kip1 expression. In agreement with previous findings, we observed that inhibition of Akt/PKB resulted in growth arrest at the G1 phase, which was associated with an increased expression of p27kip1 in RIE-iHa-Ras cells.

Oncogenic mutations in ras result in activation of multiple downstream signaling proteins including Raf/MEK/ERKs (50, 51), Rho family (52–54), and the PI3K/Akt/PKB pathway (14, 15). Activation of Raf/MEK/ERKs is known to be sufficient to transform NIH 3T3 fibroblasts (55, 56) but not sufficient for Ras transformation of RIE epithelial cells (30). These findings suggest that downstream effectors that are required for Ras-induced transformation are cell type-dependent and that additional Raf-independent pathway(s) are required to complete Ras-mediated morphological and growth transformation in intestinal epithelial cells. Our results demonstrate that ectopic expression of activated Akt/PKB did not cause significant phenotypic alterations in intestinal epithelial cells; however, expression of both active Raf and active Akt is sufficient to induce transformation of RIE cells. Our data suggest that, in cooperation with Raf, PI3K/Akt signaling promoted the EGFR-dependent autocrine growth loop that is thought to be essential for the morphological transformation of RIE cells (36, 37). Although it has been shown that Raf may be activated by Akt (57), there is no evidence to support direct activation of PI3K/Akt signaling by the Raf/MEK/ERK pathway, suggesting that they play different roles in Ras-mediated transformation. Indeed, we observed that the Raf/MEK/ERK pathway predominantly enhances cell survival and that the PI3K/Akt pathway predominantly promotes loss of contact inhibition of cell growth. Since ras mutations are found in a wide variety of human malignancies and in 50% of colorectal adenocarcinomas (26), our results are of particular interest in understanding the changes in molecular signaling pathways that occur during colorectal carcinogenesis and may have clinical significance by providing additional therapeutic targets.
