Involvement of the PI 3-kinase signaling pathway in progression of colon adenocarcinoma

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The phosphoinositide 3-kinase (PI 3-kinase) signaling pathway has been shown to play a pivotal role in intracellular signal transduction pathways involved in cell growth, cellular transformation and tumorigenesis. Analysis of several colon adenocarcinoma cell lines indicates that the PI 3-kinase signaling pathway is up-regulated in colon cancers. In particular, the protein levels and phosphorylation status of Akt and p70 S6 kinase are up-regulated in colon adenocarcinoma cell lines. More significantly, we have demonstrated for the first time that the phosphorylation of FKHR, a downstream target of Akt, is increased in these cell lines. Intriguingly, phosphorylation of three components of the PI 3-kinase signaling pathway, namely Akt, p70 S6 kinase and FKHR, are in direct correlation with the degree of tumorigenic potential of the colon cell lines tested. No differences in the protein levels of the two subunits of PI 3-kinase, p85 and p110α, and PTEN were noted. Real-time quantitative PCR indicated an increase in levels of Akt message only, and not of the other signaling pathway components. Inhibition of the PI 3-kinase with wortmannin decreased the anchorage-independent growth of colon cells in a soft agar assay. Hence, the components of the PI 3-kinase signaling pathway could serve as potential candidates for drug development in treatment of colon cancer.

Introduction

Protein kinases participate at multiple levels along signal transduction pathways. These signaling cascades are involved in diverse biological responses, ranging from cell cycle control to differentiation to cell proliferation, as well as differentiation of organ size and body growth (1–3). A vast body of literature has documented the involvement of the phosphoinositide 3-kinase (PI 3-kinase) signaling pathway in such functions (3–17). The involvement of PI 3-kinase signaling pathway effectors in various forms of cancers has been documented. PIK3CA elevation was noted in ovarian cancers (18), while deletion of the catalytic subunit of PI(3)Kα in mice resulted in colorectal carcinomas (19). Increased expression and activation of Akt was noted in primary human gastric adenocarcinomas, pancreatic ductal adenocarcinomas, colon carcinomas, glioblastomas and thyroid carcinomas, as well as prostate, breast, cervical, ovarian and small cell lung cancers (7,20–31). Similarly, elevation of p70 S6 kinase was noted in small cell lung cancer cells, pancreatic cancer cells and liver tumors (24,32–34).

PI 3-kinase, a heterodimeric protein composed of a catalytic subunit (p110α) and a regulatory subunit (p85), is implicated in suppression of apoptosis (6,9,10,35). Upon stimulation of cells with a wide range of extracellular stimuli, PI 3-kinase phosphorylates the D-3 position of phosphoinositides to form the second messengers phoshatidylinositol-3,4-bisphosphate and phoshatidylinositol-3,4,5-trisphosphate. The survival mechanism initiated by these lipid products is exerted via downstream components of this kinase, Akt, mTOR and p70 S6 kinase (36). An intracellular inhibitor of PI 3-kinase activity is PTEN, a lipid phosphatase acting on the phosphatidylinositides generated by PI 3-kinase, thereby functioning as a tumor suppressor (37–39).

Akt regulates a number of critical cellular pathways, including those leading to cellular proliferation and inhibition of apoptosis (6,9–16,40,41). The survival signals of Akt upon its phosphorylation and activation are mediated in part via identified downstream targets of this kinase. Phosphorylation of BAD (42,43), Caspase 9 (44) and Forkhead transcription factor (FKHR) (4,45–47) by Akt suppresses the pro-apoptotic function of these proteins. FKHR, together with AFX and FKHRL1, belongs to a small subset of the Forkhead family of transcription factors (48). Five phosphorylation residues have been identified in FKHR (Thr24, Ser256, Ser319, Ser322 and Ser325) (46,47,49). Phosphorylation of FKHR results in its inactivation and nuclear exclusion (44,47,50–53). Thus, Akt also promotes cell survival by negative regulation of the transcriptional activity of AFX, FKHR and FKHRL1, through their phosphorylation.

Another downstream target of Akt is mTOR. Akt phosphorylates mTOR on Ser2448 (54–56). mTOR has numerous regulatory functions, including activation of p70 S6 kinase, as a means of translational modulation. p70 S6 kinase contains several phosphorylation sites (57) which are targeted by various upstream kinases, including mTOR and PDK (58–62). Phosphorylation and activation of p70 S6 kinase results in phosphorylation of the 40S ribosomal protein S6, which in turn stimulates translation of 5' TOP mRNAs, messages encoding for ribosomal proteins and other components of the translational machinery (63–65).

This study analyses involvement of the PI 3-kinase signaling pathway effector molecules in progression of colon adenocarcinoma. Colorectal cancer constitutes the third leading cause of cancer-related death in North America (Cancer Facts & Figures 2002, American Cancer Society; Canadian Cancer Statistics 2000, Canadian Cancer Society). We examined total protein level and phosphorylation status of various

Abbreviations: DTT, dithiothreitol; FBS, fetal bovine serum; FKHR, Forkhead transcription factor; PMSF, phenylmethylsulfonyl fluoride; PI 3-kinase, phosphoinositide 3-kinase.

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members of the PI 3-kinase pathway in an array of colon adenocarcinoma cell lines which were previously shown to demonstrate varying degrees of tumorigenic potential (66). We show that protein levels and phosphorylation levels of Akt and p70 S6 kinase are elevated in colorectal carcinoma cell lines, and this elevation positively correlates with the increased tumorigenic potential of colon adenocarcinoma cell lines. More significantly, we found that increased levels of FKHR and its phosphorylation at Ser265 correlate directly with the degree of transformation potential of the cell lines tested. Inhibition of the PI 3-kinase signaling pathway with wortmannin resulted in a reduction in colony size in a soft agar assay, indicating that inhibition of this pathway plays an important role in oncogenicity, at least in colon cell lines.

### Materials and methods

#### Cell lines

Human colon adenocarcinoma cell lines: SW1417, CaCo2, Colo201, Colo205, SW1222, SW403, Lovo, LS174T and LS180 were grown in α-MEM medium supplemented with 10% fetal bovine serum (FBS). Human kidney 293A cells were grown in DMEM supplemented with 10% FBS.

#### Western blotting

To test the specificity of the phospho-specific antibodies, 293A cells were starved in DMEM medium without FBS for 16 h. For serum induction, cells were stimulated with medium containing 10% FBS for 30 min. To test rapamyacin and wortmannin sensitivity, cells were incubated with rapamycin (20 ng/ml) or wortmannin (100 nM) for 30 min and then stimulated with medium containing 10% FBS. For analysis of PI 3-kinase and PTEN, the lysis buffer contained 10 mM Tris–HCl, pH 7.5, 1% Triton X-100, 50 mM KCl, 1 mM dithiothreitol (DTT), 2 mM MgCl2; and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). For analysis of Akt, the lysis buffer contained 20 mM Tris–HCl, pH 7.5, 140 mM NaCl, 1% glycerol, 1% NP40, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μM PMSF, 20 mM NaF, 1 mM Na3VO4, and 1 mM Na2VO4. For analysis of p70 S6 kinase, the lysis buffer contained 50 mM sodium phosphate, pH 7.2, 2 mM EGTA, 25 mM NaF, 25 mM β-glycerophosphate, 0.5% Triton X-100, 100 μM Na3VO4, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM benzamidine and 2 mM DTT. Lysates were clarified by centrifugation at 14,000 r.p.m. for 10 min and total protein concentration was determined in triplicate using the Bio-Rad Protein Assay (Bio-Rad) and quantified against a standard curve of bovine serum albumin protein concentrations (Pierce). For each experiment, equal amounts of total protein, as indicated in the figure legend, were electrophoresed on 6–15% SDS–PAGE gels and electroblotted onto nitrocellulose membranes (Millipore). Filters were then blocked in Tris-buffered saline containing 0.2% Tween 20 (TBST) and 5% (w/v) dry milk at 25°C for 2 h. Membranes were incubated overnight at 4°C with one of the following antibodies at the indicated dilutions: mouse monoclonal anti-actin (ICN Biomedicals) at 1:500, anti-PI 3-kinase p85 (N-SH3 clone AB6) (Upstate Biotechnology) at 1:1,000, anti-PI 3-kinase p110ε (BD Biosciences) at 1:150, rabbit polyclonal anti-Akt, anti-PTEN, anti-p70 S6 kinase, anti-phospho-specific Akt (Ser473) (Cell Signal Technology) at 1:1,000 and anti-phospho-specific p70 S6 kinase (Thr389) (Cell Signal Technology) at 1:300. Membranes were washed with TBST three times and incubated as follows: for rabbit polyclonal antibodies, membranes were incubated with donkey anti-rabbit horseradish peroxidase-conjugated IgG (Cell Signal Technology) at 1:2,000; for mouse monoclonal antibodies membranes were incubated with goat anti-mouse horseradish peroxidase-conjugated IgG (Bio-Rad) at 1:5,000 for 1 h. Membranes were washed with TBST three times and signals were detected using an enhanced chemiluminescence kit (LumiGLO reagent, Cell Signal Technology) after exposure to X-ray film (Kodak).

#### Soft agar assay

Six-well tissue culture plates were covered with 4 ml of 0.5% Bacto Agar (Difco) growth medium containing 20% FBS in the presence or absence of 100 nM wortmannin (Calbiochem). Cells (20,000) were plated in duplicate in a 3 ml suspension of 0.35% agar medium. After 24 h, medium containing 20% FBS was added to each well and refreshed twice weekly. For wortmannin treatment of cells the drug was added to the solid underlay and overlay agar medium, as well as the liquid top layer. Cells were photographed after 2 weeks.

### Quantitative PCR

Total cellular RNA was extracted with Trizol (Gibco BRL) according to the manufacturer’s instructions. Any potential traces of genomic DNA were removed by treatment with DNA-Free DNase™ according to the manufacturer’s instructions (Ambion). A two-step RT–PCR was carried out. The first strand cDNA synthesis was generated using the Superscript™ First-Strand Synthesis System for RT–PCR (Gibco BRL) using one of the following primers according to the manufacturer’s instructions. The second strand cDNA was synthesized using gene-specific primers with the Light Cycler-FastStart DNA Master SYBER Green 1 kit (Roche), according to the manufacturer’s instructions, on a Light Cycler machine (Roche).

#### PI 3-kinase activity assay

Cells were washed with ice-cold buffer A (137 mM NaCl, 20 mM Tris–HCl, pH 7.4, 1 mM CaCl2, 1 mM MgCl2) once and lysed with buffer A containing 1% NP40 and proteinase inhibitor mixture (tablets from Roche Diagnostics). The whole cell lysates containing 2.8 mg of total protein were subjected to immunoprecipitation with 5 μg of anti-PI 3-kinase antibody (Upstate Biotechnology). After 1 h incubation at 4°C, 50 μl of a 50% slurry of protein A-Sepharose was added and the samples were incubated with rocking for another hour at 4°C. The beads were washed three times with buffer A containing 1% NP40, three times with 0.1 M Tris–HCl, pH 7.4 and 5 mM LiCl and twice with TNE buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) and suspended in 50 μl of TNE. Ten microliters of phosphatidylinositol (2 μg/μl) (Avanti Polar Lipids Inc.), 10 μl of 100 μM MgCl2 and 5 μl of [γ-32P]ATP (0.5 mM ATP containing 30 μCi of [γ-32P]ATP) were added to the immunoprecipitants and the samples were incubated with agitation for 10 min at 37°C. The phosphatidylinositol solution was prepared by suspending phosphatidylinositol in 10 mM Tris–HCl, pH 7.4, 1 mM EGTA buffer and sonicating at 4°C for 10 min with 20 x 30 s pulses. The reaction was stopped by adding 20 μl of 6 N HCl and the lipid was extracted by adding 160 μl of chloroform:methanol (1:1). The lipid in the lower phase was loaded on a silicon TLC plate (Fisher Scientific) and the plate was developed by chromatography in chlororform: methanol:H2O:NH4OH (60:47:11.3:2). The radiolabeled lipid was detected by exposure of the TLC plate to X-ray film (Kodak).

#### Northern blot

Total RNAs from the individual cell lines were extracted by the Trizol method (Life Technologies Inc.) and loaded onto agarose gels at 20 μg/lane. The separated RNA was transferred to nylon membrane and the blot used for hybridization. PTEN cDNA containing the full coding region (1.2 kb) and human β-actin cdNA were labeled with 32P using Ready-To-Go™ DNA Labelling Beads from Amersham Biosciences according to the manufacturer’s instructions. Blots were prehybridized in ULTRAhyb™ solution from Ambion Inc. for 30 min. Hybridization was performed in the same solution containing 105 c.p.m./ml 32P-labeled cdNA fragment at 42°C. Then the blots were washed twice with 2 x SSC, 0.1% SDS for 10 min and twice with 0.1 x SSC, 0.1% SDS for 15 min at 42°C and exposed to X-ray films for 24 h.

### Results and discussion

#### Akt

We initially set out to observe the protein expression level of Akt (PKB) in a panel of colon adenocarcinoma cell lines with varying degrees of transformation, as assayed previously for their tumorigenic potential in nude mice (66). We performed western blot analysis on nine colon adenocarcinoma cell lines. In cell lines displaying low tumorigenic potential, such as SW1417, CaCo2, Colo201 and Colo205, the level of Akt protein was low (Figure 1A, lanes 1–4) in comparison with cell lines with high tumorigenic potential, such as SW1222, SW403, Lovo, LS174T and LS180 (lanes 5–9). Since the activity of Akt is regulated by its phosphorylation at residues Ser473 and Thr308 (67,68), we examined its phosphorylation state using phospho-specific antibodies directed against these two sites. To demonstrate the specificity of the anti-phospho antibodies for phosphorylated Akt, serum-deprived 293A cells were stimulated with serum to induce phosphorylation. The antibodies failed to interact with Akt from serum-deprived or wortmannin-treated cells, but they bound to Akt from serum-fed or rapamycin-treated cells (Figure 1B and C, compare lanes 10 and 13).
Phosphorylation of Akt at Ser473 and Thr308 was increased in colon cell lines with high tumorigenic potency (Figure 1B and C, lanes 5–9) in comparison with cell lines with low tumorigenic potency (Figure 1B and C, lanes 1–4). Thus, with the exception of Colo201 and Colo205, both total Akt and phosphorylated Akt are correlated with increasing tumorigenic potential of the cell lines. To determine whether the increase in Akt protein was at the level of translation or transcription, we performed real-time quantitative PCR analysis of Akt. Our results indicated that Akt message was increased in the various cell lines, within a 2- to 4-fold amplification range in comparison to the level of Akt mRNA in SW1417 cells, the most non-tumorigenic colorectal adenocarcinoma cell line in our panel (Table 1). However increases in Akt message did not correlate with the tumorigenic potential of the available colon cell lines. As a control, we examined the levels of the PRL-2 gene, which is not directly involved in the PI 3-kinase signaling pathway. Levels of PRL-2 mRNA were similar in our entire panel of colon adenocarcinoma cell lines (Table I).

During preparation of this manuscript, Itoh et al. (23) and Roy et al. (27) reported similar findings upon examining the role of Akt in colon carcinomas. Itoh et al. examined Akt phosphorylation at Ser473 in two colon cell lines and 65 human colorectal carcinomas; they noted high expression of phosphorylated Akt in 46% of the tumors. Roy et al. demonstrated that Akt overexpression is an early event during sporadic colon carcinogenesis, as Akt was detected in 57% of the sporadic carcinomas (21 out of 37 tumors) and adenomas (17 out of 30) examined, with phosphorylation of Akt at Ser473 detected only in neoplastic rather than normal epithelium. Here we have noted transcriptional, translational and post-translational modification increases in Akt in colon adenocarcinoma cells. However, only increases in the latter two correlate well with the increased tumorigenic potential of the colon

Table I. Quantitative RT–PCR of PI 3-kinase signaling genes

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Akt</th>
<th>p70s6k</th>
<th>FKHR</th>
<th>PRL-2</th>
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<tbody>
<tr>
<td>SW1417</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CaCo2</td>
<td>1.4</td>
<td>1.1</td>
<td>3.5</td>
<td>1</td>
</tr>
<tr>
<td>Colo201</td>
<td>2.5</td>
<td>0.9</td>
<td>0.6</td>
<td>1</td>
</tr>
<tr>
<td>Colo205</td>
<td>2.2</td>
<td>0.5</td>
<td>1.1</td>
<td>1</td>
</tr>
<tr>
<td>SW1222</td>
<td>4.2</td>
<td>0.8</td>
<td>18.4</td>
<td>1</td>
</tr>
<tr>
<td>SW403</td>
<td>0.9</td>
<td>0.6</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>Lovo</td>
<td>2.3</td>
<td>1.6</td>
<td>1.2</td>
<td>1</td>
</tr>
<tr>
<td>LS174T</td>
<td>1.1</td>
<td>1</td>
<td>1.3</td>
<td>1</td>
</tr>
<tr>
<td>LS180</td>
<td>1</td>
<td>0.8</td>
<td>0.7</td>
<td>0.9</td>
</tr>
<tr>
<td>HT29</td>
<td>1.2</td>
<td>1</td>
<td>0.9</td>
<td>1</td>
</tr>
</tbody>
</table>

Total cellular RNA (2.5 μg) was processed for RT–PCR as described in Materials and methods. Levels of Akt, p70 S6 kinase, FKHR and PRL-2 message were normalized to those of two housekeeping genes, cyclophilin and 28S rRNA. Values reported are fold amplification of various messages in comparison to the most non-transformed cell line, SW1417.
adenocarcinoma cells, indicating that activation of Akt could play an essential role in the progression of colon carcinomas.

**FKHR**

Since the level and activity of Akt increased with the tumorigenic potential of the colon adenocarcinoma cell lines, we proceeded to examine the protein expression pattern of FKHR, a downstream target of Akt (4,48–50,52,53,69,70). Western blot analysis indicated a gradual increase in the expression of total FKHR and phosphorylation of this protein at Ser256, which correlated well with increasing tumorigenicity of the colon cell lines with the exception of a relatively low phosphorylation level in Colo201 and Colo205, a result similar to Akt (Figure 2A and B, lanes 1–9). Although the phosphorylation of FKHR at Thr24 was increased in three of the high tumorigenic cell lines (Figure 2C, compare lanes 1–6 with 7–9), we did not observe a gradual increase in phosphorylation of this site accompanying the progressive tumorigenic potential of the colon cell lines. The specificity of the phospho-specific antibodies was demonstrated as they did not detect FKHR from serum-deprived or wortmannin-treated cells, but rather from serum-stimulated and rapamycin-treated cells (Figure 2B and C, compare lanes 10 and 13 with 11 and 12). In contrast, antibodies directed against FKHR recognized the protein in 293A cells irrespective of its phosphorylation state (Figure 2A, lanes 10–13). A representative blot was immunoblotted with β-actin to demonstrate equal protein content of the samples (Figure 2D). Real-time quantitative PCR analysis indicated that the levels of FKHR message were essentially the same across the colon cell lines, with the exception of CaCo2 and SW1222, in which 3- and 18-fold elevations in FKHR mRNA, respectively, were noted in comparison with the levels observed in SW1417 cells (Table 1). High level expression of mRNA in these two cell lines may be due to their intrinsic, tumor-unrelated characteristics. Therefore, only the translational and post-translational modification increases in FKHR are correlated with the tumorigenic potential of the cells.

It is worth noting that despite elevations in FKHR levels observed in colon adenocarcinoma cells, we suspect that the majority of FKHR in these cells is inactivated due to enhanced phosphorylation. Phosphorylation of FKHR results in its exclusion from the nucleus and decreases its transcriptional activity on targeted cell cycle regulating genes (4,47,50,53,64). A number of studies have aimed at understanding the role of the various FKPH phosphorylation sites. Phosphorylation of Ser256 has been shown to inhibit transactivation, by inhibiting nuclear import through suppression of a nuclear localization signal (51,72,73). Phosphorylation of Thr24 has been suggested to induce interaction of FKHR with 14-3-3 proteins (4,73). This may sequester FKHR isoforms in the cytosol, contributing to the growth factor-induced nuclear exit of these transcription factors. Thus differences in phosphorylation pattern of FKHR at the two sites examined in colon cell lines may provide clues as to the mechanism of PI 3-kinase involvement in progression of colon carcinomas.

**PI 3-kinase and PTEN**

Since the activity of Akt and its downstream effector FKHR correlated well with the increased transformed state of the colon adenocarcinoma cell lines, we wished to determine whether up-regulation of the PI 3-kinase signaling pathway also occurred for the upstream regulator of Akt, PI 3-kinase (41,74). To this end, we examined protein levels of the regulatory and catalytic sub-units, p85 and p110α, respectively, of PI 3-kinase. Western blot analysis indicated that the levels of p110α and p85 are similar across the colon cell lines (Figure 3A and C). The only exception was that p85 was not detected in cell line SW1417 (Figure 3C, lane 1). This observation was reproducible and did not appear to be due to degradation of proteins during protein extraction or sample preparation. Again, both blots were probed with an anti-β-actin antibody to demonstrate equal loading of total cellular proteins (Figure 3B and D). We next determined if PI 3-kinase activity is involved in the tumorigenic potential of the colon cell lines. To determine PI 3-kinase activity, we immunoprecipitated PI 3-kinase with anti-p85 antibody and carried out in vitro PI 3-kinase activity assay. As shown in Figure 3E, PI 3-kinase activity appeared to display some correlations with the increased transformed state of the colon adenocarcinoma cell lines as PI 3-kinase was hardly detected in SW1417 cells but was greatly activated in cell line LS180, a cell line with the highest tumorigenic potential (Figure 3E). Undetectable activity of PI 3-kinase in SW1417 cells is consistent with lack of expression of the p85 subunit in this cell line. It is not known why p85 could not be detected by the anti-p85 antibody in the SW1417 cell line.

**Fig. 2.** Elevation of FKHR and its phosphorylation in colon cells. Total cell extracts (50 μg) were processed for western blotting as described in Materials and methods, using 10% SDS-PAGE. One membrane was probed with a rabbit polyclonal anti-FKHR antibody which recognizes FKHR irrespective of its phosphorylation state (A), while the others were probed with a rabbit polyclonal anti-phosphopeptide antibody which is specific for phospho-S256 (B) and phospho-T24 (C) in FKHR. A representative blot was probed with a mouse monoclonal anti-β-actin antibody, to normalize for protein loading.
PTEN is a lipid phosphatase acting on PI 3-kinase. We also checked the levels of PTEN, to determine whether alterations in its levels may play a role in up-regulation of the activity of downstream targets of PI 3-kinase. Western blot analysis indicated that the PTEN expression profile was not altered amongst colon adenocarcinoma cell lines (Figure 4A), with the exception that the antibody did not detect observable amounts of the protein in SW1222 cells (lane 5). This observation was reproducible and did not appear to result from degradation of proteins during protein extraction or sample preparation. Taniyama et al. (75) also reported that PTEN protein expression is maintained in sporadic colorectal tumors. Although our examination of PTEN protein levels did not evaluate loss of function due to genetic mutations, a phenomenon normally associated with cancer formation, it is possible that PTEN may be genetically modified in colon adenocarcinoma cells and rendered inactive. The molecular mechanism of lack of PTEN expression in SW1222 cells was further investigated by RT–PCR and northern blot analysis. This mRNA could not be detected by RT–PCR (data not shown) or northern blot (Figure 4B) in SW1222 cells. As controls, it was readily detectable in other cell lines (Figure 4B and data not shown). These results suggest that the lack of expression of PTEN in cell line SW1222 is very likely caused by gene deletion or promoter silencing, but not by nonsense or mismatch mutation. We also extracted genomic DNA and tried to amplify the PTEN gene using three pairs of primers. We failed to amplify PTEN genomic DNA from cell line SW1222, supporting the notion that the PTEN gene is probably deleted in this cell line.

**Soft agar**

The soft agar assay is often used to test for contact inhibition or non-adherent growth of transformed cells. Since our data indicated that the PI 3-kinase signaling pathway is up-regulated in colon cell lines, we were interested to see whether the transformation potential of the cells would be reduced by addition of wortmannin, an inhibitor of this pathway, in a semi-solid agarose medium. Cell lines with low transformation potential, SW1417, CaCo2 and Colo201, formed few and small foci in the agar medium suspension (Figure 5, left panels). In contrast, the Lovo cell line, which has high transformation potential, displayed abundant and large colonies (Figure 5, left panels). Addition of wortmannin to colon adenocarcinoma cells did not result in a reduction in colony efficiency (data not shown), but rather resulted in a decrease in colony size (Figure 5, right panels). These findings further indicate that the PI 3-kinase signaling pathway plays a role in progression of colon carcinoma and that control of the activity of the pathway may have potential in the treatment of colon carcinomas.
Another component of the PI 3-kinase signaling pathway is p70 S6 kinase. Western blot analysis for p70 S6 kinase indicated that its protein level was also up-regulated in colon cell lines, and the gradual increase in the protein content correlated well with the increased tumorigenic potential of the colon cell lines (Figure 6A, lanes 1–8). Since the activity of p70 S6 kinase is also regulated by its phosphorylation (57,62,76–78), we examined its phosphorylation state at Thr389 using a phosphospecific antibody directed against this site (Figure 6B). The phosphorylation of this residue is rapamycin sensitive and plays an important role in the regulation of p70 S6 kinase activity (79). To demonstrate the specificity of the phosphospecific antibody for phosphorylated p70 S6 kinase, serum-deprived 293A cells were stimulated with serum to induce phosphorylation. The antibodies failed to interact with p70 S6 kinase from serum-deprived, wortmannin- and rapamycin-treated cells, but it bound to p70 S6 kinase from serum-fed cells (Figure 6B, compare lanes 9, 11 and 12 with lane 10). The increase in p70 S6 kinase phosphorylation at Thr389 correlated well with the tumorigenic potential of the colon adenocarcinoma cell lines (Figure 6B, lanes 1–8). Interestingly, while total and phosphorylated Akt and FKHR were relatively low in both Colo201 and Colo205 cells, the levels of total and phosphorylated p70 S6 were elevated in Colo205 but not Colo201 cells, indicating that there is additional complexity in the expression of these downstream components in the pathway in these two cell lines. Quantitative PCR analysis did not reveal any increase in p70 S6 kinase message across the colon cell lines (Table 1), indicating that only translational and post-translational modification increases of the protein correlate with increasing tumorigenic potential of the cells.

**P70 S6 kinase**

Another component of the PI 3-kinase signaling pathway is p70 S6 kinase. Western blot analysis for p70 S6 kinase indicated that its protein level was also up-regulated in colon cell lines, and the gradual increase in the protein content correlated well with the increased tumorigenic potential of the colon cell lines (Figure 6A, lanes 1–8). Since the activity of p70 S6 kinase is also regulated by its phosphorylation (57,62,76–78), we examined its phosphorylation state at Thr389 using a phosphospecific antibody directed against this site (Figure 6B). The phosphorylation of this residue is rapamycin sensitive and plays an important role in the regulation of p70 S6 kinase activity (79). To demonstrate the specificity of the phosphospecific antibody for phosphorylated p70 S6 kinase, serum-deprived 293A cells were stimulated with serum to induce phosphorylation. The antibodies failed to interact with p70 S6 kinase from serum-deprived, wortmannin- and rapamycin-treated cells, but it bound to p70 S6 kinase from serum-fed cells (Figure 6B, compare lanes 9, 11 and 12 with lane 10). The increase in p70 S6 kinase phosphorylation at Thr389 correlated well with the tumorigenic potential of the colon adenocarcinoma cell lines (Figure 6B, lanes 1–8). Interestingly, while total and phosphorylated Akt and FKHR were relatively low in both Colo201 and Colo205 cells, the levels of total and phosphorylated p70 S6 were elevated in Colo205 but not Colo201 cells, indicating that there is additional complexity in the expression of these downstream components in the pathway in these two cell lines. Quantitative PCR analysis did not reveal any increase in p70 S6 kinase message across the colon cell lines (Table 1), indicating that only translational and post-translational modification increases of the protein correlate with increasing tumorigenic potential of the cells.
To our knowledge, this is the first report on elevated p70 S6 kinase level and activity in colon adenocarcinoma cells.

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